UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

ASPECTOS MICROBIOLÓGICOS E AMBIENTAIS DE CANDIDEMIAS EM HOSPITAL TERCIÁRIO (HC/FMB/UNESP/BOTUCATU) LOCALIZADO NA REGIÃO CENTRO-SUL DO ESTADO DE SÃO PAULO, BRASIL.

JULIANA GIACOBINO

Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração *Biologia de Parasitas e Micro-organismos*.

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BOTUCATU- SÃO PAULO

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DOUTORANDA: JULIANA GIACOBINO

ORIENTADOR: PROFESSOR TITULAR EDUARDO BAGAGLI

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“Se podemos sonhar, também podemos tornar nossos sonhos realidade”. (Walt Disney)

RESUMO

Infecções fúngicas causadas por leveduras constituem um dos maiores problemas em pacientes hospitalizados em todo o mundo e têm se tornado uma importante causa de morbidade e mortalidade. Embora *Candida albicans* seja a espécie mais frequentemente isolada e sua forma de infecção ocorra geralmente por translocação endógena, tem-se observado um aumento das espécies *não-albicans*, com destaque para o complexo *C. parapsilosis,* cuja principal forma de infecção é exógena, provavelmente pelas mãos dos profissionais de saúde. Este trabalho propôs estudar os aspectos microbiológicos e ambientais das candidemias, com especial atenção para o complexo *Candida parapsilosis,* no hospital terciário HC/FMB/UNESP, Botucatu, utilizando-se de métodos moleculares, busca ativa dos agentes no ambiente hospitalar (ar, superfícies e mãos de profissionais de saúde), bem como determinar os fatores de virulência e susceptibilidade aos antifúngicos destas espécies e associação com o desfecho clínico. Os isolados clínicos (obtidos de hemoculturas, período 2007-2015) e ambientais (período 2014-2015) de *C. parapsilosis sensu lato* foram identificados pelo meio CHROMagar *Candida*, Vitek-2, sequenciamento do rDNA e perfis dos inteins VMA e ThrRS. Fatores de virulência (produção de proteinase, fosfolipase e biofilme) e perfis de susceptibilidade aos antifúngicos anfotericina B, fluconazol, voriconazol, caspofungina e micafungina foram estimados, e dados clínicos obtidos junto aos prontuários dos pacientes. Dentre os isolados clínicos (n=45), *C. parapsilosis sensu stricto* (*s.s.*) representou 84%, *C. orthopsilosis* 16% e ausência de *C. metapsilosis*. Os perfis de inteins confirmaram o padrão híbrido incomum da espécie *C. orthopsilosis*. Dentre os ambientais (n=14), todos pertenceram à *C. parapsilosis s.s.* A produção de proteinase foi positiva ou fortemente positiva em 55% dos isolados clínicos de *C. parapsilosis s.s.*, e negativa nos isolados de *C. orthopsilosis*. Todos os isolados ambientais de *C. parapsilosis s.s.* foram produtores de proteinase (64% fortemente positivos). A produção de fosfolipase foi positiva para apenas um isolado ambiental de *C. parapsilosis s.s.* A produção de biofilme foi significativamente maior em *C. orthopsilosis*. Todos os isolados clínicos de *C. parapsilosis s.s.* foram sensíveis à anfotericina B e voriconazol, cinco foram sensíveis dose-dependentes e dois resistentes ao fluconazol, três foram simultaneamente sensíveis dose-dependentes à caspofungina e micafungina. Os isolados de *C. orthopsilosis* foram sensíveis a todos os antifúngicos. A mortalidade foi alta (51%) e as mortes ocorreram principalmente em recém-nascidos e idosos. A melhor compreensão das leveduras do complexo *C. parapsilosis* terá impactos positivos no tratamento e prevenção de novas infecções.

Palavras-chave: complexo *Candida parapsilosis*; fatores de virulência; identificação molecular; exposição ambiental; agentes antifúngicos.

ABSTRACT

Fungal infections caused by yeasts are serious problems in hospitalized patients around the globe and have become a major cause of morbidity and mortality. Although *Candida albicans* is the most frequently isolated species and its infection usually occurs by endogenous translocation, an increase of *non-albicans* species has been observed, with emphasys for the *C. parapsilosis* complex, whose main route of infection is exogenous, probably by the hands of health professionals. This work proposes to study the microbiological and environmental aspects of candidemia, with special attention to the *C. parapsilosis* complex, in a public tertiary hospital HC/FMB/UNESP, in Botucatu, using the molecular methods, active search of agents in the hospital environment (air, surfaces and hands of health professionals), as well as to determine the virulence factors and susceptibility to the antifungals of these species and correlate with the patient clinical outcomes. The clinical isolates (obtained of the blood cultures, 2007-2015 period) and environmental (2014-2015 period) of the *C. parapsilosis sensu lato* were identified by CHROMagar *Candida* medium, Vitek-2, rDNA sequencing and VMA and ThrRS inteins profiles. Virulence factors (production of proteinase, phospholypase and biofilm) and profiles of susceptibility to antifungals amphotericin B, fluconazole, voriconazole, caspofungin and micafungin were estimated, and the clinical data were obtained from patient's records. Among the clinical isolates (n=45), *C. parasilosis sensu stricto* (*s.s.*) represented 84%, *C. orthopsilosis* 16% and absence of *C. metapsilosis*. The inteins profiles confirmed the unusual hybrid pattern of the *C. orthopsilosis* species. Among the environmental isolates (n=14), all belong to *C. parapsilosis s.s.* Proteinase production was positive or strongly positive in 55% of clinical isolates of *C. parapsilosis s.s.*, and negative in the *C. orthopsilosis* isolates. All the environment isolates of *C. parapsilosis s.s.* were proteinase producers (64% of them were strongly positive). The phospholipase production was positive for only one environmental isolate of *C. parapsilosis s.s.* The biofilm production was significantly higher in *C. orthopsilosis*. All the clinical isolates of *C. parapsilosis s.s.* were sensitive to amphoterycin B and voriconazole, five were dose-dependent sensitivity and two resistant to fluconazole, three were simultaneously dose-dependent sensitivity to caspofungin and micafungin. The isolates of *C. orthopsilosis* were sensitive to all antifungals. The mortality was high (51%) and deaths occurred mainly in newborns and elderly patients. A better understanding of *C. parapsilosis* complex yeasts will have positive impacts on the treatment and prevention of new infections in our hospitals.

Keywords: *Candida parapsilosis* complex; virulence factors; molecular identification; environmental exposures; antifungal agents.

LISTA DE ABREVIATURAS:

BLAST: Basic Local Aligment Search Tool (Inglês).

Candida parapsilosis sensu stricto: (referido como *C. parapsilosis s.s.*).

CDC: Centers for Disease Control and Prevention (Inglês).

D1/D2: Região Variável da Subunidade Maior 26S do rDNA.

GLT1: Gene da Glutamato-Synthase.

HE: Homing Endonuclease (Inglês).

ITS: Internal Transcribed Spacer (Inglês) (presente na região nuclear ribossomal).

NNIS: National Nosocomial Infection Survey (Inglês).

PCR: Polimerase Chain Reaction (Inglês).

rDNA: Ribossomal DNA (DNA ribosomal).

ThrRS: RNA de transferência para o aminoácido threonil (referido como intein ThrRS).

VMA: ATPase da membrana vacuolar (referido como intein VMA).

SUMÁRIO

INTRODUÇÃO

Infecções fúngicas nosocomiais e leveduras do gênero *Candida*

As infecções fúngicas nosocomiais têm se tornado uma das principais causas de morte nas unidades de terapia intensiva (UTIs). Vale ressaltar, nas últimas décadas, um importante aumento na taxa global no número destas infecções fúngicas sistêmicas, principalmente causadas por leveduras, e este aumento constitui um importante problema de saúde pública, devido principalmente a sua gravidade, aumentos no tempo de hospitalização, no custo e nas taxas de morbidade e mortalidade (MARTIN et al., 2003; ARENDRUP, 2010; STORTI et al., 2012; MENEZES et al., 2015). Nos últimos anos, houve um notável aumento em sua incidência, devido aos avanços nas áreas de diagnóstico e tratamento com antimicrobianos de amplo espectro e à realização de transplantes de órgãos sólidos e hematopoiéticos (PERLROTH et al., 2007; COLOMBO et al., 2013). Assim, essas infecções constituem um dos maiores problemas nas UTIs tanto nos países desenvolvidos como nos países em desenvolvimento. Nos Estados Unidos, a incidência destas infecções aumentou em 2% a cada 1000 admissões hospitalares, nas últimas décadas (BAJWA & KULSHRESTTHA, 2013). Já em países em desenvolvimento, como o Brasil, que carece de recursos humanos e financeiros e de práticas de controle e prevenção da disseminação desses micro-organismos, a incidência destas infecções aumentou em 5% (SALES-JÚNIOR et al., 2006; COLOMBO et al., 2013). Outro fato importante é que em países com clima predominantemente tropical, como Brasil e Índia, algumas evidências indicam que as diversas condições climáticas podem favorecer também o aumento destas infecções fúngicas no ambiente hospitalar (BECK–SAGUE & JARVIS, 1993; STORTI et al., 2012).

Os fungos estão amplamente distribuídos na natureza e podem sobreviver em ambientes extremos. Das 500 espécies mais conhecidas e/ou comumente associadas ao homem, poucas eram consideradas patogênicas e/ou oportunistas há alguns anos atrás, mas nos últimos anos, estes números têm crescido consideravelmente, embora não se saiba ainda quantas espécies de fungos são patogênicas ao homem. A maior incidência de infecções fúngicas sistêmicas nosocomiais é observada principalmente em pacientes imunodeprimidos, sendo responsáveis por aproximadamente 60% a 90% dos casos (BECK–SAGUE & JARVIS, 1993; RICHARDSON, 2005; APERIS et al., 2006; PFALLER et al., 2007; PAPPAS et al., 2010).

Em pacientes internados em UTIs, a maioria dos patógenos fúngicos causadores de infecções sistêmicas nosocomiais e de infecções de corrente sanguínea pertencem ao gênero *Candida*, sendo responsável por cerca de 80% destas infecções (COLOMBO & GUIMARÃES, 2003; EGGIMANN et al., 2003; PFALLER & DIEKEMA, 2002). As espécies de *Candida* são leveduras pertencentes à ordem *Saccharomycetales*, da classe dos *Ascomycetes*, e muitas de suas espécies fazem parte da microbiota da pele e dos tratos gastrintestinal e geniturinário. Alterações na microbiota da pele e mucosas, bem como a perda da integridade da barreira do trato gastrintestinal podem proporcionar condições de crescimento excessivo ou a translocação dos micro-organismos através do intestino (FRIDKIN & JARVIS, 1996; EGGIMANN et al., 2003). Nos últimos 20 anos, diversos fatores têm contribuído para o aumento na incidência das candidemias, dentre eles destacam-se aqueles que comprometem as condições dos pacientes, como as deficiências imunológicas e desordens congênitas, tais como: neutropenia, neoplasia, cirurgias do tratogastrintestinal, tranplantes de órgãos sólidos, diabetes avançada, terapia com glicocorticoides, neonatos com baixo peso, idosos, pacientes infectados pelo vírus HIV, pacientes com cânceres submetidos à quimioterapia e que necessitam de procedimentos médicos invasivos (como o uso de cateter

venoso central e sondas urinária e parenteral) submetidos a longos períodos de hospitalização. Outros fatores de risco também envolvidos nesse aumento são o uso profilático e terapia com antimicrobianos de amplo espectro, principalmente quando se refere ao uso dos azóis (principalmente o fluconazol), colonização por *Candida* spp. em diferentes sítios, hemodiálise e o próprio ambiente hospitalar, como as UTIs (WEY et al., 1988; LUSATTI et al., 2000; COLOMBO & GUIMARÃES, 2003; MARCHETII et al., 2004; PERLROTH et al., 2007; STORTI et al., 2012; NUCCI et al., 2010; MENEZES et al., 2015).

