UNIVERSIDADE ESTADUAL PAULISTA
INSTITUTO DE BIOCIÊNCIAS
CÂMPUS DE BOTUCATU

SUSCEPTIBILIDADE ANTIFÚNGICA, PRODUÇÃO DE BIOFILME E CARACTERIZAÇÃO DO GENE *ALS3* EM ISOLADOS DE *Candida albicans* E NÃO-*albicans* DO HOSPITAL DAS CLÍNICAS, UNESP, BOTUCATU

ARIANE CRISTINA MENDES DE OLIVEIRA BRUDER NASCIMENTO

Dissertação apresentada ao Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Mestre no Programa de PG em Biologia Geral e Aplicada

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Ó mundo invisível, nós o vemos

Ó mundo intangível, nós o tocamos

Ó mundo não identificável, nós o identificamos

Inapreensível, nós o agarramos

(Francis Thompson, 1859-1906)

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"Tu te tornas eternamente responsável por aquilo que cativas."
(O Pequeno Príncipe - Antoine de Saint-Exupéry)

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"O essencial é invisível aos olhos..."

(O Pequeno Príncipe - Antoine de Saint-Exupéry)

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RESUMO

Leveduras oportunistas do gênero *Candida* são capazes de disseminar-se em hospedeiros susceptíveis, num processo crescente nos últimos anos. Um fator complicador destes quadros ocorre quando estas leveduras são capazes de produzir biofilme, principalmente quando associadas a cateteres ou outros dispositivos médicos, elevando o poder de penetração e invasão em órgãos do hospedeiro. Por também conferir maior resistência às drogas antifúngicas do que as células dispersas, o biofilme fúngico tornou-se um dos maiores problemas no combate a estas infecções. A base genética da produção de biofimes nestas leveduras é complexa, porém já foi determinado o envolvimento de genes da família *ALS*, codificadores de glicoproteínas de adesão. Dentre os oito genes desta família (*ALS1* ao *ALS7* e *ALS9*), destaca-se o papel de *ALS3.* O gene *ALS3*, assim como todos os outros genes da família, apresenta uma estrutura composta por 3 domínios. O domínio 5', região bem conservada; um domínio central que apresenta *motifs* de 108pb repetidos em *tandem,* com variações de tamanho entre os genes da mesma família e entre o mesmo gene em diferentes espécies, em uma mesma espécie e até mesmo entre alelos de uma mesma cepa, e o domínio 3, menos conservado que o domínio 5', que pode apresentar variações de tamanho e de algumas seqüências de aminoácidos. Tendo em vista a crescente incidência de infecções por esse microrganismo em todo o mundo, o presente estudo objetivou investigar a freqüência das diferentes espécies de *Candida* em nossa região e caracterizá-las quanto à susceptibilidade a drogas antifúngicas e produção de biofilme, e possível correlação da produção de biofilme com polimorfismos de tamanho do gene *ALS3*. Os resultados obtidos confirmam a crescente incidência de espécies não-*albicans*, principalmente isoladas de infecções invasivas como cultura de sangue e liquido peritoneal, onde C. *parapsilosis* foi a espécie mais freqüente isolada. Em relação à produção de biofilme, também os isolados de infecções invasivas apresentaram maior positividade para a produção de biofilme que os de secreção vaginal e urina. Quanto às espécies, amostras de *Candida* não-*albicans* também apresentaram maior positividade para a produção de biofilme que as de C. *albicans*. Reações da polimerase em cadeia foram realizadas para o gene *ALS3*, polimorfismos de tamanho foram detectados, mas a correlação do padrão de polimorfismos com a produção, *in-vitro,* de biofilme não foi significativa. O presente estudo também apresenta dados de susceptibilidade a quatro drogas antifúngicas, fluconazol, cetoconazol, itraconazol e anfotericina B, onde todas apresentaram excelente atividade sobre as cepas testadas e resistência foi detectada em poucos isolados. Os dados obtidos no presente trabalho podem refletir a situação das infecções por *Candida* spp. em nossa região, bem como orientar no tratamento e prevenção de infecções.

ABSTRACT

Opportunistic yeasts of the genus *Candida* are able to disseminate into the bloodstream in susceptible hosts, in an increasing course in the recent years. A complicating factor is when these yeasts are capable of producing biofilms, especially associated with catheters or other medical devices. Biofilm also confers greater resistance to antifungal drugs than dispersed cells, so the fungal biofilm has become one of the greatest problems in combating these infections. The genetic basis of the biofim production by yeasts is complex, but it has been know the involvement of *ALS* gene family, encoders of adhesion glycoproteins. Among the eight genes of this family (*ALS1* to *ALS7* and *ALS9*), the *ALS3* are considered the most important. The *ALS3* gene, such as the others members of the family, have three general domains: the 5'domain, conserved, with approximately 1300-pb; followed by a central domain consisting entirely of tandem-repeats of a 108-pb sequence, that are somewhat variable; and the 3' domain, which is least conserved in length and sequence. Considering the increase incidence of these infections worldwide, the aims of this study were identify the frequency of *Candida* species in our region, to characterize the profile of antifungal susceptibility; to quantify the biofilm production and to correlate this production with the *ALS3* gene length polymorphism. Our data confirm the increase incidence of non-*albicans* species, mainly when obtained from invasive infections, such as blood and peritoneal fluid, in which *C. parapsilosis* was the most frequent isolated species. The same was also observed to biofilm production, in which isolates obtained from invasive infections (blood and peritoneal fluid) are more biofilm producers than that obtained from vaginal secretion and urine. Among the different species, isolates of non-*albicans* also are more biofilm producers than *C. albicans*. Polimerase chain reactions are used to evaluate the *ALS3* gene length polymorphism. The number of tandem-repeats copies varied from seven to fourteen in the *C. albicans* isolates, and presented no correlation with biofilm production. The study also presents data of susceptibility tests against fluconazole, itraconazole, ketoconazole and amphotericin B. All these drugs showed a greatest activity, and resistance was observed in a few numbers of isolates. These data appear to reflect the real situation of *Candida* infection in the Brazilian public tertiary hospital, and might serve as guide for better treatment and prevention strategies.

INTRODUÇÃO

Fungos patogênicos, especialmente espécies de *Candida*, têm emergido como importantes agentes de infecções oportunistas, principalmente, em indivíduos com a imunidade comprometida, incluindo aidéticos, pacientes com câncer submetidos à quimioterapia, transplantados em terapia imunossupressora e pacientes com diabetes avançada (RICHARDSON, 2005; APERIS *et al*., 2006). Acredita-se que a maioria dos casos de candidemias seja adquirida por via endógena, pela translocação do patógeno através do trato digestivo, local de rica colonização por *Candida* spp. (COLE, HALAWA, ANAISSIE, 1996; NUCCI, ANAISSIE, 2001). No entanto, estas infecções também podem ser adquiridas por via exógena, através do contato das mãos de profissionais da saúde com pacientes portadores de cateter, implante de próteses contaminadas, bem como pela administração parenteral de soluções contaminadas (PFALLER, 1995; WENZEL, 1995, TROFA, GÁCSER, NOSANCHUCK, 2008). Acredita-se que a maior parte das candidemias é sempre precedida pelo evento colonização pela mesma espécie de levedura, o que é considerado um importante fator de risco para o desenvolvimento destas infecções (COLE, HALAWA, ANAISSIE, 1996; NUCCI , ANAISSIE, 2001).

Os principais fatores que predispõem os pacientes a infecções disseminadas incluem a colonização do trato gastrointestinal por espécies de *Candida,* resultante do uso prolongado de agentes antibacterianos de amplo espectro, ruptura da mucosa gastrointestinal por agentes citotóxicos, e neutropenia. Entretanto, o cateter venoso central, aparenta ser o fator de risco mais comum para o desenvolvimento de candidemia em pacientes não-neutropênicos ou que não apresentam imunodeficiência (REX, 1996).

A partir da década de 80, a incidência de candidemias aumentou substancialmente em hospitais terciários de todo o mundo. (WISPLINGHOFF *et al*., 2004), e atualmente essas infecções têm emergido como os maiores responsáveis pela morbidade e mortalidade nos pacientes imunodeprimidos (PFALLER *et al*., 2000, 2008). No Brasil, Colombo *et al.* (2007) conduziram um estudo epidemiológico reunindo dados sobre infecções de corrente sanguínea documentados em quatro hospitais da cidade de São Paulo. Um total de 7038 episódios de bacteremias e fungemias ocorridos no período de um ano foi avaliado, *Candida* spp. responderam por 4,3% do total das infecções de corrente sanguínea. Freqüência semelhante foi também detectada no Hospital das Clínicas da Faculdade de Medicina de Botucatu (HC/FMB), que de um total de 6417 episódios e amostras de culturas positivas avaliadas, no período de janeiro de 1991 a dezembro de 1994, *Candida* spp. foram isoladas em 222 (3,5%) culturas, as

quais foram oriundas principalmente das unidades de pediatria e berçário (SUGIZAKI *et al.*, 1998). Ruiz *et al.* (2005) relataram que, nesse mesmo hospital, as espécies mais freqüentes foram *C. albicans* (38,7%) e *C. parapsilosis* (30,7%).

Embora a *C. albicans* seja a principal espécie isolada de pacientes com fungemia (PFALLER *et al.*, 1998a,b,c; SANDVEN *et al.*, 1998; KRCMÉRY JR V, KOVACICOVÁ G, 2000), têm aumentado os relatos de infecções causadas por espécies não-*albicans* (LACAZ *et al.*, 2002; COLOMBO *et al.*, 2007). Em 1963, eram conhecidas apenas cinco espécies de *Candida* causadoras de doenças humanas, *C. albicans, C. parapsilosis, C. tropicalis, C. stellatoidea* e *C. guilliermondii*. Atualmente são conhecidas cerca de 20 espécies de *Candida* implicadas em micoses superficiais ou invasivas em humanos (DIGNANNI, SOLOMKIN, ANAISSIE, 2003).

As principais espécies de interesse clínico são: *C. albicans, C. parapsilosis, C. tropicalis, C. glabrata, C. krusei, C. guilliermondii* e *C. lusitaniae*. Entretanto, número progressivo de casos relacionados a espécies emergentes de *Candida* tem sido descrito, envolvendo isolamentos de *C. dubliniensis, C. kefyr, C. rugosa, C. famata, C. utilis, C. lipolytica, C. norvegensis, C. inconspicua* entre outras (COLEMAN *et al.*, 1998). A freqüência de espécies de *Candida* não-*albicans* é depende da população de pacientes estudada, da terapêutica utilizada, do uso de antibióticos ou outras medidas adotadas (PFALLER, 1995; ABI-SAID *et al.*, 1997; NUCCI, COLOMBO, 2007; PFALLER *et al.*, 2008).