Nos Estados Unidos (EUA), segundo dados do *National Nosocomial Infection Survey* (NNIS), as espécies pertencentes ao gênero *Candida* foram consideradas a quarta causa mais comum de infecções sanguíneas nosocomiais encontradas na década de 1990 (EDMOND et al., 1999; RANGEL-FRAUSTO et al., 1999; WISPLINGHOFF et al., 2004). Em anos mais recentes, houve um aparente crescimento destas infecções, que passaram a ocupar a terceira posição, sendo apenas superada estatisticamente pelas espécies bacterianas *Staphylococcus aureus* e *Staphylococcus epidermidis* (WISPLINGHOFF et al., 2004), sendo responsáveis por 8% a 15% das infecções sanguíneas isoladas de hemoculturas em pacientes hospitalizados em UTIs (JARVIS & MARTONE, 1992; PFALLER et al., 2002; ZAOUTIS et al., 2005). Segundo dados do ano de 2007 do *Centers for Disesase Control and Prevention* (CDC), as candidemias constituem a terceira maior causa de infecções sanguíneas associadas a cateteres nos EUA, nos últimos anos e estas taxas variam de 30% a 80% (LUSATIA et al., 2000). No Brasil, também vem sendo observado um aumento na taxa de incidência destas infecções (com média de 1,66 episódios por 1000 admissões hospitalares), números maiores do que os observados em países da Europa (0,17 a 0,76 por 1000 admissões hospitalares) e EUA (0,28 a 0,96 por 1000 admissões hospitalares) (WISPLINGHOFF et al., 2004; COLOMBO et al., 2007). Ainda no Brasil, estas infecções variam entre diferentes centros médicos de uma

mesma região, bem como em diferentes regiões geográficas (COLOMBO et al., 2007; NUCCI et al., 2010). Essas infecções representam um elevado custo hospitalar, pois aumentam o tempo de internação do paciente, e acarretam elevadas taxas de morbidade e mortalidade em todo o mundo (PATTERSON, 2005; ZAOUTIS et al., 2005; ODDS et al., 2007; PFALLER & DIEKEMA, 2002; ALANGADEN, 2011). Nos EUA, a taxa de mortalidade é estimada em 49% nas UTIs de adultos e chegam em até 75% nas UTIs pediátricas (STORTI et al., 2012; WISPLINGHOFF et al., 2014).

Candidemias (*Candida albicans* X *Candida não-albicans*)

A epidemiologia das candidemias vem sofrendo alterações nas últimas décadas. Até a década de 1980, a espécie até então mais frequentemente isolada era *Candida albicans,* também considerada a espécie mais virulenta de todas as espécies de *Candida* (BRIELAND et al., 2001; PERLROTH et al., 2007). Embora *C. albicans* seja ainda considerada a principal espécie do gênero e uma das mais importantes causadoras de infecções em muitos países, como os EUA, França e Tailândia, tem sido observado, a partir da década de 1990, um aumento na incidência das espécies *Candida não-albicans* em diversos centros médicos hospitalares em todo o mundo (PERLROTH et al., 2007; PFALLER & DIEKEMA, 2002; MARRA et al., 2011; PAPPAS et al., 2010; PFALLER et al., 2011; PARMELAND et al., 2013).

Dentre as espécies *não-albicans* mais frequentemente encontradas destacam-se: *C. parapsilosis*, *C. tropicalis*, *C. glabrata* e *C. krusei* (STORTI et al., 2012). Na literatura são encontrados estudos epidemiológicos com as mais variadas taxas de frequência das espécies, sempre dependendo da casuística e da região geográfica de cada centro avaliado. Nos EUA, Reino Unido, França, Alemanha, Noruega e China, *C. glabrata* foi a espécie *não-albicans*

mais frequentemente encontrada causadora de candidemias, enquanto que, na Espanha, Israel e em muitos países da América Latina as espécies mais encontradas foram *C. parapsilosis* e *C. tropicalis* (MARCHETTI et al., 2004; ALMIRANTE et al., 2006; PFALLER & DIEKEMA, 2002; GONZÁLEZ et al., 2008; NUCCI et al., 2013; DING et al., 2015). Alguns estudos dão ênfase a esses achados, como o observado por Ding et al. (2015) que dentre os 106 pacientes analisados, 53,8% apresentaram infecções por *Candida não-albicans*: *C. glabrata* (25,5%), *C. tropicalis* (15,1%), *C. parapsilosis* (10,4%) e *C. krusei* (0,9%). Pereira et al. (2010) realizaram um estudo em um hospital terciário na cidade de São Paulo, Brasil (Hospital Brigadeiro), num período de 5 anos (2004 a 2008), e observaram que 82% das infecções foram causadas por espécies *não-albicans*, sendo *C. parapsilosis* e *C. tropicalis*, as espécies mais comumente isoladas (26% cada). Em outro país semelhante ao Brasil, pelo clima, localização geográfica e/ou condições socioeconômicas, por exemplo, Índia, observa-se que as taxas de espécies de *Candida não-albicans* variaram de 52% a 96% dos casos, sendo a espécie *C. tropicalis* predominante em vários grupos estudados (CHAKRABARTI et al., 2002; RANI et al., 2002; VERMA et al., 2003; CHOW et al., 2008). No Peru, em estudo realizado em uma UTI neonatal, as espécies *Candida não-albicans* foram as mais frequentemente isoladas, sendo: *C. parapsilosis* (32% dos casos), *C. pellucilosa* (32%), *C. glabrata* (8%) e *C. albicans* em apenas 22% dos casos (IPEK et al., 2011). Em um estudo multicêntrico envolvendo 36 diferentes instituições na Argentina, no período de abril de 1999 a abril de 2000, observou-se também o predomínio das espécies *Candida não-albicans* (50,92%), sendo isoladas principalmente *C. parapsilosis* (28,67%), *C. tropicalis* (15,84%), *C. famata* (3,77%), *Cryptococcus neoformans* (3,77%), *C. glabrata* (2,64%) e outros (4,53%) enquanto que *C. albicans* foi isolada em 40,75% dos casos (RODERO et al., 2005).

Ainda neste tema sobre espécies *não-albicans*, é fundamental salientar sobre a emergência da espécie *Candida auris*, até então pouco conhecida e ainda não isolada no Brasil. Infecções por *C. auris* têm surgido como uma importante mudança no paradigma de pacientes admitidos em UTIs devido à ocorrência de surtos potenciais, resistência multidrogas, principalmente ao fluconazol, anfotericina B e equinocandinas, e associação a altas taxas de mortalidade, sendo que estas variam de 30 a 70%. Assim, esta espécie está relacionada à falha terapêutica e disseminação no ambiente hospitalar (CHOWDARY et al., 2013, 2014, 2017; MORALES-LÓPEZ et al., 2017; RUDRAMURTHY et al., 2017). O primeiro relato de isolamento desta espécie ocorreu no Japão em 2009 em um paciente com quadro de otite por *C. auris* (KIM et al., 2009). Atualmente, há relatos em vários países do mundo, como Kuwait, Índia, Coreia do Sul, África do Sul, Reino Unido, EUA, Israel, Europa, Colômbia e Venezuela (BEM-AMI et al., 2017; BORMAN et al., 2016; CALVO et al., 2016; CHOWDARY et al., 2013, 2014, 2017; KHILLAN et al., 2014; EMARA et al., 2015; LEE et al., 2011; LOCKHART et al., 2017; MAGOBO et al., 2014; MORALES-LÓPEZ et al., 2017; RUDRAMURTHY et al., 2017; SCHELENZ et al., 2016; VALLABHANENI et al., 2016).

Complexo *Candida parapsilosis*

Dentre este universo relativamente amplo das espécies *não-albicans* e por sua alta frequência e importância nos hospitais brasileiros, apresentamos a seguir alguns dados mais detalhados sobre o complexo *C. parapsilosis*, também referido como *C. parapsilosis sensu lato* ou grupo *psilosis* (BRUDER-NASCIMENTO et al., 2010; MONDELLI et al., 2012). Infecções causadas por leveduras pertencentes a este grupo têm sido particularmente observadas em pacientes com AIDS, câncer, transplantados, recém-nascidos com baixo peso (<1500g) e pacientes hospitalizados em Unidades de Terapia Intensiva (UTIs) que necessitam

de cirurgia invasiva do trato gastriintestinal e uso prolongado de cateteres intravasculares, tanto para administração de nutrição parenteral como para terapia antimicrobiana (COLOMBO, 2003; CHANG et al., 2008; TROFA et al., 2008; COLOMBO et al., 2013; PAMMI et al., 2013; QUINDÓS, 2014; ALENCAR et al., 2017).

Baseados em estudos moleculares e filogenéticos, *C. parapsilosis* foi reclassificada em três espécies crípticas distintas: *C. parapsilosis sensu stricto* (antigo grupo I), *C. orthopsilosis* (antigo grupo II) e *C. metapsilosis* (antigo grupo III) (TAVANTI et al., 2005). No entanto, a definição de espécies dentro do grupo *psilosis* pode ser muito mais complexa do que se conhece. Estudos recentes de sequenciamento de ITS, locus MAT, inteins e genômica comparativa vêm demonstrando que isolados de *C. orthopsilosis* são geneticamente heterogêneos, com a ocorrência de pelo menos dois subgrupos distintos (subespécies) (SAI et al., 2011; PRANDINI et al., 2013; PRYSZCZ et al., 2014), originadas por pelo menos quatro eventos distintos de hibridização (SCHRÖDER et al., 2016).

Embora as reais prevalência e distribuição de espécies do complexo *C. parapsilosis* não estejam completamente claras para as diferentes regiões geográficas, vários estudos indicam que *C. parapsilosis s.s.* ocorre em maior frequência do que *C. orthopsilosis* e *C. metapsilosis* em casos clínicos (TAVANTI et al. 2005, TROFA et al., 2008, NÉMETH et al., 2013), sendo responsável por 7% a 15% das candidemias na maioria das séries publicadas nos EUA e Europa, e ainda diversos estudos afirmam que é a segunda espécie mais comumente isolada de fungemias em várias regiões do mundo, sendo encontrada em maior frequência que *C. albicans* em vários hospitais da Europa, Ásia e América Latina, principalmente em recém-nascidos (VOSS et al., 1996; PFALLER et al., 2001; ALMIRANTE et al., 2005; MORAN et al., 2002; NAKAMURA & TAKAHASHI, 2006; MEDRANO et al., 2006; COSTA-DE-OLIVEIRA et al., 2008; TROFA et al., 2008; DASILVA et al; 2015).

Em relação aos aspectos ecológicos ou ambientais, a literatura indica que *C. parapsilosis sensu lato* é patógeno comensal humano, podendo ocorrer como habitante normal ou transitório da pele, bem como de mucosas. Existem poucos estudos ecológicos com a diferenciação das três espécies e a maior parte dos dados refere-se à espécie *C. parapsilosis s.s*. Estes organismos apresentam potencial capacidade de formar biofilme em superfícies bióticas e abióticas, como células do tecido epitelial, cateteres e outros dispositivos médicos, e de proliferar-se em soluções contendo glicose, como soluções de nutrição parenteral e de uso em diálise peritoneal (COLOMBO, 2003; TROFA et al., 2008; SABINO et al., 2011; PAMMI et al., 2013). Outro fato importante que está relacionado à sua disseminação no ambiente hospitalar, principalmente nas UTIs é que diferente da maioria das espécies de *Candida* cuja principal via de transmissão de infecções é a endógena, *C. parapsilosis* é transmitida frequentemente de modo horizontal pelas mãos dos profissionais de saúde que entram em contato com pacientes que requerem uso intensivo de implantes médicos (TAVANTI et al., 2005; TROFA et al., 2008). Há estudos que já demonstraram o isolamento desta espécie em mãos de profissionais de saúde e aparelhos por eles manuseados (HUANG et al., 1999; LUPETTI et al., 2002; TROFA et al., 2008). Quando comparada a outras espécies de *Candida*, possui uma extensa distribuição na natureza, e têm sido também isolada de animais domésticos, insetos, solo, ambientes marinhos e hospitalares (assim como ar, sistemas de ventilação, construções hospitalares, carpetes, água, alimento entre outras superfícies) (WEEMS, 1987; SABINO et al., 2011). Em estudo realizado no Brasil por Cordeiro et al. (2010), em um total de 240 amostras de ar envolvendo diferentes ambientes hospitalares, foram isoladas 34 amostras de *C. parapsilosis s.s*, sendo a espécie predominante quando comparada a outras espécies, como *C. tropicalis* (n=8), *C. albicans* (n=4), *C. guilliermondii* (n=1), *C. krusei* (n=1), *Trichosporon asahii* (n=11) e *Sacharomyces cerevisiae* (n=1)

Aplicação de métodos moleculares na identificação e caracterização de leveduras

Embora as diversas espécies tendam a apresentar diferentes perfis clínicos e epidemiológicos, a correta identificação destes agentes tem se tornado um grande desafio na prática laboratorial, principalmente quando baseado apenas nas características morfofisiológicas destas espécies. A identificação de patógenos fúngicos por métodos convencionais (fenotípicos), além do uso de sistemas comerciais denominados VITEK e API-32C, está se tornando cada vez mais difícil e inconclusiva, especialmente para as espécies fúngicas menos frequentes e/ou raras. Isto se dá, principalmente, pelas condições de crescimento das culturas que levam dias ou semanas, além de uma difícil interpretação de suas estruturas morfológicas e de seus perfis de assimilação e fermentação de carboidratos (CHEN et al*.,* 2000; LEAW et al*.,* 2006).

Com o advento da biologia molecular, surgiram novas possibilidades de detecção e identificação desses patógenos, sem que haja a necessidade de isolamento e cultivo do mesmo, utilizando para isso técnicas de reação de polimerase em cadeia, como a PCR (SAIKI et al., 1985). Informações de sequências gênicas e de genomas completos vêm sendo geradas em crescimento exponencial e depositadas nos respectivos bancos de dados (GenBank, EMBL-EBI, BroadInstitute, Sanger Institute e outros). Espécies podem ser discriminadas com base nas suas sequências de nucleotídeos; e as ferramentas de bioinformática, para a realização das análises comparativas, evoluíram para um grande poder de análise, associadas também à relativa simplicidade para o usuário, como por exemplo, o BLAST (Basic Local Aligment Search Tool) (http://blast.ncbi.nlm.nih.gov/Blast). Algumas regiões gênicas em particular, como as de rDNA que contém os fragmentos de ITS1/ITS2 e D1/D2, demonstram ser de grande utilidade para os protocolos de identificação rápida pela reação da PCR, devido ao fato de apresentarem, concomitantemente, regiões conservadas, isto é, não-variáveis

presentes em todos os fungos e regiões variáveis (única para cada espécie ou grupo de espécies próximas) (BAGAGLI & MARQUES, 2010). Diversos laboratórios já vêm empregando este tipo de abordagem na identificação de leveduras do gênero *Candida,* com excelentes resultados (CHEN et al., 2000, 2001; LEAW et al. 2006; CIARDO et al., 2006). A análise das regiões de ITS1/ITS2, juntamente com a do gene 5.8S rDNA, também serviu para indicar a ocorrência de três grupos genéticos distintos (grupos I, II e III) em *C. parapsilosis sensu lato* (LIN et al., 1995), que, posteriormente, confirmou serem de fato três espécies crípticas distintas, como já mencionado acima (TAVANTI et al., 2007).

Outros alvos moleculares, denominados inteins, também vêm sendo utilizados na identificação fúngica (KASUGA et al., 2003; BUTLER & POULTER, 2005; CARRERO et al., 2008) e mostraram ser particularmente úteis na distinção de espécies filogeneticamente próximas (PRANDINI et al., 2013). Inteins são considerados elementos genéticos parasitas, normalmente inseridos em genes codificadores de proteínas, evolutivamente conservados e importantes para o funcionamento celular, os denominados "house-keeping genes" (BONEN & VOGEL, 2001). Caracteristicamente, os inteins são transcritos e traduzidos juntamente com a sequência flanqueadora (extein), e apresentam propriedades de auto-excisão (self-splicing) e religação das sequências flanqueadoras, de forma a minimizar seus efeitos deletérios à célula hospedeira (BONEN & VOGEL, 2001; GOGARTEN et al., 2002). Existem três tipos de inteins na natureza: os inteins completos ou bi-funcionais (também conhecidos como large-inteins ou full-lenght inteins), os mini-inteins e os split inteins. Os inteins bi-funcionais são constituídos de um domínio responsável pelo splicing proteico (Spl) cujas porções N e C terminais são separadas por um domínio central codificador de uma homing endonuclease (HE) que confere ao intein mobilidade, resultando na ocupação de alelos vazios e duplicação do elemento genético parasita (LIU et al; 2000). Os mini-inteins não possuem o domínio HE

tendo, portanto um domínio Spl contínuo ou os dois domínios Spl separados por pequena sequência (linker, formada restos da HE degenerada). Os split-inteins constituem mini-inteins cujas partes N e C terminais estão separadas ao longo do cromossomo bacteriano (só foram obsevados em bactérias e arqueas), fragmentando o gene em que está inserido/associado. Após a tradução, as porções N e C terminais se unem, sofrem o splicing e ligam seus exteins em uma reação de *trans-splicing* proteico (WU et al., 1998). Inteins, em especial os bi-funcionais, proporcionam excelentes informações filogenéticas, pois apresentam um maior polimorfismo de sequência no domínio HE do que no domínio Spl devido a uma seleção mais "frouxa", especialmente no caso do domínio HE não estar mais ativo na população (GOGARTEN & HILÁRIO, 2006; THEODORO & BAGAGLI, 2009). Três inteins, presentes nos genes de uma membrana vacuolar ATPase (VMA), da threonyl-tRNA synthethase (ThrRS) e da glutamate synthase (GLT1), são particularmente importantes nas leveduras do gênero *Candida* (FERNANDES et al., 2016). Trabalho recente desenvolvido pelo nosso grupo demonstrou que a análise destes inteins representa uma maneira relativamente simples e eficiente para discriminar as três espécies do grupo *psilosis* (*C. parapsilosis sensu stricto, C. orthopsilosis* e *C. metapsilosis*), além de também indicar a ocorrência de dois genótipos distintos em isolados de *C. orthopsilosis* (PRANDINI et al., 2013; FERNANDES et al., 2016; SCHRÖDER et al., 2016).

Paralelamente aos métodos de identificação molecular, métodos de tipagem molecular para a diferenciação e detecção dos genótipos vêm evoluindo muito em leveduras e micro-organismos em geral. Técnicas como a do cariótipo obtido por eletroforese com alternância do campo elétrico (PFGE, "pulse-field gel electrophoresis"), da análise de polimorfismo de DNA gerado pela amplificação de fragmentos com uso de *primers* arbitrários (RAPD, "ramdom amplified polymorphic DNA"), ou as obtidas pelo polimorfismo de fragmentos

gerados com enzimas de restrição (RFLP, "restriction fragment length polimorphysm"), dentre outras, estão sendo frequentemente empregadas na tipagem destes micro-organismos, e vários genes e/ou regiões genômicas, ou o genoma todo, podem ser empregados nestes estudos. A combinação do uso de enzimas de restrição de cortes relativamente pouco frequentes em células na forma de esferoplastos, seguido de PFGE, tem se mostrado bastante útil no rastreamento de genótipos associados a surtos epidêmicos, por possibilitar comparações seguras entre isolados da mesma espécie, porém obtidos de diferentes fontes (CHEN et al., 2005; FILIPPIDI et al., 2014).

Fatores de Virulência (proteinase, fosfolipase e biofilme) em leveduras do complexo *C. parapsilosis*

Em relação aos fatores de virulência, estudos têm demonstrado diferenças significativas na secreção de enzimas hidrolíticas (fosfolipases e proteinases) e produção de biofilme entre essas espécies (RUCHEL et al., 1986; CÁNTON et al., 2011; PRANDINI et al., 2013, NÉMETH et al., 2013; PAMMI et al., 2013). Alguns estudos realizados *in vitro* demonstraram que *C. metapsilosis* é menos virulenta que *C. parapsilosis sensu stricto* e *C. orthopsilosis* (TAVANTI et al., 2005; SILVA et al., 2009; CANTÓN et al., 2011; SAI et al., 2011; NÉMETH et al., 2013; TOSUN et al., 2013). Associados a esses achados, a publicação recente do genoma de *C. orthopsilosis* mostrou que houve uma redução na família de genes associados à patogenicidade quando comparado ao da espécie *C. parapsilosis s.s.* (RICCOMBENI et al., 2012). Portanto, esse fato corrobora com os achados de que um número menor de infecções nosocomiais seja causado por *C. orthopsilosis* e *C. metapsilosis*, e sugere assim, que essas espécies são menos virulentas que *C. parapsilosis s.s.* (TAVANTI et al., 2005). Estudos recentes descreveram a presença de *C. orthopsilosis* e *C. metapsilosis* em

menos de 10% dos isolados clínicos. Também já foi reportado que ambas as espécies (*C. orthopsilosis* e *C. metapsilosis*) não estão relacionadas ao comensalismo humano (TAVANTI et al., 2005). Blanco-Blanco et al., 2014, em estudo realizado na Espanha, em um hospital terciário, durante o período de junho de 2007 a junho de 2009, observaram que em 36,5% dos casos de candidemias foram isoladas as espécies do grupo *psilosis* (sendo *C. parapsilosis s.s.* em 25% dos casos e *C. orthopsilosis* em 11,5%), seguida de *C. albicans* (30,8%), *C. tropicalis* (11,5%) e *C. glabrata* (11,5%). Embora *C. parapsilosis s.s* ocorra com maior frequência dentro do grupo *psilosis* e seja mais virulenta que *C. orthopsilosis* e *C. metapsilosis,* observou-se em uma UTI pediátrica no Brasil três casos de candidemias com óbitos, sendo dois casos por *C. orthopsilosis* e um por *C. metapsilosis* (OLIVEIRA et al., 2014).

A secreção de enzimas hidrolíticas (proteinases e fosfolipases) tem um importante papel na patogênese de doenças causadas pelas espécies pertencentes ao complexo *C. parapsilosis* o que facilita a sua aderência e invasão no tecido hospedeiro e dificultam a ação do sistema imunológico contra a ação dos antimicrobianos (NÉMETH et al., 2013). Em termos de atividade de proteinase e fosfolipase dentro do complexo *C. parapsilosis* há achados contraditórios que enfatizam a hipótese de que a expressão dos fatores de virulência é dependente da amostra (ZICCARDI et al., 2015). Horváth et al. (2012) demonstraram que a secreção de proteinase desempenha um importante papel na virulência de *C. parapsilosis s.s.*, o que parece ser um fator mais prevalente nesta espécie. Outros estudos também detectaram uma maior proporção de secreção de proteinase em isolados de *C. parapsilosis s.s* com taxas variando de 66,1 a 100% (TAVANTI et al., 2010; GE et al., 2011; ABI-CHACRA et al., 2013; NÉMETH et al., 2013). A produção de fosfolipase parece não ser um fator de virulência significativo dentro das espécies *não-albicans*, pois estudo demonstrou que a

atividade desta enzima ocorre em maiores porcentagens em isolados de *C. albicans* (54 a 73%) quando comparada às demais espécies de *Candida* (2 a 17%) (TAY et al., 2011).