Paralelamente ao aumento das infecções causadas por leveduras do gênero *Candida*, especialmente a nível hospitalar, tem sido observado o aparecimento de resistência aos antimicóticos, assim como a seleção de espécies não-*albicans*. Há alguns anos, considerava-se que os fungos eram regularmente sensíveis aos antimicóticos, apenas algumas espécies *Candida* podiam adquirir resistência à 5-fluorcitosina (DIASSIO, BENETT, MYERS , 1978), observando-se mais tarde o surgimento de resistência de *C. albicans* em pacientes com candidíase granulomatosa crônica sob tratamento prolongado com cetoconazol (HORSBURGH, KIRKPATRICK, 1983). Até esse momento, o problema não parecia ter maior repercussão, mas uma década mais tarde a situação mudaria drasticamente ao observar-se um aumento na freqüência de candidemias devido não apenas à *C. albicans* resistentes, mas também para espécies diferentes da *C. albicans*, geralmente menos suscetíveis aos antifúngicos. Entre estas últimas, destacavam a *C. krusei* que é intrinsicamente resistente ao fluconazol, e *C. glabrata*, cuja suscetibilidade aos azóis é muito variável (NGUYEN *et al*., 1996; PFALLER *et al*., 2000; REX, RINALDI, PFALLER, 1995). Porém, mais notórias foram as falhas de tratamento observadas a partir de 1985 em pacientes com AIDS e mucosite

candidiásica sob tratamento com fluconazol, que desenvolveram candidíase orofaringeana crônica refratária à terapia (REVANKAR *et al*., 1998; NG, DENNING, 1993; SANGEORZAN *et al*., 1994).

Diferentemente dos países da América do Norte, onde a emergência de espécies não-*albicans* parece estar associada à pressão seletiva do uso do fluconazol, no Brasil as espécies não-*albicans* mais prevalentes são sensíveis a esta droga (REX *et al*., 2001). Na ultima década, é crescente o numero de trabalhos documentando alterações na susceptibilidade das leveduras do gênero *Candida* às drogas antifúngicas. Embora muitos sejam os trabalhos relatando que a maioria dos isolados apresenta-se sensível ao fluconazol, já foi observado o desenvolvimento de resistência em pacientes previamente expostos aos azóis, seja por uso profilático ou indiscriminado da droga em determinadas populações (NOLTE *et al*., 1997; SAFDAR *et al*., 2001; KERSUN *et al*., 2008). Muitos programas de vigilância vêm documentando dados da distribuição das espécies e perfis de susceptibilidade às drogas. Consideráveis variações vêm sendo demonstradas entre diferentes hospitais ou diferentes países a respeito da incidência das espécies como etiologia de infecções e perfis de susceptibilidade dos microrganismos isolados. Em países desenvolvidos, existem acordos entre as instituições para unir dados epidemiológicos a fim de confirmar a magnitude das infecções, principalmente de corrente sanguínea, por *Candida* spp., bem como os perfis de susceptibilidade a drogas, gerando um importante banco de dados sobre as tendências das infecções e características mais freqüentes de cada espécie, o que não acontece na América Latina, onde esses estudos geralmente são limitados a uma única instituição (PFALLER *et al*., 1998a,b,c; RANGEL-FRAUSTRO *et al*., 1999; PFALLER *et al*., 2008). Essa situação é menos agravante em nosso país, onde já foi conduzido um grande estudo sobre candidemias, envolvendo 11 instituições, o qual apresentou consideráveis taxas de morbidade e mortalidade, embora a presença de amostras resistentes aos antifúngicos tenha sido rara (COLOMBO *et al*., 2006).

O *National Committe for Clinical Laboratory Standards* (NCCLS) dos Estados Unidos, denominado, a partir de 2005, *Clinical and Laboratory Standards Institute* (CLSI) publicou um método de referência para testes de susceptibilidade antifúngica em leveduras, o M27-A2. Trata-se de um método quantitativo, que contem técnicas de diluição em meio líquido, para se determinar a concentração inibitória mínima (CIM), em leveduras, frente à anfotericina B, 5-fluorcitosina e derivados azólicos, incluindo cetoconazol, fluconazol, itraconazol, voriconazol, além de posaconazol e ravuconazol, estes últimos ainda não comercializados no Brasil (CLSI, Documento MA 27-A2).

Assim como acontece com bactérias, já foram relatados casos de infecção por fungos multi-resitentes, como por exemplo, algumas cepas de *C. glabrata*, que por sua vez ocorrem em 20-24% das candidemias nos EUA (RICHARDSON, 2005; PFALLER, DIECKEMA, MERZ, 2007). A multi-resistência a drogas é um sério fator complicador no tratamento de infecções fúngicas oportunistas que, frequentemente, ocorrem nos pacientes imunocomprometidos (THAKUR *et al*., 2008).

Entre os atributos relacionados com o potencial patogênico da *C. albicans,* bem como de outras leveduras do mesmo gênero, está a incrível capacidade de adesão destes microrganismos (CALDERONE, BRAUN, 1991; RAMAGE *et al.,* 2005; TROFA, GÁCSER, NOSANCHUCK, 2008), podendo levar à formação de biofilme. A capacidade de formação do biofilme pode ser considerada um potente fator de virulência, podendo estar presente em todas as espécies de *Candida* (REX, 1996). Como os biofilmes geralmente são mais resistentes aos mecanismos de defesa do hospedeiro e às drogas antimicrobianas do que as células dispersas, eles representam um fator predisponente de infecção para muitos pacientes (BAILLIE, DOUGLAS, 1998; DONLAN, 2001).

A formação de biofilme em *C. albicans* vem sendo descrita como um processo gradual que se inicia com a aderência a um substrato, seja ao próprio tecido do hospedeiro ou ao dispositivo médico, resultando na formação de uma confluente camada basal de células que se dividem e produzem hifas, como projeções tubulares direcionadas para a região superior do biofilme (SOLL, 2008). Estas células durante o desenvolvimento do biofilme produzem uma matriz extracelular estável de substâncias poliméricas (DOUGLAS, 2003; CHANDRA *et al*., 2001; SOLL, 2008). O estágio final do desenvolvimento do biofilme é a maturação, quando ocorre menor crescimento das leveduras e elevado crescimento das hifas, nesta fase ocorre o envolvimento do biofilme pela matriz extracelular. A estimulação da produção da matriz extracelular durante o desenvolvimento do biofilme de *C. albicans* ainda é de causa desconhecida, o que se sabe, no entanto, é que a composição da matriz inclui: carboidratos, proteínas, fósforo, glicose e hexosaminas, mas a maior parte desse conteúdo ainda não foi identificada (BAILLIE, DOUGLAS, 2000; BLANKENSHIP, MITCHELL, 2006).

Em resumo, os diferentes estágios que fazem parte do processo de desenvolvimento do biofilme incluem: estágio da aderência, célula-substrato e célula-célula; estágio de formação e desenvolvimento das hifas; e estágio de maturação, onde ocorre a produção da matriz extracelular que vai envolver e proteger as células do biofilme. Em estudo recente sobre *quorum-sensing*, Blankenship, Mitchell (2006) sugeriram um novo estágio no ciclo de vida do

biofilme de *C. albicans*, o estágio da dispersão, no qual células filhas se desenvolvem como células não aderentes, podendo ser facilmente liberadas do biofilme.

Diversos estudos vêm confirmando que a produção de biofilme por espécies não-*albicans* é significativamente mais freqüente do que em *C. albicans* (SHIN *et al.,* 2002; TUMBARELLO *et al.*, 2007). Em *C. parapsilosis,* a produção de biofilme constitui-se em importante fator de virulência. *C. parapsilosis* é bem conhecida como causadora de fungemia e candidíase invasiva associada à hiperalimentação parenteral, dispositivos intravasculares e soluções oftálmicas contaminadas (PLOUFFE *et al.*, 1977; SOLOMON *et al.*, 1984; O'DAY, HEAD, ROBINSON, 1987; WEMMS *et al.*, 1987; WEMMS, 1992). Vários fatores dão à *C. parapsilosis* uma vantagem seletiva, incluindo a capacidade de proliferar-se em altas concentrações de glicose e de aderência a materiais protéticos (CRITCHLEY, DOUGLAS, 1985; WEMMS *et al.*, 1987). Pfaller, Messer, Hollis (1995), estudaram a produção de biofilme por amostras clínicas de *C. parapsilosis* crescidas em meio de cultura contendo glicose e verificaram que as isoladas de sangue e de cateter apresentaram maior produção de biofilme do que amostras de outros sítios anatômicos.

A base molecular da formação e do desenvolvimento do biofilme destes fungos ainda não está completamente compreendida, porém, já está bem estabelecida que a interação da *C. albicans* com as células do hospedeiro ou superfícies inertes resulta em alterações na expressão de diferentes genes. Diferentes estudos têm descrito mudanças nos níveis de expressão gênica durante o desenvolvimento do biofilme (MARCHAIS *et al.*, 2005, MURILLO *et al.,* 2005). Estudos sobre a base genética da produção de biofilme têm se beneficiado significativamente com os avanços recentes observados na biologia molecular e genômica. Isto tem sido particularmente observado com a *C. albicans*, cujo genoma já foi totalmente seqüenciado (JONES *et al*., 2004; ODDS, BROWN, GOW, 2004; BRAUN *et al.*,, 2005; http://candida.bri.nrc.ca). *C. albicans* é uma levedura que se desenvolve na forma diplóide, com o material genético organizado em 8 cromossomos (1-7 e cromosssomo R), e o genoma haplóide é constituído de 14.851 kb (kilobases), contendo 6.419 ORFs (*open reading frame*, sequências de leituras, ou seja, genes codificantes) com mais de 100 codons de tamanho e 224 introns, (JONES *et al*., 2004; BRAUN *et al*., 2005; ODDS *et al*., 2007). Sistemas ou plataformas de *microarray* também já foram desenvolvidos para *C. albicans*, o que permite analisar a expressão gênica de todos os genes simultaneamente, nas diversas condições fisiológicas (CAO *et al*., 2005; http://genome.wustl.edu/ activity/ma/c*albicans*/).