Biofilmes são comunidades de micro-organismos que crescem associados a superfícies ou aderem a elas produzindo uma matriz extracelular (ECM) que é predominantemente formada por polissacarídeos, contendo resíduos de glicose e manose que conferem proteção (CHANDRA et al., 2001; RAMAGE et al., 2012). Podem ser encontrados aderidos a superfícies biológicas, tais como da via oral, mucosa e implantes médicos, os quais atuam como fontes de infecções persistentes. Recentemente, tem sido demonstrado que micro-organismos produtores de biofilme têm uma grande associação com mortalidade quando comparados às células fúngicas planctônicas, pois confere resistência significativa aos antifúngicos e protege as células de leveduras de respostas imunes do hospedeiro (CHANDRA et al., 2001; RAMAGE et al., 2012; TREVIÑO-RANGEL et al., 2015). Assim, os micro-organismos em condições de biofilme normalmente são mais resistentes aos diversos fatores ambientais, ao estresse físico e químico e ainda podem apresentar cooperação metabólica. Os biofilmes também servem como reservatórios de fontes persistentes de infecções em pacientes e têm efeito na saúde de um número aumentado de indivíduos imunocomprometidos (RAMAGE et al., 2012).

Espécies de *Candida,* em geral, são capazes de aderir a superfícies bióticas e abióticas, podendo levar à formação de biofilme, que se comporta como importante fator de virulência nesses micro-organismos (CALDERONE & BRAUN, 1991). Biofilmes em espécies de *Candida* podem se formar em superfícies inertes de dispositivos implantados como cateteres, próteses de válvulas cardíacas, próteses de articulações, entre outros materiais médicos (BRANCHINI et al., 1994; HAWSER & DOUGLAS, 1994; KUMAR & ANAND, 1998; DONLAN, 2001; FUX et al., 2005). Em relação à *C. parapsilosis s.s.*, vários fatores conferem

vantagem seletiva a esta espécie, incluindo as capacidades de proliferar-se em altas concentrações de glicose e de aderência a materiais protéticos (DOUGLAS, 2003).

A produção de biofilme pode ser estimada por vários métodos, incluindo o XTT, que mensura a atividade metabólica das células formadoras de biofilme (CHANDRA et al., 2008; RAMAGE et al., 2012). Kuhn et al. (2002) em estudo envolvendo a produção de biofilme utilizando-se de discos de silicone e XTT comprovaram que *C. albicans* produzem quantidades maiores de biofilme tanto quantitativamente quanto qualitativamente quando comparadas às espécies *não-albicans*, em particular *C. parapsilosis s.s*. Já, as variações nos resultados de produção de biofilme entre as espécies do complexo *C. parapsilosis* podem estar relacionados às diferentes metodologias utilizadas nos estudos ou também ao fato dos isolados serem provenientes de regiões geográficas distintas (TOSUN et al., 2013).

Perfis de susceptibilidade e resistência aos antifúngicos

Os tratamentos das candidemias representam verdadeiros desafios na prática médica, apesar de poder contar com fármacos como os polienos (anfotericina B) e seus derivados lipídicos (anfotericina B lipossomal), os triazóis de amplo espectro (fluconazol, voriconazol, posaconazol), as pirimidinas fluoradas (5-fluorcitosina) e as recentes equinocandinas (caspofungina, anidulafungina e micafungina), as quais apresentam maior segurança e melhores perfis farmacocinéticos e farmacodinâmicos (GARCÍA-AGUDO & GARCÍA-MARTOS, 2009). O fármaco anfotericina B apresenta vários efeitos adversos, destacando-se o seu alto potencial nefrotóxico, sendo muitas vezes, necessários ajustes em suas dosagens quando administrados em pacientes com disfunção renal. Na tentativa de diminuir seus efeitos adversos, foi lançado no mercado, na década de 1990, o fármaco anfotericina B lipossomal (comercialmente são utilizadas duas preparações lipossomais Fungisome e Ambisome), o qual

é menos nefrotóxico que a anfotericina B convencional (RESENDE, 2002; SIDRIM & ROCHA, 2004). Em estudo multicêntrico, Pachón et al. (2006) avaliaram a eficácia e segurança dos fármacos anfotericina B e fluconazol e obtiveram uma taxa de eficácia equivalente para ambos os fármacos testados (70% para fluconazol X 79% para anfotericina B), porém o fluconazol apresenta uma toxicidade renal significativamente menor quando comparado à anfotericina B (2% fluconazol X 37% anfotericina B), confirmando assim um melhor perfil de segurança (GARCIA-EFFRON et al., 2009).

Com os avanços na terapia antifúngica e na tentativa de diminuir seus efeitos adversos, novos fármacos foram introduzidos no mercado, a partir de 2001, como as equinocandinas (caspofungina, anidulafungina e micafungina). A introdução desses fármacos contribuiu com um importante avanço no tratamento das infecções fúngicas por reduzir os problemas de resistência cruzada aos azois e toxicidade associada aos fármacos polienos. Esta nova classe de antifúngicos possui um mecanismo inovador, pois são os únicos que atuam na inibição irreversível da síntese de (1,3)-beta-D-glucana, que é um polissacarídeo essencial presente na parede de fungos patogênicos, e, além disso, possuem uma atividade fungicida contra a maioria das espécies de *Candida,* sendo, portanto, um dos fármacos de escolha utilizados no tratamento das candidemias (SIDRIM & ROCHA, 2004; GARCÍA-AGUDO & GARCÍA-MARTOS, 2009; MURRAY et al., 2010, PFALLER et al., 2011). O crescente uso desses agentes tem proporcionado o aparecimento de espécies resistentes às equinocandinas. Essa resistência, embora raramente encontrada em espécies de *Candida* (cujas taxas de prevalência variam entre 2,9 e 3,1% dos casos), está relacionada a mutações nos genes Fks1 e Fks2, com substituições de aminoácidos em regiões conservadas das proteínas Fks, como as observadas em duas regiões conservadas (*hot spot* 1 e *hot spot* 2) de Fks1p (uma das subunidades presentes na enzima D-glucana sintase, com função catalítica) que resulta em isolados com

MICs elevados, susceptibilidade diminuída e consequente falha na terapêutica (PERLIN, 2007; GARCIA-EFFRON et al., 2008, 2009; PFALLER et al., 2011; BEYDA et al., 2012). É importante salientar que as equinocandinas, em especial a caspofungina, apresenta eficácia diminuída contra *C. parapsilosis* e *C. guilliermondii*, o que está relacionada à ocorrência de variantes naturais em Fks1 (MURRAY et al., 2010; BEYDA et al., 2012; FLEVARI et al., 2013). Assim, torna-se necessário avaliar o perfil de susceptibilidade aos antifúngicos nas espécies pertencentes ao complexo C. *parapsilosis*, pois essas espécies tendem a apresentar valores altos de MIC para as equinocandinas (caspofungina, micafungina e anidulafungina) (GARCIA-EFFRON et al., 2008; PFALLER et al., 2008; PFALLER et al., 2010; TORTORANO et al., 2013). Além disso, estudos moleculares têm indicado a presença de uma mutação natural que induz a substituição de um resíduo (prolina para alanina) na posição 660 de Fks1 que está associada à resistência às equinocandinas (GARCIA-EFFRON et al., 2008).

JUSTIFICATIVAS DO TRABALHO

Diante do exposto, nota-se que o espectro de candidemias tem mudado com a emergência das espécies *Candida não-albicans*, levando a um aumento na morbidade e mortalidade e na resistência às drogas antifúngicas em pacientes hospitalizados em UTIs. Com isso, a correta identificação das espécies de *Candida* e o conhecimento dos possíveis fatores ambientais associados poderão contribuir de forma significativa na elucidação das diferentes formas de aquisição da infecção, tanto as adquiridas por ativação endógena, e principalmente as adquiridas de fontes exógenas (ar, superfícies e mãos de profissionais de saúde). De particular interesse neste trabalho foi aprofundar o conhecimento da ecoepidemiologia do complexo *C. parapsilosis*, dado a sua ocorrência relativamente alta em

nossos hospitais. Dessa forma, procurou-se aqui organizar e responder pelo menos parte das seguintes perguntas: a) teriam todas as espécies do grupo *psilosis* a mesma capacidade de colonização das mãos de profissionais da saúde e também do ambiente físico hospitalar? b) quais são os principais fatores de virulência associados a cada uma destas espécies que explicariam suas diferentes incidências e sua provável influência no desfecho clínico? c) qual/quais os perfis de susceptibilidade aos antifúngicos nos diferentes isolados deste grupo e possíveis mecanismos moleculares de resistência, em especial para as equinocandinas? d) quais são os possíveis significados (biológico e clínico) da provável ocorrência de dois subgrupos geneticamente distintos dentro de *C. orthopsislosis*? Espera-se que os resultados forneçam informações relevantes para o entendimento dos aspectos microbiológicos e ambientais das candidemias em nosso meio, em particular das espécies pertencentes ao complexo *C. parapsilosis* que ainda é pouco compreendido, e assim contribuir para a melhoria da qualidade de vida e sobrevida destes pacientes.

OBJETIVO GERAL

Caracterizar os aspectos microbiológicos e ambientais das candidemias, com especial atenção para o complexo *Candida parapsilosis,* no hospital terciário HC/FMB/UNESP, Botucatu, localizado na região Centro-Sul do estado de São Paulo.

OBJETIVOS ESPECÍFICOS

a) Estimar as frequências de candidemias causadas pelo complexo *C. parapsilosis* das diferentes enfermarias e das UTIs Central e Neonatal do HC/FMB/UNESP, Botucatu, retrospectivamente, no período de janeiro de 2007 a dezembro de 2013; e prospectivamente, num período de 12 meses (de julho de 2014 a julho de 2015);

b) Identificar as leveduras pertencentes ao complexo *C. parapsilosis* isoladas de mãos de profissionais de saúde (equipe de enfermagem), do ar e superfícies das UTIs Central e Neonatal do HC/FMB/UNESP, Botucatu, SP, prospectivamente em um período de 12 meses (de julho de 2014 a julho de 2015);

c) Identificar por métodos morfofisiológicos e moleculares pelo sequenciamento de regiões de rDNA (ITS1/ITS2 ou D1/D2) e pelo uso de inteins VMA e ThrRS as espécies pertencentes ao complexo *C. parapsilosis*;

d) Determinar o perfil de virulência (produção de proteinase, fosfolipase e biofilme) em isolados do complexo *C. parapsilosis* de hemoculturas, do ar, superfícies e mãos dos profissionais de saúde;

e) Determinar o perfil de susceptibilidade aos antifúngicos anfotericina B, fluconazol, voriconazol, caspofungina e micafungina nos isolados clínicos pertencentes ao complexo *C. parapsilosis* e sua correlação com os padrões gênicos de Fks1 (para a verificação de diminuição da susceptibilidade ou resistência às equinocandinas);

f) Correlacionar os dados dos pacientes (sexo, idade, enfermarias, recidivas, fatores de virulência, susceptibilidade aos antifúngicos e espécies do complexo *C. parapsilosis*) com os desfechos clínicos dos pacientes.

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Capítulo 1. "*Candida parapsilosis* complex in a public tertiary hospital in Brazil: molecular and environmental aspects, virulence factors, antifungal susceptibilities and clinical outcomes." *

GIACOBINO, J.[1]; GARCES, H.G.[1]; TRINCA[2], L.; RUIZ, L.S[3].; MONDELLI[4], A.L; MONTELLI, A. C. [1]; BAGAGLI, E[1].

[1] Departamento de Microbiologia e Imunologia, Instituto de Biociências de Botucatu, Universidade Estadual Paulista Júlio de Mesquita Filho, São Paulo, Brasil.