O envolvimento de alguns genes no processo de formação e desenvolvimento do biofilme parece já estar bem estabelecido (RAMAGE *et al*., 2005; NOBILE, MITCHELL

2006). Dentre os vários grupos de genes implicados neste fenótipo, constatou-se que os da família *ALS* (*agglutinine like sequence*) presentes em *C. albicans* e espécies relacionadas desempenham papel chave neste processo, por codificar proteínas com características de glicoproteínas de adesão à superfície da célula (HOYER, PAYNE, HECHT, 1998; HOYER, 1998). Já foi demonstrado que genes *ALS* estão com sua expressão aumentada durante a formação do biofilme (CHANDRA *et al*., 2001; GARCIA-SANCHEZ *et al*., 2004; GREEN *et al*., 2004; O'CONNOR *et al*., 2005).

O gene *ALS1* em *C. albicans* foi descrito pela primeira vez por Hoyer *et al.* (1995) e, desde então, pesquisadores vêm tentando entender sua relação com o restante da família *ALS* e explorando suas proteínas e funções. A família *ALS* presente em *C. albicans* inclui oito genes (*ALS1-ALS7* e *ALS9*) que codificam muitas glicoproteínas de superfície (HOYER *et al.*, 2008). Cada gene da família *ALS* apresenta uma estrutura similar composta por três domínios: um dominio 5', na extremindade N, composto por 1299 a 1308pb, que apresenta 55-90% de similaridade entre os diferentes genes da família; um domínio central variável, organizado em *tandem repeats,* com *motifs* de 108pb que se repetem ao longo do domínio; e um domínio 3', extremidade C, que é relativamente variável em tamanho e seqüência de nucleotídeos entre os genes da mesma família (HOYER, HECHT, 2001). Os genes da família *ALS* estão localizados em três cromossomos distintos: *ALS1, ALS2, ALS4, ALS5* e *ALS9* estão localizados no cromossomo 6, *ALS6* e *ALS7* estão localizados no cromossomo 3, e *ALS3* no cromossomo R (HOYER *et al.*, 1995; HOYER, PAYNE, HECHT*,* 1998; HOYER, HECHT 2001).

O tamanho de um mesmo gene *ALS* freqüentemente varia dentro de uma mesma espécie e entre alelos de uma mesma cepa devido a diferenças no número de cópias dos *motifs* de 108pb organizados em *tandem repeat,* presentes no domínio central de cada gene (HOYER, HECHT, 2001). É comum, por exemplo, uma mesma cepa apresentar padrões diferentes (duas bandas) para o gene *ALS1* devido à variabilidade do número de repetições dos *motifs* na região do domínio central em cada alelo (HOYER, 2001).

Os genes *ALS* exibem diversos níveis de variabilidade, incluindo espécie-específica e alelo-específica, diferenças de tamanho para um mesmo gene, diferenças na regulação gênica espécie-específica, ausência de um gene *ALS* particular em certos isolados, e regiões codificadoras adicionais em outros (HOYER, HECHT, 2001). Estudos moleculares sobre a expressão de genes de *ALS* demonstraram que os mesmos são regulados e expressos diferencialmente em função de processos fisiológicos celulares, tais como o estágio de crescimento e morfologia da célula, ou seja, predominantemente leveduriforme ou na forma de hifas e pseudo-hifas (HOYER *et al.*, 1995; HOYER *et al.*, 1998; HOYER, PAYNE,

HECHT,1998). Constatou-se que destes genes, o *ALS1*, que codifica glicoproteínas de superfície celular, apresenta-se em alta expressão em células do biofilme de *C. albicans* (GARCÍA-SÁNCHEZ *et al.*, 2004). O gene *ALS3* também mostrou alta expressão, porém, aparentemente associado à produção de hifas de *C. albicans* (HOYER *et al.*, 1998). Nailis *et al.* (2006) compararam a expressão gênica de *ALS1* e *ALS3* entre as células do biofilme de *C. albicans* formadas sobre superfície de silicone e as células em suspensão (planctônicas) e constataram um significativo aumento da expressão de *ALS1* nas células do biofilme, e uma diminuição da expressão de *ALS3.* Por outro lado, em um estudo recente, Nobile *et al*. (2008) concluíram, após vários testes com mutantes *als1*/*als1 als3*/*als3*, que *ALS3* e *ALS1* são essenciais para a formação do biofilme *in-vivo* e a redução na expressão dessas proteínas acarreta na formação de um biofilme frágil, suas funções no biofilme são compatíveis com sua estrutura e propriedade bioquímica.

Em outros estudos, o papel do produto do gene *ALS1* na aderência das *C. albicans* às células humanas, Fu *et al.* (1998, 2002) constataram que o gene *ALS1* codifica uma proteína de superfície celular responsável pela aderência as células endoteliais e epiteliais, e o rompimento de ambas as cópias deste gene acarretou em redução de 35% na aderência às células endoteliais, e o aumento da expressão de *ALS1* elevou a aderência para 125%. Zhao *et al*, (2005) demostraram que a redução na expressão da proteína *Als2* acarretou na redução da biomassa do biofilme, sugerindo que *Als2* contribui com os estágios mais avançados do desenvolvimento do biofilme e não com o estágio da aderência.

Num modelo experimental de infecção de cateter *in-vivo*, *Als1* e *Als3* também apresentaram funções redundantes; e a alta expressão de outros genes da família – *ALS5, ALS6, ALS7* e *ALS9* – foram capazes de substituir parcial ou completamente a ausência de *ALS1* e/ou *ALS3* facilitando o desenvolvimento do biofilme nesse tipo de modelo experimental, enquanto que *ALS2* e *ALS4* não foram capazes, e ainda, todos os genes *ALS* puderam ser substituídos por *ALS3* ou *ALS1* em modelos *in-vivo* e *in-vitro* (NOBILE *et al*., 2008).

Em outros estudos também envolvendo cepas mutantes nocauteadas, principalmente com deleção de genes *ALS,* constatou-se a importância de alguns fatores transcripcionais, como *Tec1* e *Bcr1*, bem como de outros genes codificadores, como de *HWP1(hyphal wall protein)* (SCHWEIZER *et al*., 2000; GARCIA-SANCHEZ *et al*., 2004; NOBILE, MITCHELL, 2005; NOBILE *et al*, 2006; SCHWEIZER *et al*., 2008; SOLL, 2008). O fato de alguns destes mutantes para *ALS3* e fatores transcripcionais ainda serem capazes de formar biofilme, mesmo rudimentar e com menor espessura, sugere que essas proteínas podem não desempenhar papel fundamental durante o estágio de adesão à superfície do substrato, e sim em estágios mais

avançados, como por exemplo, no estágio de adesão célula-célula ou célula-hifa (BLANKENSHIP, MITCHELL, 2006). Outros autores também observaram que a expressão das *Als3* e *Hwp1* ocorre somente durante o estágio de hifa (STAAB, FERRER, SUNDSTROM, 1996; HOYER, PAYNE, HECHT, 1998), e que essas proteínas podem ser as mediadoras da aderência célula-hifa ou hifa-hifa (BLANKENSHIP, MITCHELL, 2006).

Nobile *et al*. (2008) sugerem que a função de *Als1* e *Als3* possam ser complementares à função de *Hwp1* das células vizinhas. Estes estudos também indicaram uma interessante analogia entre as adesinas de C. *albicans* com as *mating*-aglutininas de *S. cerevisae*, particularmente devido à similaridade estrutural de *Als1* e *Als3* com as α-aglutininas de *S. cerevisae,* proteínas estas relacionadas com a atividade sexual (*mating* de *S. cerevisiae*). Parte da estrutura da *Als*, incluindo a porção N-terminal, é similar à estrutura das α-aglutininas (Sag1) de *S. cerevisae*. As proteínas *Als* apresentam especificidades diferentes da *Sag1*, porém afinidades similares ao análogo a-aglutinina de *S. cerevisae*. Também foi observada analogia entre *Hwp1* e a-aglutinina (*AGA1 e AGA2*) do *S. cerevisae* (Nobile *et al*., 2008). Essa analogia entre o tipo de resposta sexual (*mating reaction)* e biofilme já havia sido descrita por Daniels *et al*., (2006) que mostraram que o fator *mating* pode simular a formação do biofilme em um correspondente genético de *C. albicans*.

Portanto, as funções complementares de *Hwp1*, *Als1* e *Als3* na formação do biofilme são análogas às funções das aglutininas sexuais durante a *mating reaction*. Essa associação entre biofilme e *mating reaction* foi também sugerida por Soll (2008), o qual ainda especula que este processo pode estar presente em outros organismos, como em *Escherichia coli* na qual a conjugação (*mating*) ocorre com freqüência 1000 vezes maior durante o biofilme do que em condições de células dispersas (GHIGO, 2001). Em resumo, esses dados sugerem que a complementaridade pode ser uma relíquia evolutiva, ou seja, uma reorganização ou aquisição de uma nova função do produto gênico de um ancestral "sexualmente mais ativo" que a *C. albicans* de hoje. Essa complementaridade das adesinas sugere a importância da presença de uma única espécie no biofilme, uma vez que o biofilme depende do contato intra-específico (NOBILE *et al*., 2008).

JUSTIFICATIVA

O Laboratório de microbiologia Médica do Instituto de Biociências de Botucatu-UNESP possui uma importante coleção de amostras de leveduras do gênero *Candida* obtidas de pacientes do Hospital das Clínicas da Faculdade de Medicina de Botucatu, UNESP, com alguns isolados já previamente constatados como resistentes e outros sensíveis a alguns dos principais antifúngicos, e também produtores e não produtores de biofilme. Desse modo, o presente trabalho propôs aprofundar o estudo de caracterização destes isolados em relação a estes aspectos (sensibilidade aos antifúngicos e produção de biofilme), bem como iniciar estudos sobre a base genética deste fenótipo complexo que é a produção de biofilme, considerado fundamental na determinação da gravidade e evolução clínica das infecções causadas por estes germes. Os dados aqui obtidos poderão repercutir positivamente para o melhor entendimento e monitoramento destas infecções em nosso próprio HC, como também poderá servir de modelo e referencial comparativo para este grave problema mundial representado por essas leveduras nos ambientes hospitalares.