[2] Departamento de Bioestatística, Instituto de Biociências de Botucatu, Universidade Estadual Paulista Júlio de Mesquita Filho, São Paulo, Brasil.

[3] Núcleo de Ciências Biomédicas, Instituto Adolfo Lutz, Bauru, São Paulo, Brasil.

[4] Hospital das Clínicas de Botucatu (HC/UNESP/Botucatu), Universidade Estadual Paulista Júlio de Mesquita Filho, São Paulo, Brasil.

Short Title: *Candida parapsilosis* complex in Botucatu, Brazil**.**

Keywords: *Candida parapsilosis* complex; environmental isolates; molecular identification; virulence factors; antifungal susceptibility.

Corresponding author:

Eduardo Bagagli. Department of Microbiology and Immunology – Institute of Biosciences, UNESP – Botucatu, São Paulo, Brazil.

Phone: (014) 3880 0418.

E-mail: bagagli@ibb.unesp.br.

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SUMMARY

This work aimed to investigate *Candida parapsilosis* complex in the public tertiary hospital of Botucatu, Brazil. Clinical (blood cultures) and environmental isolates (air, surfaces and hands of health professionals) were identified by rDNA sequencing and intein profiles; virulence factors (proteinase, phospholipase and biofilm) and antifungal susceptibilities were determined and correlated with the patient clinical outcomes. Concerning the clinical isolates, *C. parapsilosis sensu stricto* (*s.s.*) (n=45) represented 84% and *C. orthopsilosis* 16%, with no isolation of *C. metapsilosis*. All environmental isolates (n=14) were identified as *C. parapsilosis s.s*. The intein profiles confirm the uncommon hybrid pattern of *C. orthopsilosis* species. Proteinase production was positive or strongly positive in 55% of the clinical *C. parapsilosis s.s*. isolates, and negative for *C.orthopsilosis* isolates; all environmental *C. parapsilosis s.s.* isolates were proteinase producers. Phospholipase production was negative for all clinical isolates and positive for one environmental *C. parapsilosis s.s*. isolate. *C. orthopsilosis* produced more biofilm than *C. parapsilosis s.s*. All clinical isolates of *C. parapsilosis s.s*. were sensitive to amphotericin B and voriconazole, five showed dose-dependent susceptibility and two resistance to fluconazole, three presented dose-dependent susceptibility to caspofungin and micafungin simultaneously; all *C. orthopsilosis* isotes were sensitive to all antifungals. The mortality was high (51%) and deaths occurred mainly among newborns and elderly patients. A better comprehension of heterogeneous aspects of *psilosis* group will contribute for treatments and prevention of new infections, with positive impacts on the life quality and survival of the patients.

Keywords: *Candida parapsilosis* complex; environmental isolates; molecular identification; virulence factors; antifungal susceptibility.

INTRODUCTION

Fungal infections caused by yeasts are one of the greatest problems worldwide in hospitalized patients and have become an important cause of morbidity and mortality. Most of the fungal pathogens causing nosocomial and bloodstream infections are those belonging to the genus *Candida*, accounting for about 80% of these infections [1,2]. Until the 1980s, the most frequently isolated species was *Candida albicans*, being also considered the most virulent species of all *Candida* species [3,4]. Although *C. albicans* is still considered the main species of the genus and one of the most important causes of infection in many countries, such as the USA, France and Thailand, an increase in the incidence of *non-albicans Candida* species has been observed since the 1990s in various hospital medical centers around the globe, with emphasis on *Candida parapsilosis* complex, also denominated the *psilosis* group [1–8].

Infections caused by the *C. parapsilosis* complex have been particularly observed in patients with AIDS, cancer, transplant recipients, neonates with low weight (<1500g) and patients hospitalized in Intensive Care Units (ICUs) who require invasive surgery of the gastrointestinal tract and prolonged use of intravascular catheters for both the administration of parenteral nutrition and antimicrobial therapy [9,10].

Based on molecular and phylogenetic studies, the *C. parapsilosis* complex was reclassified into three distinct cryptic species, namely *C. parapsilosis sensu stricto* (former group I, now herein referred to as *C. parapsilosis s.s.*), *C. orthopsilosis* (former group II) and *C. metapsilosis* (former group III) [11]. However, species definition within the *psilosis* group might be more complex than had been supposed. Recent studies of the ITS sequencing, MAT locus, inteins (internal proteins) and comparative genomics have identified *C. orthopsilosis* isolates as being genetically heterogeneous,

with the occurrence of distinct subgroups (subspecies) [12–14], originated by at least four hybridization events [15].

While the real prevalence and species distribution of the *C. parapsilosis* complex are not completely clear in relation to different geographic regions, several studies have indicated that *C. parapsilosis s.s.* occurs much more frequently than *C. orthopsilosis* and *C. metapsilosis* in clinical cases [10,11,16]. Unlike most *Candida* species whose main route of infection transmission is endogenous, *C. parapsilosis* complex species are supposed to be horizontally transmitted through the hands of health professionals that are in contact with patients requiring intensive use of medical devices [10,11]. *C. parapsilosis s.s* .is considered a human commensal and a normal or transient inhabitant of the skin, being found mainly in epithelial and mucosal tissues. There are still few data on the ecological aspects of *C. orthopsilosis* and *C. metapsilosis*.

Concerning to virulence factors, studies have demonstrated significant differences in the biofilm production and secretion of hydrolytic enzymes (phospholipases and proteinases) among these species [9,13,16–19]. As expected, the less prevalent *C. metapsilosis* species is also the least virulent in the *psilosis* group [16].

Antifungal susceptibilities in *C. parapsilosis* complex species also need to be properly evaluated, since when compared to other *Candida* species, they tend to present higher MIC values for the echinocandins (caspofungin, micafungin and anidulafungin) [20–22]. In addition, molecular studies have indicated the presence of a natural mutation inducing a residue substitution (proline to alanine) at the 660 position of the Fks1 gene, which is associated with resistance to echinocandins[20].

Herein, we characterized a collection of clinical and environmental isolates of *C. parapsilosis* complex from a public tertiary hospital, located in Botucatu, Brazil, in relation to their molecular profiles, virulence factors and antifungal susceptibilities, and also attempting to correlate

with the patient clinical outcomes. The data might contribute for a better understanding of why this complex yeast group occurs so frequently in our hospitals and also provide additional strategies in the treatment and prevention of new infections.

PATIENTS AND METHODS

1. Patient, isolates and morphophysiological identification:

The main criterion for inclusion in this study is that the yeast isolates belong to the *C. parapsilosis* complex. The clinical isolates were obtained from bloodstream infections of patients from the University Hospital of Botucatu Medical School, São Paulo State, Brazil, which were stored in the culture collection of the Department of Microbiology and Immunology, Biosciences Institute of Botucatu, UNESP, covering the periods of January 2007 to December 2013 (retrospectively) and July 2014 to July 2015 (prospectively). The patient data (sex, age, antifungal treatment, clinical ward, relapse occurrence and the clinical outcome) were obtained from the medical records for most episodes of candidemia caused by *Candida psilosis* group (data on 35 patients from a total of 45 clinical case/isolates) (Table 1 and Table S2, Supplementary Material).

The environmental isolates (*psilosis* group) were obtained in 12 monthly collections from the air, surfaces and health professional hands of two Intensive Care Units (ICUs), the Central ICU and the Neonatal ICU of the same above mentioned hospital, in the prospective period from July 2014 to July 2015. The air samples were obtained by using the MAS-100 Air Sampler (Merck®, Darmstadt, Germany), employing 100 liters of air for each plate containing Sabouraud Dextrose Agar (Difco®, Maryland, USA) plus chloramphenicol (Sigma-Aldrich®, St. Louis, USA), in duplicate, in three distinct sites in each ICU. The surface samples were obtained by the technique using Rodac Plates containing SDA plus chloramphenicol, in duplicate, sampling the door handles,

boards and/or desks utilized to prepare medications, walls and floors near patient beds. The hand samples were obtained by using sterile swabs soaked in saline, which were scrubbed on the fingers and nails of three health professionals, who had volunteered or been randomly chosen, and seeded in SDA plus chloramphenicol plates, and all incubated at 35ºC.

All the clinical and environmental isolates were screened visually and microscopically in Lacto-phenol cotton blue preparations, followed by identification in CHROMagar (Difco®, Maryland, USA), the automatic VITEK-2 system (bioMérieux®, Hazelwood, MO, USA) molecular methods, as described below. This study was approved by The Research Ethics Committee (protocol: CAAE 30275814.1.0000.5411).

2. Molecular Identification:

DNA extraction: the protocol was carried out as described by McCullough et al. 2000 [23], and standardized in our laboratory. After the extraction, the DNA was quantified using NanoVue equipment (GE Healthcare®, Buckinghamishie, UK), and concentrations were adjusted to 10ng/µl. DNA remained stored in a freezer at -20°C until use.

Sequencing of ITS1-5.8S-ITS2 or D1/D2 regions: the PCR amplification was performed using the Go TaqGreen Master Mix (Promega®, Madison, USA) in a Verity thermocycler (Applied Biosystems, Foster City, USA). The cycling conditions using primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'), described by White et al. (1990)[24], were: a first denaturation cycle for 5 min at 95°C, followed by 35 denaturation cycles at 95°C for 1 min, annealing at 55°C for 1 min, elongation at 72°C for 2 min, and final extension at 72°C for 10 min. For D1/D2 regions, the primers utilized were NLS1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NLS4 (5'-GGTCCGTGTTCAAGACG-3'), as described by Kurtzman and Robnett (1998) [25], with the same cycling conditions except for an

annealing temperature of 60°C. The amplicons were purified with EXOSAP-IT (GE Healthcare®, Affymetrix, Cleveland, OH, USA), quantified in 1.5% agarose gel and sent for sequencing in the Sequencing Service of the Department of Microbiology and Immunology (Botucatu Biosciences Institute, UNESP). The sequences were viewed and edited using the software Mega v6.0. The sequences obtained (≥400bp) were submitted for identification by using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast), considering the alignments that presented greater identity and/or similarity with deposited sequences, which originated from reference isolates (ATCC and CBS strains or other equivalent collection). For species identification, only the values greater than 98% and E-values equal to and/or very close to zero were considered. The sequences were deposited at the GenBank (accession numbers on Table S1, Supplementary Material).

Analysis of the inteins VMA and ThrRS: the amplification of the inteins Vacuolar Membrane ATPase (VMA) and Threonyl-tRNA Synthetase (ThrRS) intein were performed using the primers TP1fwd (5'-ACTGCTGATTAYCCATTGTTG-3') and TP2rev (5'-AGATTGAWGCTTCTCTKGCAG-3') and TP3fwd (5'-GAARGARGCTGCTGAAAGAG-3') and TP4rev (5'-TCTTGTTGGAAACGACGAAC-3'), respectively, as proposed by Prandini et al. (2013)[13]. Each PCR reaction had a final volume of 25 µl, containing 23 µl of PCR reaction 200 mmol l^{-1}Tris-HCl, pH 8.4, 1.5 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1}KCl, 0.2 mmol l^{-1} dNTP (deoxynucleoside triphosphate), 0.4mmol l^{-1} of each primer and 1 unit of Taq Polymerase (Invitrogen®, Cincinnati, USA) and 2µl of *Candida* spp. DNA. The cycling conditions for both primers were: the first denaturation cycle for 5 min at 95°C, followed by 40 denaturation cycles at 95°C for 1 min, annealing at 55°C for 1 min, elongation at 72°C for 2 min, and final extension at 72°C for 10 min. The PCR products were identified in 1.5% agarose gel electrophoresis. The VMA intein polymorphic profiles permit the differentiation of the three cryptic species, since in *C. parapsilosis s.s.* the VMA intein is absent (319bp fragment), *C. orthopsilosis* presents a larger

(1909bp fragment) and *C. metapsilosis* a shorter intein (1681bp fragment). The ThrRS intein polymorphic profiles permit the differentiation of the two distinct genetic groups of *C. orthopsilosis*: *C. orthopsilosis* group A (979bp, mini-intein) and *C. orthopsilosis* group B (1756bp, full-length intein).