OBJETIVOS

Considerando a escassez de informações relacionada às infecções por leveduras do gênero *Candida*, relativa à nossa região, este estudo teve os seguintes objetivos:

-caracterizar o espectro de espécies de leveduras isoladas de culturas de sangue, urina, secreção vaginal e líquido peritoneal de pacientes ambulatoriais e/ou internados no Hospital das Clínicas da Faculdade de Medicina de Botucatu;

-determinar os padrões de suscetibilidade antifúngica destes isolados;

-quantificar a produção de biofilme nos mesmos isolados;

-caracterizar polimorfismos de tamanho da região do domínio central do gene *ALS3* em isolados obtidos de hemocultura e fazer sua correlação à produção de biofilme.

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Species distribution and susceptibility profile of *Candida* species in a Brazilian Public Tertiary Hospital

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Abstract

Background: Since opportunistic yeast infections are increasing worldwide, we carried out species identification and antifungal tests in 327 *Candida* isolates obtained from bloodstream infections (102 isolates), urine (85), vulvovaginal secretion (115) and peritoneal fluid (25), from a Brazilian Tertiary Clinical Hospital (UNESP School of Medicine at Botucatu, São Paulo State) from 1998 to 2005.

Results: We observed 153 (46.8%) isolates of *Candida albicans*, 66 (20.2%) of *Candida parapsilosis,* 37 (11.3%) of *Candida tropicalis*, 29 (8.9%) of *Candida glabrata*, 12 (3.7%) of *C. krusei* and 30 (9.2%) of others species. In blood culture, *C. parapsilosis* was the most frequently encountered species (43.1). The resistance to antifungal agents was relatively low, while only five (3.3%) isolates of *C. albicans* were resistant to fluconazole, twenty one (72.4%) isolates of *C. glabrata,* six (50%) of *C. krusei,* seven (18.9%) of *C. tropicalis,* and two (3%) of *C. parapsilosis* were resistant to this drug. Resistance to itraconazole was found in eleven (7.2%) isolates of *C. albicans,* twenty six (89.7%) of *C. glabrata,* eleven (91.7%) of *C. krusei,* three (4.6%) of *C. parapsilosis,* and ten *(*27%) of *C. tropicalis*. Ketoconazole exhibited great activity against all isolates, with only two (1.3%) isolates of *C. albicans* being resistant. Eight amphotericin B resistant isolates were non-*albicans Candida* species, with six (9.1 %) being *C. parapsilosis* and two (10.5%) *Candida* spp..

Conclusions: Both the species distribution and antifungal susceptibility observed herein appear to reflect the real incidence of these opportunistic yeasts in the tertiary hospitals of Latin American countries, in which *C. parapsilosis* is the species most frequently encountered in bloodstream infections, while *C. albicans* continues to occur in an important

number of cases, although with a low number of resistant isolates. *C. glabrata* is emerging with a high number of resistant isolates, as also observed in developed countries.

Background

Infections caused by opportunistic pathogens, such as yeasts, are becoming important causes of morbidity and mortality in many patients, because of alterations in the immune system and invasive hospital procedures (White *et al*., 1998; Yang and Lo, 2001). Candidemia is commonly associated with high morbidity and mortality resulting in significant increases in the length of patients' hospitalization and in healthcare costs (Colombo *et al*., 2006; Girão *et al*., 2008).

In the past two decades, nosocomial yeast infections have increased significantly worldwide (Almirante *et al*., 2005; Asmundsdottir *et al*., 2002; Wisplinghoff *et al*., 2004). In the United States, yeast infection ranks as the 4th most common cause of nosocomial bloodstream infection (BSIs) (Wisplinghoff *et al*., 2004). In Brazil, *C. albicans* was the most common species isolated, followed by *C. tropicalis* and *C. parapsilosis*. In addition, the study revealed that antifungal resistance was rare (Colombo *et al*., 2006). There has been an important shift in the species causing nosocomial candidemia, with the emergence of non-*albicans* species particularly more resistant to antifungal drugs (Abi-Said *et al*., 1997; Clark & Hajjed, 2002, Trick *et al*., 2002; Snydman, 2003; Sobel, 2006).

Several antifungal drugs have been used to control such infections, and as a result of broad prophylactic usages and long-term treatments with those drugs, the prevalence of drug resistance has become an important issue in various yeast infections, thus profoundly affecting human health (Marr *et al*., 2001; Pfaller *et al*., 2003; Yang *et al*., 2004). *Candida* species have various degrees of susceptibility to the frequently used antifungal drugs. *Candida krusei* is intrinsically resistant to fluconazole, and *Candida glabrata* is less susceptible or has higher MICs than other *Candida* species (Akova *et al*., 1991; Orozco *et al*., 1998; Yang *et al*., 2004). In the present work, we present data on species frequency and antifungal susceptibility of *Candida* isolates obtained in a Brazilian public tertiary hospital.

Methods

Origin of the isolates: All the yeast cultures were obtained from patients of the Clinical Hospital of the UNESP School of Medicine, Botucatu, São Paulo State, between 1998 and 2005. The isolates were stored in vial tubes containing Brain Heart Infusion plus 10% glycerol, in a freezer at -80°C. At the moment of the study each isolate was cultured on Sabouraud Dextrose Agar plates at 35ºC.

Species identification: Species identification was based on the colony morphology on Chromogenic Agar (CHROmagar *Candida*, Difco), microscopy features on Corn-meal Agar slide culture, as well as the assimilation and fermentation tests, according to Kurtzman & Fell (1998). Isolates that not fit any recognized taxon were considered *Candida* spp.

Susceptibility tests: Minimal inhibitory concentrations (MIC) of fluconazole (Pfizer, São Paulo, Brazil), itraconazole (Janssen, Beerse, Belgium), ketoconazole (Janssen, Beerse, Belgium) and amphotericin B (Sigma, St. Louis, MO, USA) were determined by broth microdiluition according to the Clinical and Laboratory Standard Institute (CLSI) document guidelines for the susceptibility testing of yeasts (CLSI, M27-A2, 2002). *C. parapsilosis* ATCC 22019 and *C. krusei* 6258 were used for quality control on each test run. For the azole drugs, the MIC was defined as the lowest concentration corresponding to 50% inhibition compared with growth in the drug-free control well (CLSI, M27-A2, 2002; Espinell-Ingroff *et al*., 2005). For amphotericin B, the MIC was considered the lowest concentration showing growth inhibition (CLSI, M27-A2, 2002). For susceptibility to fluconazole, isolates with MIC ≥64 μg/mL were considered resistant, whereas those with MIC ≤8 μg/mL were susceptible. For susceptibility to itraconazole, isolates with MIC ≥1 μg/mL were defined as resistant, whereas those with MIC ≤0.125 μg/mL were susceptible. Isolates with MICs falling between 16 and 32 μg/mL in relation to fluconazole, 0.25–0.5 μg/mL to itraconazole were defined as dose-dependent susceptibility. With regard to ketoconazole, isolates with MIC >16 μg/mL were defined as resistant, whereas those with MIC ≤ 0.03 μg/mL were susceptible while those with MIC between 0.03-16 μg/mL presented dose-dependent susceptibility, according to the E-test® recommendation. For susceptibility to amphotericin B, isolates with MIC ≥2 μg/mL were considered resistant, and those with MIC ≤1 μg/mL were susceptible (Nguyen *et al*., 1998, Yang *et al*., 2008). Where ten or more species were tested, the MIC_{50} and the MIC_{90} were calculated.

Results

Species identification: In a total of 327 yeast cultures, 153 (46.8%) were isolates of *C. albicans*, sixty six (20.2%) *C. parapsilosis*, thirty seven (11.3%) *C. tropicalis*, twenty nine (8.9%) *C. glabrata*, twelve (3.7%) *C. krusei*, nine (2.8%) *C. guilliermondii,* one (0.3%) *C. lusitaniae*, one (0.3%) *C. pelliculosa* and nineteen (5.8%) *Candida* spp.(Table 1). With regard to the clinical materials, while *C. albicans* continued as the most frequent species in urine (34.1%) and in vulvovaginal secretions (80.9%), *C. parapsilosis* was the most frequent in blood (43.1%) and in peritoneal fluid (40.0%). *C. tropicalis*, the third most frequent species, occurred mainly in urine and vulvovaginal secretions (Table 1).

Susceptibility tests: Susceptibility tests for fluconazole, itraconazole, ketoconazole and amphotericin B were performed on 327 isolates of *Candida* species. Table 2 shows the MIC ranges delimiting inhibition of isolates at proportions of 50% and 90%, as well as the percentages of isolates resistant to the four antifungal drugs tested. Overall, the resistance to antifungal agents was relatively low, especially for *C. albicans*. Among the 327 evaluated isolates, forty-one (12.5%) were resistant to fluconazole, fifty-eight (17.7%) to itraconazole, two (0.6%) to ketoconazole and eight (2.4%) to amphotericin B.

Fluconazole exhibited the greatest activity against *C. albicans* with resistance shown by only five (3.3%) isolates. Twenty-one (72.4%) isolates of *C. glabrata,* six (50%) of *C. krusei,* seven (18.9%) of *C. tropicalis,* and only two (3%) of *C. parapsilosis* were resistant to fluconazole. The fluconazole MIC_{50} and MIC_{90} for *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis, C. tropicalis* and *Candida* spp. were 0.5 and 8, 64 and 64, 32 and >64, 2 and 8, 4 and >64, and 2 and 16 μg/mL, respectively.

Resistance to itraconazole was found in eleven (7.2%) isolates of *C. albicans,* twenty-six (89.7%) of *C. glabrata,* eleven (91.7%) of *C. krusei,* three (4.6%) of *C. parapsilosis,* and ten *(*27%) of *C. tropicalis*. The MIC_{50} and MIC_{90} for itraconazole against *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis, C. tropicalis* and *Candida* spp. were 0.03 and 0.12, 2 and >16, 2 and 2, 0.03 and 0.12, 0.12 and >16, and 0.03 and 0.12 μg/mL, respectively.

Ketoconazole exhibited great activity against all isolates, with only two (1.3%) isolates of *C. albicans* resistant. The MIC_{50} and MIC_{90} for ketoconazole against *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis, C. tropicalis* and *Candida* spp. were 0.03 and 1, 2 and 4, 2 and 4, 0.06 and 0.12, 1 and 8, and 0.06 and 0.25 μg/mL, respectively.

The eight amphotericin B-resistant isolates were non-*albicans* *Candida* species, consisted of six (9.1%) *C. parapsilosis* and two (10.5%) *Candida* spp.. The MIC_{50} and MIC_{90} for amphotericin B against all isolates were 1 μg/mL, except for *C. krusei* (0.5μg/mL), *C. parapsilosis* (0.12 and 1 μg/mL) and *Candida* spp (0.5 and 0.25 μg/mL) (Table 2).