3. Virulence Factors

Enzyme production (proteinase and phospholipase): was evaluated in all clinical and environmental isolates of the *psilosis* group. Proteinase production was carried out according to the protocol described by Rüchel et al. (1982) [26]. Briefly, 5 µl aliquots of *Candida* suspension adjusted to a concentration of $1x10^7$ CFU/mL were placed in a proteinase-inducing medium for 96 hours at 35°C. The inducer medium contained 0.2% bovine serum albumin (Sigma-Aldrich®, St. Louis, USA) and a solution of vitamins (Sigma-Aldrich®, St. Louis, USA) was supplemented with Yeast Carbon Base (YCB, Sigma-Aldrich®, St. Louis, USA), adjusted to pH 5, sterilized by filtration and added with 2% autoclaved agar. The presence of the enzyme was observed through the formation of a translucent halo of protein degradation around the yeast colony. The enzymatic activity (Pz) was calculated using the ratio of colony diameter (dc) to colony diameter plus degradation zone (ddc), that is Pz = dc/ddc, according to Price et al. (1982) [27]. It was considered negative when values of Pz =1, positive for Pz < 1.0 and ≥ 0.64, and strongly positive for Pz < 0.64. Evaluation of phospholipase production was performed according to Price et al. (1982) [27]. Five µl *Candida* suspension adjusted to a concentration of $1x10^7$ CFU/ml was inoculated into Petri dishes containing 20ml of Sabouraud dextrose agar (Difco®, Maryland, USA) supplemented with 1M NaCl,5mmol l^{-1} $CaCl_2$ and 10% sterile egg yolk. Plates were incubated at 35°C and the reading was performed after 96 hours. The enzymatic activity (Pz) was calculated as it was for proteinase. *C. albicans* ATCC 64548 strain was used as a positive control.

Biofilm Production: *in vitro* biofilm production was determined for all isolates of the *psilosis* group, being quantified by their metabolic activity, as described by Pierce et al. (2008) [28]. Briefly, the 96-well polystyrene plates were inoculated with 100 µl of bovine serum albumin (Sigma-Aldrich®, St. Louis, USA) and kept overnight, after which they were washed twice with sterile PBS buffer. The cells previously cultured in yeast peptone dextrose (YPD, Sigma-Aldrich®, St. Louis, USA) medium were collected by centrifugation, washed with PBS and re-suspended in 10^6 cells/mL in RPMI-1640 medium (Cultilab®, Campinas, Brazil); 100 µl of this yeast suspension was incubated at 37°C for 48h. The wells were washed three times in PBS Tween-20 at 0.05% for removal of the non-adherent cells. The fungal cells that adhered to the plastic surface were considered biofilm forming. The biofilm adhered to the well plates was quantified using XTT (2,3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide innersalt) (Sigma-Aldrich®, St. Louis, USA) and menadione (Sigma-Aldrich®, St. Louis, USA) for measuring the metabolic activity of the biofilm, and the reading was performed in a spectrophotometer with the plate reader Power Wave XS2 (Biotek®, Winooski, USA), in a 492 nm filter. Positive and negative controls were represented by *C. albicans* SC 5314 isolate and a blank reference (without inoculum), respectively. The results were expressed in optical densities (ODs), obtained in quadruplicate for each isolate, which were classified as low, medium and high biofilm producers, according to the respective tertiles, which were arbitrarily estimated from the arithmetic averages of all the isolates and species, adapted from Marcos-Zambrano et al. (2014) [29].

Attempts were made to correlate the profiles of virulence factors (production of proteinases, phospholipases and biofilm) and antifungal susceptibility with the patient clinical outcomes.

4. Susceptibility to antifungals and FKS genes profiles

Susceptibility profile analysis: the susceptibility antifungal profiles were determined in all clinical isolates of the *psilosis* group, for the following antifungal agents: amphotericin B (Sigma-Aldrich®, St. Louis, USA), fluconazole (Sigma-Aldrich®, St. Louis, USA), voriconazole (Sigma-Aldrich®, St. Louis, USA), caspofungin (Merch Co®, New Jersey, USA) and micafungin (AstellasFarma®, Takaoka, Japan). The minimum inhibitory concentration (MIC) was determined according to the liquid microdilution technique described by the Clinical and Laboratory Standards Institute, protocols CLSI M27-A3 and M27-S4 [30,31]. The breakpoint values of susceptibility to fluconazole, voriconazole, caspofungin and micafungin were established by CLSI M27-S4 protocol. The breakpoint values for fluconazole, caspofungin and micafungin were sensitive (S) ≤ 2 μg/mL, dose-dependent susceptibility (DDS) = 4 μg/mL and resistance (R) ≥ 8 μg/mL, while for voriconazole S ≤ 0,12 μg/mL, DDS 0,25 - 0,5 μg/mL and R ≥ 1 μg/mL. In the case of amphotericin B, susceptibility (S) and resistance (R) were considered values ≤ 2μg/mL and > 2μg/mL, respectively, according to the CLSI M27-A3 protocol. The ATCC 22019 (*C. parapsilosis*) and ATCC 6258 (*C. krusei*) strains were used as quality control and for standardizing the test.

Fks1 gene analysis: the hot spot 1 region of the FKS1 gene was determined in order to correlate with echinocandin resistance [20,32]. Thus, the primers CpaFKS1F (5'-ATA CTC CAA GTC CTA ATA TGC-3 ') and CpaFKS1R (5'-AAT CCC AAC ACA ATC TTG GC-3') were designed and employed in the amplification reactions with the use of Go Taq Green Master Mix (Promega®, Madison, USA). The cycling conditions were the same employed for amplifying *Candida* spp. nuclear ribosomal region (above described) with an annealing temperature of 60°C. After amplification, the amplicons of 330bp were detected by a 1.5% agarose gel electrophoresis. The product was purified with EXOSAP-IT (GE Healthcare®, Affymetrix, Cleveland, OH, USA) and subjected to the sequencing of both forward and reverse sequences on a 3500 analyzer (Applied

Biosystems®, Foster City, CA, USA) according to the manufacturer's instructions. The sequences were edited using the software MEGA v6.0 to determine the mutation responsible for echinocandin resistance.

5. Statistical Analysis: Fisher's Exact Test was employed to study associations between clinical outcomes with the several clinical and fungal species data, as well as between species with virulence factors and antifungal susceptibilities.

RESULTS

1. *Candida parapsilosis* complex species occurrence on patients and hospital environment

The patients presenting bloodstream infection by *Candida* species that make up the *psilosis* group originated mostly from the ICU (Central or Neonatal), 57% were female, while neonates and the elderly represented nearly 30% and 20%, respectively. Relapses were observed in 16% and death occurred in 51% of the patients, mainly in the neonate and elderly groups (P = 0.04, Fisher's Exact Test). As to the species of clinical isolates, obtained in the periods from January 2007 to December 2013 and from July 2014 to July 2015, *C. parapsilosis s.s.* and *C. orthopsilosis* represented, respectively, 84% and 16% of the cases, with no occurrence of *C. metapsilosis* (Table 1).

Table 1. Patient data (n=45) and association with the clinical outcome in bloodstream infections caused by *Candida parapsilosis* complex at the University Hospital of Botucatu Medical School, São Paulo state, Brazil, years 2007 to 2013 and 2014, july to 2015, july.

Patient/ Isolate**	Sex	Age	Clinical Ward	Relapse	Species	Clinical Outcome
1C		27 days	Neonatal ICU	N	*C. parapsilosis s.s.*	Death
2C	M	44years	Emergency Room	N	*C. parapsilosis s.s.*	Death
4C	M	74years	Urology	N	*C. parapsilosis s.s.*	Death
17C	M	15years	Pediatrics	Y	*C. parapsilosis s.s.*	Cure
21C			Neonatal ICU	Y	*C. parapsilosis s.s.*	Cure
22C	M	71years	Emergency Room ICU	N	*C. parapsilosis s.s.*	Death
26C	M	52years	Emergency Room	N	*C. parapsilosis s.s.*	Death
30C	F			N	*C. orthopsilosis*	Cure
32C	M	1year	Pediatrics	Y	*C. orthopsilosis*	Cure
38C	F	4months	Pediatrics	N	*C. parapsilosis s.s.*	Cure
39C	M	42years	Gastroclinic	N	*C. parapsilosis s.s.*	Death
40C	F	74years	Urology	N	*C. orthopsilosis*	
44C	M	37years	Central ICU	N	*C. parapsilosis s.s.*	Cure
50C	F	60years	Urology	N	*C. orthopsilosis*	Death
55C	F	34years	Ginecology	Y	*C. parapsilosis s.s.*	Death
59C	M	7months	Pediatrics	N	*C. parapsilosis s.s.*	
60C		15 days	Neonatal ICU	N	*C. parapsilosis s.s.*	Death
64C	M	72years	Neurosurgery	N	*C. parapsilosis s.s.*	Death
77C	M	10years	Pediatrics	N	*C. orthopsilosis*	
83C	M	59years	Emergency Room ICU	N	*C. parapsilosis s.s.*	
84C	F			N	*C. parapsilosis s.s.*	Cure
85C	F	30years	Central ICU	N	*C. parapsilosis s.s.*	
86C	F			N	*C. parapsilosis s.s.*	Cure
87C	F	9days	Neonatal ICU	N	*C. parapsilosis s.s.*	Cure
90C		28 days	Neonatal ICU	N	*C. orthopsilosis*	Death
91C	F	61years	Hematology	N	*C. parapsilosis s.s.*	Death
94C	F	28years	Ginecology	N	*C. parapsilosis s.s.*	
99C	F	86years	Central ICU	N	*C. parapsilosis s.s.*	Death
100C	F			N	*C. parapsilosis s.s.*	Cure
107C	F	71years	Cardiac Surgery	N	*C. parapsilosis s.s.*	Cure
109C	M	4months	Pediatrics	Y	*C. parapsilosis s.s.*	Death
113C	F	57years	Emergency Room	N	*C. parapsilosis s.s.*	
117C		27 days	Neonatal ICU	N	*C. parapsilosis s.s.*	Death
118C	F	16years	Ginecology		*C. parapsilosis s.s*	
120C	F	72years	Cardiac Surgery	N	*C. parapsilosis s.s.*	Death
126C	M	11 months	Pediatrics	Y	*C. parapsilosis s.s.*	
130C	M	4months	Pediatrics	Y	*C. orthopsilosis*	Cure
134C	F	40years	Emergency Room	N	*C. parapsilosis s.s.*	Cure
138C	F			N	*C. parapsilosis s.s.*	Cure
154C	F	36years	Central ICU	N	*C. parapsilosis s.s.*	
155C	M	48years	Urology	N	*C. parapsilosis s.s.*	Cure
158C	M	48years	Urology	N	*C. parapsilosis s.s.*	Cure
165C	F	78years	Emergency Room	N	*C. parapsilosis s.s.*	Death
174C	F	6months	Pediatrics	N	*C. parapsilosis s.s.*	Cure
2 (H-6)C	F	57years	Central ICU	N	*C. parapsilosis s.s.*	Death
Fisher's Test	NS	P=0.04	NS	NS	NS	

C: clinical isolate (bloodculture isolate); **All patient received treatment with fluconazole. Sex (M: male; F: female); Relapse (Y: Yes; N: No); *C. parapsilosis s.s.* (*Candida parapsilosis sensu stricto*); N.I.: Not informed; *: significant by Fisher's Test; NS: non-significant by Fisher's Test.