All isolates of *C. guilliermondii* (nine), *C. lusitaniae* (one) and *C. pelliculosa* (one) were susceptible to amphotericin B and to the three azoles, except one isolate of *C. guilliermondii* that was resistant to fluconazole and intraconazole.

Discussion

The frequency of infections caused by yeasts, especially *Candida* spp., has increased dramatically worldwide in recent years (Pfaller & Diekema., 2002; Hajjeh *et al*., 2004). Although *C. albicans* remains as the most frequent species, several other *Candida* species are emerging, and in some casuistic cases non-*albicans* ones are the most frequent (Colombo *et al*., 2006; Caggiano, *et al*., 2007; Celebi *et al*., 2007; Shivaprakasha *et al*., 2007; Costa-de-Oliveira *et al*., 2008; González *et al*., 2008; Kersun *et al*., 2008). In the present study, carried out in a Brazilian public tertiary hospital, the frequency of non-*albicans* species represented 53.2% of all *Candida* isolates. *C. parapsilosis* was the most frequent non-*albicans* species recovered, occurring in 20.2% of all isolates, followed by *C. tropicalis* (11.3%) and *C. glabrata* (8.9%). Other studies have also indicated an important occurrence of *C. parapsilosis*, in Brazil, mainly associated with bloodstream infections (Colombo *et al*., 1999; Passos *et al*., 2007) and, in the present case, also found in peritoneal fluid. Ruiz *et al.* (2005) and Medrano *et al.* (2006) showed that *C. parapsilosis* was the species most frequently isolated from bloodstream infections, both in the southeast and northeast regions of Brazil. The real reasons why *C. parapsilosis* occurs more frequently in Latin American countries is not completely understood. *C. parapsilosis* is considered a commensal of human skin since it has been isolated from the hands of health workers (Asbeck *et al*., 2007; Trofa, et al., 2008), which have been identified as the major vectors in the infection acquisition (Trofa *et al*., 2008). Some important virulence factors have been observed in *C. parapsilosis*, such as adherence to host cells, biofilm formation and production of hydrolytic enzymes (Branchini *et al*., 1994; Trofa *et al*., 2008). Furthermore, this species presents selective growth capability in hyperalimentation solutions and an affinity for intravascular devices and prosthetic materials (Clark, et al., 2004; Trofa *et al*., 2008). The lead occurrence of *C. parapsilosis* in the peritoneal fluid in our casuistic case

also comes as no surprise, and this fact has also been observed in different countries in patients receiving peritoneal dialysis (Wang *et al*., 2000; Manzano-Gayoso *et al*., 2003). The adoption of good infection control practices, with adequate asepses of health workers' hands and medical devices, especially in catheters, may substantially minimize infection by *C. parapsilosis*.

While in developed countries *C. glabrata* has been considered the most frequent among non-*albicans* species, in the present study *C. glabrata* was only the fourth most frequent, occurring in blood, urine and vulvovaginal secretions. Under our casuistic, *C. krusei* was isolated only from vulvovaginal secretions, at a frequency similar to those observed in other distant areas, such as in Asia (Chong *et al.* 2007). Invasive infections by *C. krusei* in Brazil appear to be less frequent than in developed countries, as already observed in other local studies (Matsumoto *et al*., 2002; Antunes, *et al*., 2004; Colombo *et al*., 2006). In a worldwide surveillance study, Pfaller *et al*., (2008) indicated that *C. krusei* represents 3.3% of all *Candida* spp. isolated in Europe and North America and 1.7% in Latin America.

C. guilliermondii, considered a normal component of human skin and mucosal flora (Mok and Barreto Silva, 1984), is rarely associated with invasive infections like candidemia and peritonitis (Pasqualotto *et al*., 2006). In the present work, *C. guilliermondii* was isolated from blood, peritoneal fluid and urine. Recent reports have shown cases of candidemia by *C. guilliermondii* in several countries including Brazil (Colombo *et al*., 2006; Caggiano *et al*., 2007; Lee *et al*., 2007; Odds *et al*., 2007; Passos *et al*., 2007; Gonzáles *et al*., 2008).

Other reports have also found few cases of candidemia by *C. lusitaniae* and *C. pelliculosa* in Brazil (Colombo *et al*., 2006; Passos *et al*., 2007; França *et al*., 2008) and other countries (Caggiano *et al*., 2007; Odds *et al*., 2007; Kersun *et al*., 2008).

Paradoxically, the increased attention, monitoring and use of antifungal drugs to treat yeast infections have coincided with both the emergence of non-*albicans* species and an augmented number of resistant strains. Fluconazole has been considered the antifungal of choice for the empirical treatment of suspected infection caused by any species of *Candida.* However, several studies have clearly indicated the necessity of correct species identification, since they may differ substantially in relation to drug susceptibility (Mensa *et al*., 2008). In the present study, most of the isolates were susceptible to the antifungal drugs tested. Fluconazole and itracoanazole resistance were observed in few isolates of *C. albicans*, *C. parapsilosis*, *C. tropicalis* and *C. guilliermondii*. Similar to other studies, the percentage of isolates resistant to fluconazole was smaller than to itraconazole (Dóczi *et al*., 2002; Cheng *et al.,* 2004; Laverdiere *et al*., 2007; González *et al*., 2008). As expected, *C. krusei* and *C. glabrata* isolates

displayed high resistance to fluconazole and itraconazole, since they are considered intrinsically resistant to these drugs (Mensa et al., 2008). Bloodstream infections by *C. krusei* and C. glabrata are associated with high mortality, because of their poor response to conventional therapy (Costa-de-Oliveira *et al*., 2008; González *et al*., 2008). Although the incidences of these two species under our case casuistic have been relatively low, it is important to be vigilant in monitoring these agents, mainly in the patients receiving antifungal azole drugs.

The high susceptibility to ketoconazole shown by all isolates in our casuistic cases might also explained by the fact that this drug has not been prescribed in our hospital in recent years (data provided by the local control infection committee – Mondelli, personal communication)

In our study, resistance to amphotericin B was observed only in non-*albicans* species, mainly in *C. parapsilosis*, the most frequent non-*albicans* species. Although the majority of pertinent studies report a lack of amphotericin B-resistant isolates (Colombo *et al*., 2006; Odds *et al*., 2007; Arendrup *et al*., 2008; Chen *et al*., 2008; Kersun *et al*., 2008), another Brazilian study also found resistance by *C. parapsilosis* to amphotericin B (Passos *et al*., 2007). Amphotericin B is particularly used in several Brazilian public tertiary hospitals in the treatment of systemic mycosis, in which the patients remain hospitalized for long periods of treatments, as in our hospital for paracoccidiodomycosis (Dillon *et al.*, 1986). The possible effect of this drug against selectively resistant Candida species should not be excluded and merits proper evaluation.

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Table 1 – Distribution frequency of Candida species obtained from different clinical materials at the Brazilian Tertiary Hospital (Clinical Hospital of the UNESP School of Medicine, Botucatu, São Paulo State), from 1998 to 2005.

Species identification	Blood % (n)	Urine % (n)	Vulvov. % (n)	Per. fluid % (n)	Total % (n)
C. albicans	22.5 (23)	34.1 (29)	80.9 (93)	32.0 (8)	46.8 (153)
All non-*C. albicans* species	77.5 (79)	65.9 (56)	19.1 (22)	68.0 (17)	53.2 (174)
C. glabrata	4.9 (5)	23.5 (20)	3.6 (4)	-	8.9 (29)
C. guilliermondii	5.9 (6)	1.2 (1)	-	8.0 (2)	2.8 (9)
C. lusitaniae	1.0 (1)	-	-	-	0.3 (1)
C. parapsilosis	43.1 (44)	8.2 (7)	4.5 (5)	40.0 (10)	20.2 (66)
C. pelliculosa	1.0 (1)	-	-	-	0.3 (1)
C. tropicalis	2.9 (3)	32.9 (28)	0.9 (1)	20.0 (5)	11.3 (37)
C. krusei	-	-	10.4 (12)	-	3.7 (12)
Candida spp.	18.6 (19)	-	-	-	5.8 (19)
Total	102	85	115	25	327

Table 2. *In vitro* activity of antifungal agents against *Candida* spp. isolates from different clinical materials at the Brazilian Tertiary Hospital (Clinical Hospital of the UNESP School of Medicine, Botucatu, São Paulo State), from 1998 to 2005.

Isolates (n)	Drugs	MIC (µg/ml)			
		Range	MIC_{50}	MIC_{90}	R n(%)
C. albicans (153)	FLU	0.03->64	0.5	8	5 (3.3)
	ITR	0.03->16	0.03	0.12	11 (7.2)
	KET	0.03->16	0.03	1	2 (1.3)
	AMB	0.06-1	1	1	0
C. glabrata (29)	FLU	2->64	64	64	21 (72.4)
	ITR	0.06->16	2	>16	26 (89.7)
	KET	0.03-16	2	4	0
	AMB	0.5-1	1	1	0
C. krusei (12)	FLU	32->64	32	>64	6 (50)
	ITR	0.5-4	2	2	11 (91.7)
	KET	1-4	2	4	0
	AMB	0.5-1	0.5	0.5	0
C. parapsilosis (66)	FLU	0.12-64	2	8	2 (3)
	ITR	0.03->16	0.03	0.12	3 (4.6)
	KET	0.03-4	0.06	0.12	0
	AMB	0.25-2	1	1	6 (9.1)
C. tropicalis (37)	FLU	0.12->64	4	>64	7 (18,9)
	ITR	0.03->16	0.12	>16	10 (27)
	KET	3-16	0.12	8	0
	AMB	0.5-1	1	1	0
Candida spp. (19)	FLU	0.25-16	2	16	0
	ITR	0.03-0.25	0.03	0.12	0
	KET	0.03-0.25	0.06	0.25	0
	AMB	0.5-2	0.5	2	2 (10.5)

FLU: fluconazole, ITR: itraconazole, KET: ketoconazole, AMB: amphotericin B

Biofilm production and *ALS3* central domain polymorphism in *Candida* species from different clinical sources.