During the prospective study, in the period from July 2014 to July 2015, 14 environmental isolates of *C. parapsilosis s.s.*, were obtained in 12 monthly samplings of the air, surfaces and hands of health professionals from the Central and Neonatal Intensive Care Units (ICUS) of HC, with no occurrence of *C. orthopsilosis* or *C. metapsilosis*. In the Central ICU, six isolates were obtained from surfaces and three from the hands of health professionals, while in the Neonatal ICU, four isolates were obtained from surfaces and one from the air (Table S1, Supplementary Material).

All the isolates belonging to the *psilosis* group (45 clinical and 14 environmental) were identified by sequencing the ITS or D1/D2 rDNA regions. In addition, we also performed the analysis of VMA and ThrRS intein profiles, which has been proposed for differentiating species of the *psilosis* group without sequencing (Table S1, Supplementary Material). The VMA intein profiles allowed the differentiation of all isolates of *C. parapsilosis s.s.* that typically do not present this intein (bands with 319 pb). As to *C. orthopsilosis,* with one exception (#50) that was inconclusive, all the remaining isolates presented the VMA intein (bands with 1,909bp) in at least one allele (they are diploid); furthermore, three of them also presented one allele without this intein (bands with 319bp), in a heterozygous condition, that is, in the presence of two bands (319 and 1909bp) (Figure 1). The analysis of ThrRS inteins, in turn, facilitated the differentiation of the two *C. orthopsilosis* subgroups (A and B). The species belonging to subgroup A (four isolates) showed 979bp fragments, reflecting the presence of a mini-intein, while the species belonging to subgroup B (two isolates) showed fragments of 1756bp, reflecting the presence of a full-length intein (Figure 2 and Table S1, Supplementary Material).

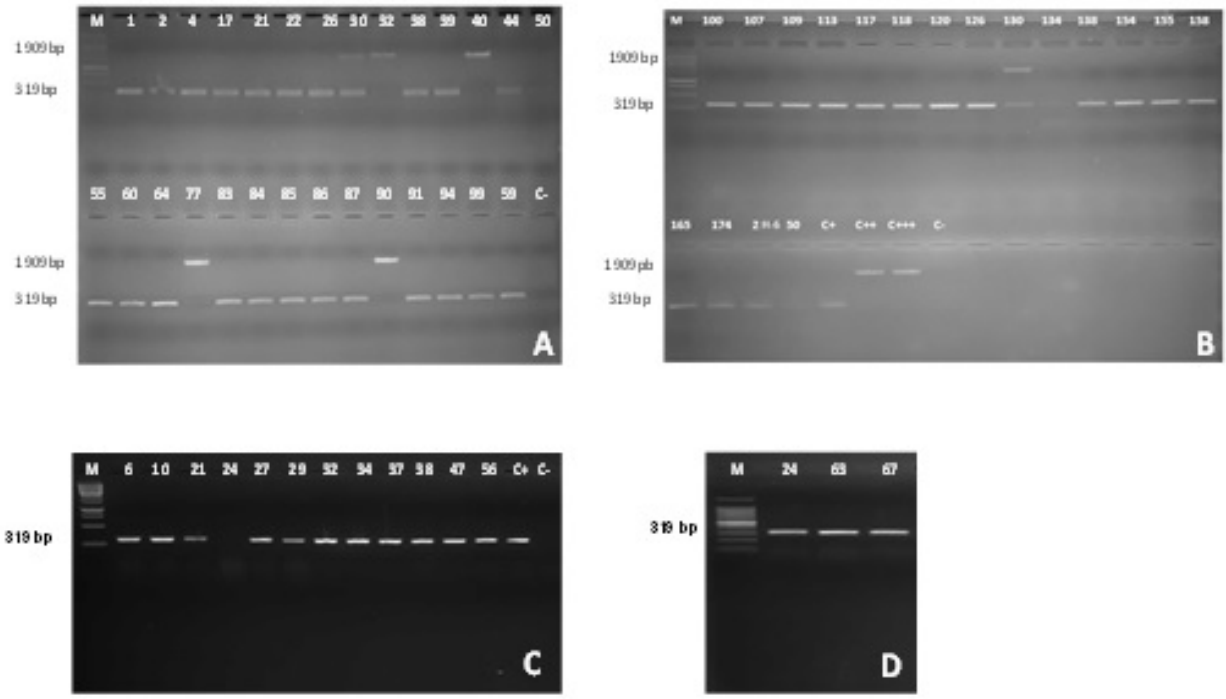

Figure 1. Vacuolar membrane ATPase (VMA) intein profiles of clinical (A and B) and environmental (C and D) *Candida parapsilosis* complex isolates. In **A,** Lines 30, 32, 40, 77 and 90 are *C. orthopsilosis* isolates, and the remaining numerated lines are *C. parapsilosis s.s.* isolates; C- (negative control); M (molecular marker, 1 kb, Promega). In **B,** all lines are *C. parapsilosis s.s.* isolates, except line 130 (*C. orthopsilosis*); C+ (positive control for *C. parapsilosis s.s.*); C++ and C+++ (positive control for *C. orthopsilosis*); C- (negative control) and M (molecular marker, 1 kb, Promega). In **C** and **D**, all lines contain distinct environmental isolates of *C. parapsilosis s.s.*; C+ (positive control for *C. parapsilosis,* ATCC 90018); C- (negative control); M (molecular marker, 1 Kb, Promega). Each lane number corresponds with each isolate's denomination.

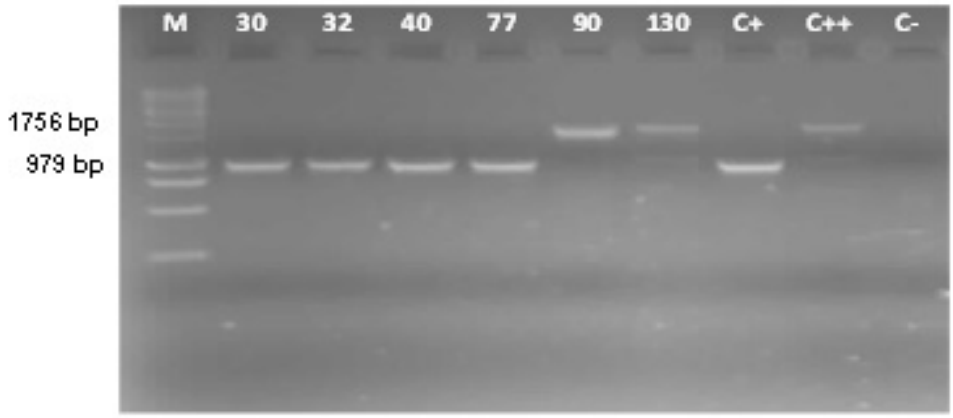

Figure 2. Threonyl-tRNA Synthetase (ThrRS) intein profiles of clinical *C. orthopsilosis* isolates. Lines 30, 32, 40 and 77 are group A *C. orthopsilosis* isolates; 90 and 130 are group B *C. orthopsilosis* isolates; C+ (positive control, group A *C. orthopsilosis*); C++ (positive control, group B *C. orthopsilosis*); C- (negative control) and M (molecular marker, 1Kb, Promega).

2. Virulence factors in the clinical and environmental isolates of *C. parapsilosis* complex species.

Proteinase production was positive or strongly positive in 55% of the clinical *C. parapsilosis s.s.* isolates, but was negative for all *C. orthopsilosis* clinical isolates herein evaluated (P = 0.03, Fisher's Exact Test); all environmental *C. parapsilosis s.s.* isolates were proteinase producers (64% of them strongly positive). Phospholipase production was negative for all clinical *C. parapsilosis s.s.* and *C. orthopsilosis* isolates*;* it was positive for only one environmental *C. parapsilosis s.s.* isolate. In relation to biofilm production, determined by the XTT procedure and the classification of isolates as low, medium or high producers, it was observed that, proportionally, *C. orthopsilosis* isolates were greater producers than *C. parapsilosis s.s.* (P= 0.02 Fisher's Exact Test) (Table 2 and Table S2, Supplementary Material).

Table 2. Virulence factors (proteinase, phospholipase and biofilm production) of *Candida parapsilosis sensu stricto* (clinical and environmental) and *Candida orthopsilosis* (clinical) isolates, obtained from the University Hospital of Botucatu Medical School, São Paulo, Brazil.

Species (number of isolates)	Proteinase			Phospholipase			Biofilm Production		
	N	P	SP	N	P	SP	Low	Medium	High
C. orthopsilosis C (n=7)	7	0	0	7	0	0	4	2	1
C. parapsilosis s.s. C (n=38)	17	9	12	38	0	0	36	2	0
C. parapsilosis s.s E (n=14)	0	5	9	13	1	0	14	0	0
Fisher's Test	P=0.03*			NS			P=0.02*		

C: clinical isolate (blood culture isolate), *C. parapsilosis s.s.: C. parapsilosis sensu stricto*; E: Environmental isolate. Proteinase and Phospholipase activity: Pz=1 (N: negative); 0,64 ≤ Pz <1 (P: positive) and Pz < 0,64 (SP: strongly positive); Biofilm values (ODs): values of average of optical densities; Biofilm production: high, medium and low production (based on average of optical densities: < 0,176 low production; 0,176 – 0,353 medium production; > 0,353 high production). *: significant by Fisher's Test; NS: non-significant by Fisher's Test.

3. *In vitro* antifungal susceptibility and FKS1 gene profiles in the clinical isolates of *C. parapsilosis* complex species.

The *in vitro* antifungal susceptibility profile was performed in all clinical isolates, for amphotericin B, fluconazole, voriconazole, caspofungin and micafungin. The breakpoint values and interpretation as sensitive, dose-dependent-susceptibility or resistant isolates are represented in

Table 3. As to *C. parapsilosis s.s.*, all the isolates were sensitive to amphotericin B and voriconazole; five isolates presented dose-dependent susceptibility and two showed resistance to fluconazole; three isolates displayed dose-dependent susceptibility simultaneously to caspofungin and micafungin. *C.orthopsilosis* isolates were sensitive to all antifungals tested (Table 3 and Table S2, Supplementary Material). All patients had been treated with fluconazole.

Table 3. *In vitro* antifungal susceptibility profiles of clinical *Candida parapsilosis* complex isolates obtained from bloodstream infections of patients (clinical isolates) from the University Hospital at Botucatu Medical School, São Paulo, Brazil.

Isolate (n)	Antifungals (µg/ml) range MIC	S	DDS	R
Candida parapsilosis s.s. C (n=38)	Am B (0.25 - 0.5)	38	-	-
	Flu (0.25 - 8)	31	5	2
	Vor (0.03 - 0.125)	38	-	-
	Cas (0.25 - 4)	35	3	-
	Mica (0.25 - 4)	35	3	-
Candida orthopsilosis C (n=7)	Am B (0.25 - 0.5)	7	-	-
	Flu (1 - 2)	7	-	-
	Vor (0.03 - 0.06)	7	-	-
	Cas (0.25 - 0.5)	7	-	-
	Mica (0.25 - 0.5)	7	-	-

n: number of isolates; *C. parapsilosis s.s.*: *C. parapsilosis sensu stricto*; C: clinical isolates; AmB: amphotericin B; Flu: fluconazole; Vor: voriconazole; Cas: caspofungin; Mica: micafungin; S: sensitive; DDS: dose-dependent susceptibility; R: resistant.

The genetic sequences of the hot spot 1 region of the FKS1 gene were determined for three *C. parapsilosis s.s.* isolates (85, 86 and 2 H-6), which were sensitive in a dose-dependent manner both to caspofungin and micafungin (MIC = 4). A point mutation that causes a substitution of the amino acid proline for alanine in this region, as described by Garcia-Effron et al. (2008) [20], was observed in these isolates.