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Abstract

Biofilm production was quantified in 327 *Candida* species isolated from the bloodstream, urine, vaginal secretion and peritoneal fluid obtained from a Brazilian tertiary public hospital (Clinical Hospital of UNESP, School of Medicine, Botucatu, São Paulo State). Of the 198 total biofilm-positive isolates, 72 and 126 were considered low and high biofilm producers, respectively. Biofilm production by *C. albicans* isolates was significantly lower than that by non-*albicans* isolates, and among the biofilm-positive isolates, the non-*albicans* isolates were classified mainly as high-biofilm producers, while *C. albicans* isolates presented low biofilm production. Such production was most frequently observed in *C. tropicalis* isolates, followed by *C. parapsilosis*, *C. glabrata*, and *C. albicans*. Among biofilm-positive isolates, the highest production intensity was observed in *C. tropicalis* isolates. Biofilm production was more frequent among bloodstream isolates than any other clinical source. In urine, the isolates displayed a peculiar distribution by presenting two distinct peaks, one containing biofilm-negative isolates and the other containing isolates with intense biofilm production, which must have originated from systemic infection, reflecting the close association between biofilm production and virulence. The *ALS3* central domain polymorphism was also evaluated in the bloodstream isolates, including *C. albicans* and non-*albicans* species, both biofilm-positive and -negative producers. The numbers of tandem-repeat copies were divergent among the isolates, which presented homozygosity and heterozygosity, and varied from 7 to 14 copies. The numbers of tandem-repeat copies per allele appear to be unassociated with biofilm production while the respective mean numbers of copies, in biofilm-negative and biofilm-positive isolates, were 11.6 ± 1.4 and 10.7 ± 1.7.

Introduction

Candida species are human commensals that can cause both superficial and systemic disease, mainly in immunocompromised individuals (Kojic & Darouiche, 2004). These organisms have emerged as important agents of opportunistic infections worldwide, primarily in immunocompromised persons (Richardson, 2005; Aperis *et al*.,2006). Although *Candida albicans* is considered the most common fungal pathogen, an increased number of non-*albicans Candida* species infections have been described (Redding, 2001; Krcmery & Barnes., 2002). *Candida* species can colonize human tissues and medical devices, such as central venous catheters, prosthetic heart valves and other devices, resulting in biofilm formation and biofilm-related infections (Douglas, 2003; Andes et al., 2004; Kojic & Darouiche, 2004). Biofilms are microbial communities of surface-attached cells embedded in a self-produced extracellular polymeric matrix (Donlan and Costerton, 2002). They can cause significant problems in many areas, mainly in medical settings as persistent and recurrent device-related infections (Flemming, 2002; Fux et al., 2005; Kumar & Anand, 1998). Biofilms are more resistant than planktonic cells, and in most cases, antifungal therapy is not effective (Douglas, 2003; Kumamoto, 2002).

The implanted device that is most commonly infected is the central venous catheter, which is used to administer fluids and nutrients as well as cytotoxic drugs. Infections can arise at any time during the use of this type of catheter. (Goldmann &. Pier, 1993; Douglas, 2002). However, endogenous infections also can occur if *Candida* spp. colonizing the gastrointestinal tract as commensals are able to penetrate the intestinal mucosa and invade the bloodstream, after which circulating yeasts can contact the catheter tip internally (Goldmann &. Pier, 1993). But non-device-related infections can also involve biofilms, for example in *Candida* endocarditis and *Candida* vaginitis (Donlan & Costerton, 2002; Douglas, 2002).

Biofilm formation in *Candida* spp. is a complex process involving multiple regulatory mechanisms (Nobile & Mitchell, 2006) and once established, *Candida* biofilms serve as a persistent reservoir of infection and, in addition, offer greater resistance to antifungal agents compared to planktonic phase yeasts (Chandra et al., 2001a,b; Samaranayake, et al., 2005; Parahitiyawa et al., 2006).

Several different biofilm *in-vitro* systems have been developed to study and quantify biofilm, including yeast development on intravascular catheter discs, acrylic discs, cylindrical cellulose filters, microtiter plates and others (Douglas, 2002; McLean et al., 2004).

Crystal violet staining, a basic dye that binds to negatively charged surface molecules and polysaccharides in the extracellular matrix, is commonly utilized to quantify biofilms formed by a broad range of microorganisms, including yeasts (Christensen et al., 1985; Jin et al., 2003; Li et al., 2003; Peeters et al., 2008).

Besides phenotypical assays to study biofilm formation in *Candida* species, some genotypical techniques have been used to characterize this phenomenon. The *Als* (agglutinin-like sequence) proteins have long been considered excellent candidates for biofilm adhesions (Green et al., 2004, Hoyer et al., 1998; Zhao et al., 2006). Eight *ALS* genes (*ALS1* to *ALS7* and *ALS9*) encode large, cell surface glycoproteins, some of which promote adhesion to host surfaces (Fu, et al., 2002; Gaur et al., 1997; Hoyer, 2001, Zhao et al., 2003; Zhao et al., 2004). *ALS* genes have three general domains: the 5'domain, conserved, with approximately 1300-pb; followed by a central domain consisting entirely of tandem repeats of a 108-pb sequence, that are somewhat variable, and the 3' domain, which is least conserved in length and sequence (Hoyer, 2001; Hoyer *et al*., 2008). Although they share a similar three-domain structure, sequence differences among the *Als* proteins can be large, suggesting that the proteins may present different functions (Hoyer, 2001). Much of the allelic variation in *ALS* genes occurs within the tandem repeat domain and is manifested as differing numbers of the 108-pb tandem repeats in *ALS* alleles. It has recently been suggested that *ALS3* is one of the most important genes associated with *C. albicans* biofilm production (Zhao et al., 2006, Hoyer, 2001; Hoyer *et al*., 1998).

The aim of the present study was to quantify biofilm production in a collection of different *Candida* species, isolated from the bloodstream and other different clinical sources, as well as to detect the polymorphisms in the *ALS3* tandem repeat domain and their possible correlation with the biofilm production profiles.

Materials and Methods

Microorganisms: A total of 327 *Candida* species isolates recovered from clinical specimens as part of routine diagnostic procedures, stored in vial tubes containing Brain Heart Infusion plus 10% glycerol, frozen at -80°C, were re-cultured and tested for biofilm production. The isolates were obtained from patients from the Clinical Hospital of UNESP (State University of Sao Paulo) School of Medicine, Botucatu, São Paulo State (HC/UNESP), between 1998 and 2005. The isolates were obtained from the bloodstream (102), urine (85),

vulvovaginal secretion (115) and peritoneal fluid (25). The *Candida* species studied included 153 *C. albicans,* 66 *C. parapsilosis*, , 37 *C. tropicalis*, 29 *C. glabrata*, 12 *C. krusei*, 9 *C. guilliermondii*, 1 *C. pelliculosa*, 1 *C. lusitaniae* and 19 *Candida* spp. (species belonging to the genus *Candida* that did not fit any recognized taxon). The identification of *Candida* species was conducted by chlamydospore formation, sugar assimilation and fermentation patterns as well as chromogenic agar (CHROMagar *Candida*, Difco). The species distribution in different materials is summarized in Table 1.

Biofilm formation assay: Tests for biofilm quantification were performed according to Li *et al*. (2003) and Jin *et al*. (2003), with few modifications. Growth conditions: Briefly, to prepare inoculum, all isolates were first streaked onto Yeast-extract peptone dextrose agar (YEPD) and incubated at 37 °C for 48 h. For each isolate, a large loop of actively growing cells was transferred to sterile Yeast Nitrogen Base (YNB) broth (Difco) containing 0.9 % D-glucose. After incubation at 37 °C for 24h, the yeast cells were centrifuged and washed twice with 0.5 mL PBS (0.14 M NaCl, 2.7 mM KCl, 8.5 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) by vortexing and centrifuging at 5000g for 5 min. The washed cells were then re-suspended in 1mL YNB broth and the concentration was adjusted to 10^7 cells/mL, according to 0.5 McFarland scale. Biofilm formation: For each isolate, 100 µL of the suspension was inoculated into individual wells of polystyrene 96-well plates (TPP). Four repetitions were performed for each isolate. YNB broth containing no inoculum was used as negative control. The plates were incubated at 37 °C for 90 min (adhesion period). Supernatant including planktonic cells and liquid medium was then discarded and wells were gently washed twice with PBS to eliminate any non-adherent cells. For biofilm growth, 100 µL of fresh YNB broth was then added to each well. The plates were incubated at 37 °C for 48h. After biofilm formation and growth, planktonic cells were discarded through three rounds of washing with 200 µL sterile PBS buffer, and the plates dried at room temperature for 45min. For staining with Crystal Violet (CV), 150µL of 0.4 % CV, diluted in water, was added to each well, and after 45 min at room temperature, all the supernatant was discarded before adding 150 µL of 95 % ethanol and maintained for 45 min, to dissolve and/or elute the dye from the biofilm cells. Next, 100 µL of each well was transferred to a new 96-well microplate and the absorbance determined using a microplate reader at 540nm filter (MultisKan EX, Labsystems). The wells containing only YNB broth with no yeasts were used as negative controls. The absorbance values were converted into transmittance percentages (%T). The %T values for each test was subtracted from the %T for the reagent blank to obtain a measure of light blocked when passing through

the wells ($\%T_{bloc}$), and the biofilm production scored as either negative ($\%T_{bloc}$ <10), positive 1+ ($\%T_{bloc}$ 10 to 20), positive 2+ ($\%T_{bloc}$ 20 to 35), positive 3+ ($\%T_{bloc}$ 35 to 50) or positive 4+ ($\%T_{bloc}$ ≥50), and the positives further categorized as low-biofilm (1+) or high-biofilm producers (2+, 3+, or 4+), according to Tumbarello *et al.* 2007.

***ALS3* characterization:** The gene *ALS3* was studied in all *C. albicans* isolates, biofilm producers and non-producers, obtained from bloodstream culture (n=23), as well as in 7 isolates of *C. parapsilosis*, 3 of *C. tropicalis*, 1 of *C. guilliermondii* 1 of *C. glabrata* and 4 of *Candida* spp., all also isolated from the bloodstream. DNA extraction: The DNA was extracted according to McCoullogh *et al.* (2000) with few modifications. *Yeasts* were grown for 24h on Sabouraud dextose agar at 37°C. Colonies were suspended in 1 mL of 1 M sorbitol and 125 mM of EDTA. The suspension was centrifuged (10 min at 13000 *g*), the supernatant was discarded, and the pellet was resuspended in 0.5 mL of lysing solution (1 M Tris-HCl, pH 8.0, with 250 mM of EDTA and 5% SDS) plus 10 µL of proteinase K (Invitrogen) and incubated for 1 h at 65°C. Next, 500 µL 5 M potassium acetate was added, incubated on ice for 2 h and then centrifuged (10 min at 13,000 *g*). The supernatant was transferred to an Eppendorf tube containing 1 mL of 100% ethanol. This was mixed by inversion and centrifuged (10 min at 13,000 *g* and 4°C). The supernatant was discarded, the pellet was washed with 500 µL of cooled 70% ethanol and centrifuged (10 min at 13,000 *g* and 4°C). The supernatant was discarded and the pellet was resuspended in 0.5 mL of sterilized MiliQ water. PCR conditions: The size of the tandem repeat domain in each *ALS* allele was determined by PCR using two independent primer pairs as described by Oh *et al.* (2005). Two primer pairs provided an additional control for the accuracy of the results. Each primer pair contained one that annealed 5' and another 3' of the tandem repeat domain. The first primer pair was *ALS3*GenoF (5'-ACC TTA CCA TTC GAT CCT AAC C-3') and *ALS3*GenoR (5'-GAT GGG GAT TGT GAA GTG G-3'). The second primer pair was *ALS3*GenoF2 (5'-CCA CAA CAC ATA CTA ATC CAA CTG A-3') and *ALS3*GenoR2 (5'-TGT AGA CCA CAA AGT TGT ATG GTT G-3'). Taq polymerase (Invitrogen) was used with both primer pairs. Reactions with the first primer pair (*ALS3*GenoF and *ALS3*GenoR) used Invitrogen Taq polymerase buffer with 1 mM $MgCl_2$. Reactions were heated for 5 min at 94 °C followed by 35 cycles of 94 °C (30 s), 57 °C (30 s) and 72 °C (3 min). A final 72 °C (7 min) extension completed the reaction. The second primer pair (*ALS3*GenoF2 and *ALS3*GenoR2) was used under similar conditions except for a difference in buffer (10 mM Tris/HCl, pH 8·8, 25 mM KCl, 1·5 mM $MgCl_2$) and annealing temperature (65 °C). When the first pair of primers provides no clear amplification,

the second pair was used. PCR products were separated on 0.7 % agarose (TBE) gels stained with ethidium bromide. The gels were analyzed in the equipment AlphaImager^{R} EC that captures the digital image whereas the sizes of the amplicons were determined by the software AlphaEase^{R} FC. To estimate, for each isolate, the numbers of motifs present in the tandem repeats in the *ALS3* gene, the primers positions were aligned with the deposited genomic sequences of strain SC5314 DNA (GenBank Accession No. AY223552.1), large and small alleles that present twelve and nine motifs, using Mega software. The numbers of motifs for each isolate evaluated were then calculated, considering 108 bp the mean size for each motif. The amplicon of one homozygous isolate was also purified by the commercial kit GFX PCR DNA and Gel Band (GE, Healthcare), sequenced using the DYEnamic ET Dye Terminator Kit (with Thermo Sequenase™ II DNA Polimerase) in a MegaBACE 1000 DNA Analysis System, and the chromatogram visualized by the Chromas program. The consensus sequence was sent to blastn for comparison with the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST).

Statistical analysis. Chi-square analysis was used to compare biofilm positivity among different *Candida* species or among isolates recovered from different sources. The Kolmogorov-Smirnov test was used to compare the numbers of tandem repeat copies between biofilm-positive and biofilm-negative isolates. Differences between groups were considered to be significant for $P < 0.05$.

Results

Biofilm production: Biofilm production for 327 isolates of *Candida* species obtained from different sources is summarized in Figure 1 and Table 2. A total of 198 of 327 (60.6%) *Candida* species isolates were biofilm-positive. Of these, 72 and 126 isolates were low and high biofilm producers, respectively. Biofilm production by *C. albicans* isolates (43.1%, 66 of 153) was significantly lower than that by non-*albicans* isolates (75.8%, 132 of 174; $P < 0.001$), and among the biofilm-positive isolates, the non-*albicans* isolates were classified mainly as high-biofilm producers, with *C. albicans* isolates defined as low-biofilm producers ($P<0.001$).

Considering the species separately, biofilm production was most frequently observed in *C. tropicalis* isolates (94.6%, 35 of 37, $P < 0.001$), followed by *C. parapsilosis* (80.3%, 53 of 66), *C. glabrata* (44.8%, 13 of 29), and *C. albicans* (43.1%, 66 of 153). Among biofilm-

positive isolates, the highest biofilm production intensity ($\%T_{bloc} > 35$) was observed in *C. tropicalis* isolates (81.1%, 30 of 37, $P < 0.001$).

In relation to the sources, biofilm production was positive in 79.4% (81 of 102) of the isolates obtained from the bloodstream, 63.5% (54 of 85) from urine, 37.4% (43 of 115) from vaginal secretion and 80% (20 of 25) from peritoneal fluid. Biofilm production among bloodstream isolates was more frequent than any other source ($P < 0.001$), except peritoneal fluid isolates, which are also prolific biofilm producers. Biofilm production was most frequent in non-*albicans* isolates ($P < 0.001$) in all sources, also with the exception of peritoneal fluid isolates ($P > 0.05$). In urine, the isolates displayed a peculiar distribution by presenting two distinct peaks, one containing biofilm-negative isolates (36.5%) and the other containing isolates (37.6%) with intense biofilm production ($\%T_{bloc} > 35$).

***ALS3* characterization:** Polymerase chain reaction was performed on a total of 39 isolates, including 23 *C. albicans* and 16 non- *albicans* species. In 19 of 23 *C. albicans* isolates, the expected fragments of the *ALS3* gene were amplified by using the first or the second pair of primers (Figure 2). In the 16 non-*albicans* evaluated, the PCR products were not amplified with both pairs of primers.

The consensus sequence of a fragment containing approximately 700 bp in the Blast analysis presented 93% identity with the *C. albicans* large allele *ALS3* gene (GenBank Accession No. AY223552.1; *E* value 0.0).

The numbers of copies of the central tandem repeat domain were divergent among the isolates, which presented 74% homozygosity and 26% heterozygosity. Alleles in the examined isolates encoded between 7 and 14 copies of tandemly repeated 108-bp sequence (Table 3). The mean numbers of tandem repeat copies per allele in biofilm-negative and biofilm-positive isolates were 11.6 ± 1.4 and 10.7 ± 1.7, respectively. The difference in the number of tandem-repeat copies between biofilm-positive and biofilm-negative isolates was not significant ($P > 0.05$).

Discussion

Biofilm production has been considered an important virulence factor among *Candida* species (Ramage et al., 2005). A broad range of methods has been employed to evaluate this phenomenon in yeasts, mainly in *Candida* species (Peeters et al., 2008). In the present study,

the biofilm production was quantified in 327 clinical isolates of *Candida* species by the crystal violet assay, a widely used method to quantify biofilm production in several microorganisms, including yeasts (Christensen et al. 1985; Stepanovic et al., 2000; Li et al., 2003; Peeters et al., 2008). Although this method is not able to differentiate between living and dead cells (Pitts et al., 2003; Peeters et al., 2008), the results obtained by the crystal violet assay have correlated highly with other assays that differentiate between living and dead cells, such as the 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) assay (Jin et al., 2003; Peeters et al., 2008). The data obtained herein clearly confirm that different *Candida* species have different abilities to produce biofilm *in-vitro* and that non-*albicans* species were the highest biofilm producers (Shin *et al.,* 2002; Tumbarello *et al.*, 2007). Among biofilm producers, *C. tropicalis* showed the highest intensity of biofilm production. In other studies involving similar numbers of *Candida* species isolates, *C. tropicalis* was the species with the highest percentages of biofilm positivity, while *C. albicans*, ranked third or forth among the biofilm-positive isolates (Shin et al., 2002; Tumbarello et al., 2007).

We also examined the associations between the source and the biofilm positivity of isolates. Isolates obtained from the bloodstream and peritoneal fluid typically are prolific biofilm producers. Considering that isolates from invasive infections produce more biofilm than those from non-invasive infections (Kuhn et al., 2002), we suggest that some isolates obtained from urine presenting intense biofilm production may have originated from systemic candidiasis and not from urinary tract infections.

Although important advances have been achieved in outlining the genetic basis of biofilm production, the subject is far from being completely understood. After the *C. albicans* genome was sequenced (Jones et al., 2004), the biofilm production by this yeast was better comprehended, although biofilm formation in non-*albicans* species remains poorly understood (Weber et al., 2008). Several genes are involved in the biofilm formation by *Candida* spp. (López-Ribot, 2005; Ramage *et al*., 2005; Nobile & Mitchell 2006). It was demonstrated that the expression of several *ALS* genes is upregulated during biofilm formation; furthermore, the *Als* proteins have long been considered excellent candidates for biofilm adhesins (Green et al., 2004; Blakenship & Mitchell, 2006). The present study analyzed the association between biofilm production and polymorphisms in the *ALS3* central domain. Results from previous studies already showed that *ALS3* gene expression is altered in *C. albicans* sessile cells compared to planktonic cells (García-Sánchez et al., 2004; Nailis et al., 2006; Chandra et al., 2001a,b). Maximal *ALS3* expression is associated with formation of germ tubes and hyphae

(Hoyer *et al.*, 1998) and a recent work demonstrates an overexpression of *ALS3* in the initial stages of biofilm formation (Nailis et al., 2009). In our results, the association between the number of tandem-repeat copies per allele and biofilm production was not significant. However, biofilm-negative isolates showed the majority of alleles with 13 tandem-repeat copies, while the majority of alleles in biofilm-positive isolates showed 10 or 11 copies. The fact that both primer pairs tested herein had been previously designed to study *C. albicans* certainly explains why none of the studied non-*albicans* species produced PCR products, due to the different sequences at the annealing sites, between *C. albicans* and non-*albicans* species. The absence of amplification in four *C. albicans* isolates suggests the presence of sequence polymorphisms at the priming sites or even the absence of the *ALS3* locus in these isolates. Evidence of the spontaneous deletion in other genes of the same family, such as *ALS5* and *ALS6* genes, was already observed by Hoyer & Hetch (2001) and Zhao et al. (2007).

In conclusion, we showed that *Candida* non-*albicans* species were more prolific biofilm producers than *C. albicans* and that the source of isolates may influence the biofilm production, in which more invasive isolates show greater biofilm productivity. The polymorphism of the *ALS3* central domain in *C. albicans,* detected by the different numbers of tandem-repeat copies, appears not to be directly related to biofilm production.

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Table 1: Distribution of *Candida* species obtained from different sources in a Brazilian tertiary public hospital (HC/UNESP), 1998-2005.

Species identification	Blood % (n)	Urine % (n)	Vaginal [a] % (n)	Peritoneal [b] % (n)	Total % (n)
C. albicans	22.5 (23)	34.1 (29)	80.9 (93)	32.0 (8)	46.8 (153)
All non-*C. albicans* species	77.5 (79)	65.9 (56)	19.1 (22)	68.0 (17)	53.2 (174)
C. glabrata	4.9 (5)	23.5 (20)	3.6 (4)	-	8.9 (29)
C. guilliermondii	5.9 (6)	1.2 (1)	-	8.0 (2)	2.8 (9)
C. lusitaniae	1.0 (1)	-	-	-	0.3 (1)
C. parapsilosis	43.1 (44)	8.2 (7)	4.5 (5)	40.0 (10)	20.2 (66)
C. pelliculosa	1.0 (1)	-	-	-	0.3 (1)
C. tropicalis	2.9 (3)	32.9 (28)	0.9 (1)	20.0 (5)	11.3 (37)
C. krusei	-	-	10.4 (12)	-	3.7 (12)
Candida spp.	18.6 (19)	-	-	-	5.8 (19)
Total	102	85	115	25	327

[a] Vaginal secretion; [b]Peritoneal fluid

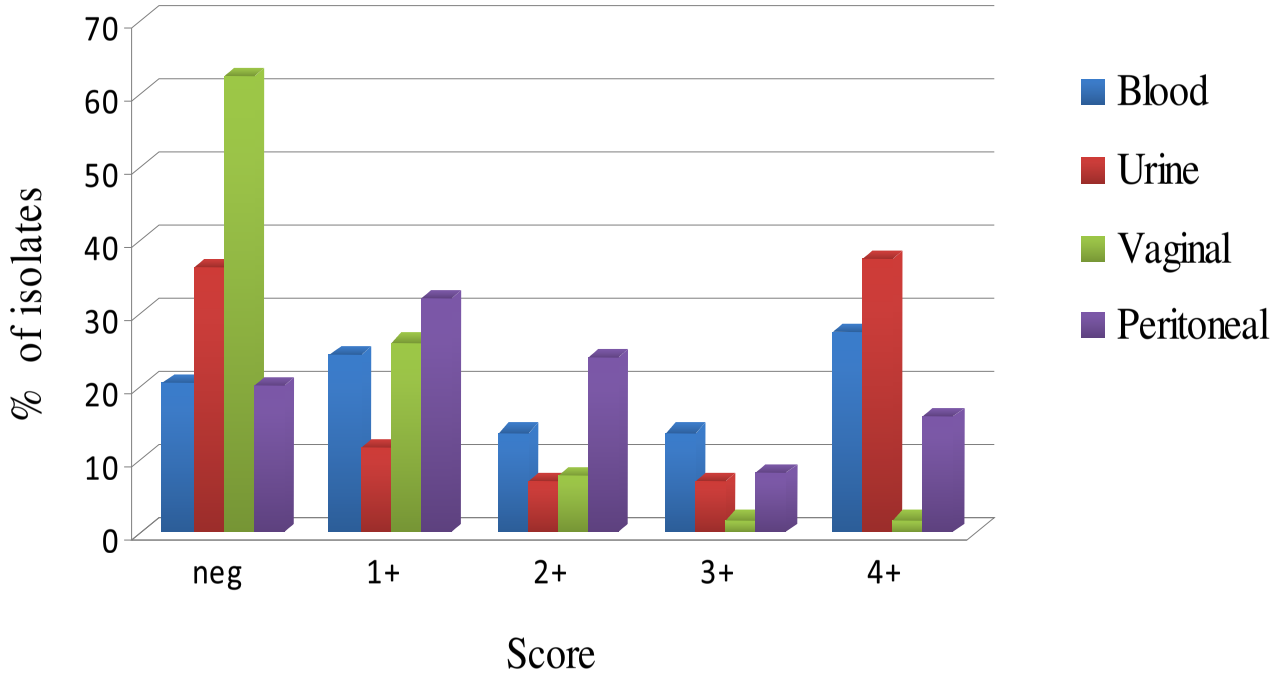


Figure 1. Frequency distribution of *Candida* species in relation to the clinical sources and biofilm production scores, categorized according to Tumbarello et al., 2007.

Table 2: Comparison of biofilm production by *Candida* species isolates from blood, urine, vaginal secretion and peritoneal fluid obtained in a Brazilian tertiary public hospital (HC/UNESP), 1998-2005.

Candida species	No. of biofilm positive/no.total (%)				
	Total	Blood	Urine	Vulvov	Perit
C. albicans	66/153 (43.1)	13/23 (56.5)	12/29 (41.4)	34/93 (36.6)	7/8 (87.5)
All non-*albicans* species	132/174 (75.9) [a]	68/79 (86.1) [a]	42/53 (79.2) [a]	9/22 (40.9) [a]	13/17 (76.5)
C. parapsilosis	53/66 (80.3)	40/44 (90.9)	5/7 (71.4)	1/5 (20.0)	7/10 (70.0)
C. tropicalis	35/37 (94.6)	3/3 (100.0)	28/28 (100.0)	0/1 (0.0)	4/5 (80.0)
C. glabrata	13/29 (44.8)	3/5 (60.0)	10/19 (52.6)	1/4 (25.0)	-
Other *Candida* species	31/42 (73.8)	24/27 (88.9)	-	7/12 (58.3)	2/2 (100.0)
Total	198/327 (60.6)	81/102 (79.4)	54/85 (63.5) [b]	43/115 (37.4) [c, d]	20/25 (80.0)

[a] $P < 0.001$, *C. albicans* versus *non-albicans*
[b] $P < 0.02$, blood versus urine
[c] $P < 0.001$, blood versus vaginal secretion
[d] $P < 0.001$, peritoneal fluid versus vaginal secretion

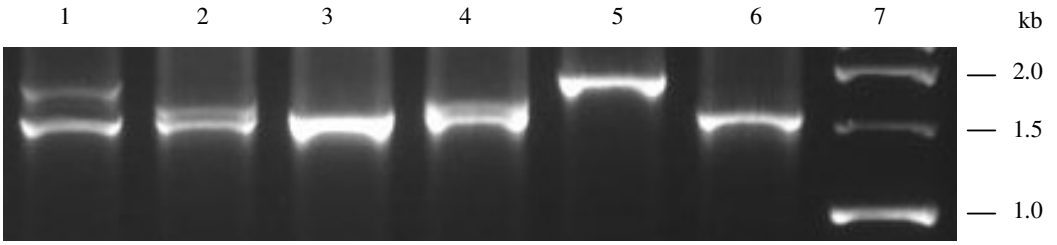


Figure 2. Ethidium bromide-stained agarose gel highlighting amplification of PCR products from the tandem repeat region of *ALS3* gene of 6 *C. albicans* isolates with *ALS3*GenoF and *ALS3*genoR primers.1- alleles with 10 and 13 motifs; 2- alleles with 10 and 11 motifs; 3- alleles with 10 motifs each; 4- alleles with 11 motifs each; 5- alleles with 14 motifs each; 6- alleles with 11 motifs each; 7- Ladder 1kb (Promega).

Table 3: Distribution of allele per *ALS3* gene tandem-repeat copies in *C. albicans* isolates from the bloodstream obtained in a Brazilian tertiary public hospital (HC/UNESP), 1998-2005.

Biofilm production (n)	Percent (n) alleles in each tandem repeat copy group						Mean no of repeat copies/allele[a]
	7	10	11	12	13	14	
Biofilm negative (16)	0 (0)	31.3 (5)	25.0 (4)	0 (0)	43.8 (7)	0 (0)	11.6 ± 1.4
Biofilm positive (22)	9.1 (2)	36.4 (8)	36.4 (8)	4.5 (1)	4.5 (1)	9.1 (2)	10.7 ± 1.7

[a]Mean number of repeat copies per allele ± standard deviation. *P* value not significant.

CONSIDERAÇÕES FINAIS

Infecções noscomiais por leveduras constituem em sério problema de saúde pública, com altos custos econômicos e sociais, e com forte evidência que tenderá a aumentar nos próximos anos. Já está bem estabelecido que diferentes espécies, além de *C. albicans,* são os agentes envolvidos neste processo. Tendo em vista que diferentes espécies apresentam diferentes perfis epidemiológicos e de resposta aos tratamentos, é fundamental que se faça estudos adequados de identificação e caracterização destes agentes, principalmente nos hospitais terciários que atendem os pacientes particularmente mais susceptíveis.

A produção de biofilme é um fenômeno importante na patogenicidade de vários microrganismos, e pode acarretar em várias implicações, tanto em ambientes hospitalares, contaminando próteses, cateteres e equipamentos médicos, como industriais, aderidos a equipamentos para processamento de água e alimentos. Atualmente sabe-se que a produção de biofilme, tanto fúngico quanto bacteriano, compreende processos controlados geneticamente. No entanto, enquanto os estudos genéticos e moleculares da produção de biofilme em *C. albicans* avançaram significativamente, o mesmo ainda não ocorreu em outras espécies de *Candida,* como por exemplo, *C. parapsilosis* e *C. tropicalis,* notáveis pela produção de biofilme. Fundamental, portanto, aprofundar estes estudos de forma a proporcionar um melhor conhecimento e entendimento das peculiaridades de cada espécie, bem como características comuns entre as mesmas, que podem gerar métodos mais eficientes de controle.

Um melhor entendimento sobre o processo de formação e desenvolvimento do biofilme associados a estudos mais aprofundados dos genes envolvidos podem proporcionar importantes avanços terapêuticos e técnicas para minimizar ou até mesmo impedir infecções decorrentes da formação de biofilme.

CONCLUSÕES

1. *C. albicans* continua sendo a espécies mais freqüente em infecções fúngicas no Hospital das Clínicas da faculdade de Medicina de Botucatu-UNESP, embora, a proporção de espécies não-*albicans* é maior que de *C. albicans.*

2. A grande maioria de *C. albicans* foi isoladas de infecções não invasivas, e *C. parapsilosis* de infecções invasivas com diferença significativa em relação a *C. albicans.*

3. De modo geral, as drogas antifúngicas utilizadas exerceram boa atividade sobre as amostras estudadas.

4. A produção de biofilme foi mais positiva entre isolados de espécies não-*albicans* em relação à *C. albicans*.

5. Isolados de infecções invasivas apresentaram maior positividade para a produção de biofilme.