DISCUSSION

Human infections caused by species belonging to the *C. parapsilosis* complex have increased in recent years throughout the world [5,6]. In several countries or geographical regions, the *C. parapsilosis* species complex ranks second or third in number of isolations from blood cultures, and in some cases, such as in Brazil, other Latin countries and Iran this group of yeasts ranks first in candidemia [10,33–40]. Particularly in our institution, a public 461-bed tertiary hospital, the *C. parapsilosis* species complex has been identified as the leading group causing bloodstream infections and fungal peritonitis in patients undergoing peritoneal dialysis, ahead of *C. albicans* species [41–43]. Understanding why the *C. parapsilosis* complex is increasingly relevant and occurs in the hospital environment was the main motive for conducting the present work.

Since 2005, when *C. parapsilosis* was recognized as a species complex composed of *C. parapsilosis s.s.*, *C. orthopsilosis* and *C. metapsilosis*, it has become mandatory to obtain the correct identification of these species, a task not only difficult but frequently inconclusive when using only conventional methods (phenotypic), despite advances in the use of commercial systems as VITEK-2 and API-32C [44,45]. By using rDNA sequencing (ITS and D1/D2 regions) as the gold standard, we succeeded in identifying all 59 clinical and environmental isolates belonging to the *C. parapsilosis* complex. Of these, 52 isolates (38 clinical and 14 environmental) were identified as *C. parapsilosis*

s.s., and 7 (all clinical) as *C. orthopsilosis*, which is similar to other studies that indicate *C. parapsilosis s.s* as the main species of the *psilosis* group [37,46–49].

We have also employed one additional molecular approach for *psilosis* species differentiation, based on intein profiles that do not require DNA sequencing. Inteins (internal proteins) are considered parasitic genetic elements, normally inserted in housekeeping coding genes, that have proven to be valuable for fungal identification, particularly in the differentiation of phylogenetically close species [13,50–52]. Three inteins – present in the genes of vacuolar membrane ATPase (VMA), threonyl-tRNA synthethase (ThrRS) and glutamate synthase (GLT1) – are relevant in yeasts of the genus *Candida* [53]. The analysis of VMA and ThrRS inteins has been suggested as a relatively simple and efficient method to discriminate among the three species of the *C. parapsilosis* complex, as well as to indicate the occurrence of two distinct genotypes (A and B subgroups) in *C. orthopsilosis* isolates [13,53]. We confirmed herein that the VMA intein was absent in all isolates of *Candida parapsilosis s.s.*, as expected. On the other hand, in *C. orthopsilosis*, the VMA intein was detected in all isolates, in at least one allele and, in three isolates, it was absent in one of the alleles (they are diploid), in a heterozygous condition, a finding not observed previously for this intein, but well documented for the ThrRS intein [13,15]. In fact, when the ThrRS intein was also herein analyzed, it was possible to detect the presence of two subgroups (A and B), confirming the recent findings indicating that the species *C. orthopsilosis*, in fact, is not a "typical species" with a single evolutionary origin but has emerged by hybridization processes between two distinct parental species, which must have occurred at a minimum of four distinct moments [15].

Since it becomes clear that the *psilosis* group harbors distinct biological entities, certainly with distinct habitats and ecological niches, it becomes necessary to carry out studies on virulence factors, antifungal susceptibility and geographic distribution of these species, and also on their ecological presence in the environment. In our data, the prevalent clinical species *C. parapsilosis*

s.s. was the only species recovered from hospital environmental sources, including surfaces (10/14), hands of health professionals (3/14) and air, corroborating other similar studies, including one in Portugal that also did not detect the environmental presence of *C. orthopsilosis* or *C. metapsilsosis* species [37,54]. Therefore, we should consider and properly evaluate whether the more abundance of *C. parapsilosis s.s*. in patients is in fact the result of its greater presence in the hospital environment (an ecological virulence factor), besides the additional traditional virulence factors.

The secretion of hydrolytic enzymes plays an important role in the pathogenesis of diseases caused by the *C. parapsilosis* complex, facilitating their adhesion, invasion and cellular damage in the host tissue. Significant differences and some contradictory findings in terms of proteinase and phospholipase activity have been observed within the *C. parapsilosis* complex [16,55–57]. In our data, proteinase activity was observed in clinical and environmental isolates of *C. parapsilosis s.s.* with positivity rates of 55.26% in clinical isolates (9 positive and 12 strongly positive) and 100% in the environmental isolates (14 isolates), respectively. Similar results were reported in a study by Tosun et al. (2013) [58], in which 34.2% of the isolates of *C. parapsilosis s.s.* (13/38) were proteinase-positive, whereas none of the isolates of *C. metapsilosis* (3 isolates) or *C. orthopsilosis* (1 isolate) showed activity by this enzyme. Other studies have also identified a higher proportion of proteinase production in *C. parapsilosis s.s.* isolates, with rates varying from 66.1% to 100% [16,59–62]. In relation to phospholipase, our findings agree with results from previous studies that detected little or no activity within the *C. parapsilosis* complex species [59,63]. In our study, it was found that all clinical isolates of *C. parapsilosis s.s.* and *C.orthopsilosis* presented negativity for phospholipase production, while only one environmental isolate of *C. parapsilosis s.s.* showed positivity (1/14) for this enzyme, reinforcing the existence of biological variation for this characteristic, particularly in environmental samples [55,64,65].

Biofilms are communities of microorganisms that grow in association with surfaces or adhering to them to produce an extracellular matrix (ECM) that is predominantly formed by polysaccharides, containing glucose and mannose residues that give them protection [66,67]. It is one of the most important virulence factors produced by yeast in patients using catheters or other medical devices. Its production can be estimated by several methods, including XTT, which measures the metabolic activity of biofilm-forming cells [67,68]. Kuhn et al. (2002) [69] in a study involving biofilm production using silicon disks and XTT showed that *C. albicans* produces larger amounts of biofilm, both quantitatively and qualitatively, when compared to *non-albicans* species, in particular *C. parapsilosis s.s*. Our results demonstrate that the *in vitro* biofilm production varies within isolates and between *C. parapsilosis s.s* and *C.orthopsilosis* species. Curiously, biofilm production was more prevalent in *C. orthopsilosis*, since in the seven clinical samples isolated, two were medium producers and one was a high producer, while in the 38 clinical isolates of *C. parapsilosis s.s*, only two were medium and the others were low producers. All the environmental isolates of *C. parapsilosis s.s* presented low biofilm production. Our findings are contrary to those of de Toro et al. (2011) [47] in which only *C. parapsilosis s.s.* were able to produce biofilm (65 of 111 isolates), but corroborate those of Treviño-Rangel et al. (2014) [56], which showed that *C.orthopsilosis* is the largest biofilm producer when determined by the same *in vitro* XTT reduction test. Variations in the results of studies involving biofilm production may be related to the different methodologies used or to the fact that the isolates come from different geographic regions [58]. In addition, the variation within *C. orthopsilosis* can also be explained by the existence of distinct genetic hybrid groups within the "same species" [13,15].

In relation to the antifungal susceptibility profile of the 45 clinical isolates, it was observed that all isolates (7/45) of *C. orthopsilosis* were sensitive to the antifungal agents tested (amphotericin B, fluconazole, voriconazole, caspofungin and micafungin). All isolates (38/45) of *C.*

parapsilosis s.s. were sensitive to amphotericin B and voriconazole, while five displayed dose-dependent susceptibility and two resistance to fluconazole. Studies report a decrease in susceptibility to fluconazole in *C. parapsilosis.*, probably as the result of the intensive use of this antifungal either for prophylaxis or treatment [25,70–72]. In our study three isolates of *C. parapsilosis s.s.* also presented dose-dependent susceptibility simultaneously to caspofungin and micafungin. It was already observed that the increased use of caspofungin in the University of Maryland Hospital is significantly correlated with the increased incidence of *C. parapsilosi*s candidemia [73]. In addition, the occurrence of isolates with low susceptibility to echinocandins has been reported while resistance to these drugs has been associated with mutations in conserved regions of Fks1 gene, the target enzyme for this antifungal group [20,74,75]. We sequenced the hot spot 1 region of the Fsk1 gene in the three isolates of *C. parapsilosis s.s.* that demonstrated dose-dependent susceptibility to caspofungin and micafungin, and confirmed the presence of the natural mutation at the 660 position that results in the replacement of a proline by an alanine, which is indicated as responsible for resistance or decreased susceptibility to these antifungals [20,75].

The mortality rate for yeasts belonging to the *C. parapsilosis* complex (*C. parapsilosis s.s.* and *C. orthopsilosis*) was high in our study (51%), and deaths occurred mainly in newborns and in those aged more than 60 years. These findings are very similar to a previous work by Mondelli et al. (2012) [43] accomplished in our same hospital, though covering a distinct period (2000 to 2006 year), in which the *C. parapsilosis* mortality rate was 51.4%, and affected mainly premature infants with deficiencies in the immune system and patients over 60 years of age. Related works carried out in other geographically close Brazilian public hospitals (less than 250km distant) also indicate high mortalities rates by *C. parapsilosis* complex that might exceed those of other countries [38,46,76,77]. In our data, it was not possible to correlate the clinical outcomes with species (*C. parapsilosis s.s.* versus *C. orthopsilosis*), patient sex, clinical ward, relapses, antifungal susceptibility or virulence

factors (proteinase, phospholipase and biofilm production), probably due to patient'sinsufficient records for further statistical comparisons.

CONCLUDING REMARKS

C. parapsilosis s.s. was the most prevalent species causing candidemia and also the most recovered from the hospital environment. The identification by molecular methods, such as the sequencing of the ITS1-5.8S-ITS2 or D1/D2 regions and the VMA and ThrRS inteins have been shown to be useful for differentiating these cryptic species and also to confirm the uncommon hybrid pattern of *C. orthopsilosis* species. As for virulence profiles, proteinase production is much more common in *C. parapsilosis s.s.,* including the environmental isolates. Phospholipase production seems to be unimportant in the *C. parapsilosis* complex. Biofilm production was low or infrequent in *C. parapsilosis s.s.* species, but unexpectedly more intense in *C. othopsilosis*. As to the clinical outcome, a high mortality rate (51%) was observed among yeasts of the *C. parapsilosis* complex (*C. parapsilosis s.s*. and *C. orthopsilosis*), especially in neonates and in individuals over 60 years of age. A correct species differentiation within the *C. parapsilosis* complex and a better understanding of their ecology, virulence factors and antifungal susceptibilities should provide better strategies in the treatments and prevention of new infections, with positive impacts on the life quality and survival of the patients.

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Table S1. Molecular identification of clinical and environmental *Candida parapsilosis* complex isolates from University Hospital of Botucatu Medical School, São Paulo, Brazil, by DNA sequencing of ITS1-5.8S-ITS2 or D1/D2 rDNA regions, and intein profiles of Vacuolar Membrane ATPase (VMA) and Threonyl-tRNA Synthetase (ThrRS) inteins.

Isolate	ITS1-5.8S-ITS2 or D1/D2 sequencing	Gen Bank Access (ID – access number)	Intein Profiles	
			VMA	ThrRS
1C	*C. parapsilosiss.s.*	KX682400	*C. parapsilosiss.s*	
2C	*C. parapsilosiss.s.*	KX682401	*C. parapsilosiss.s*	
4C	*C. parapsilosiss.s.*	KX682402	*C. parapsilosiss.s*	
17C	*C. parapsilosiss.s.*	KX682403	*C. parapsilosiss.s*	
21C	*C. parapsilosiss.s.*	KX682404	*C. parapsilosiss.s*	
22C	*C. parapsilosiss.s.*	KX682405	*C. parapsilosiss.s*	
26C	*C. parapsilosiss.s.*	KX682406	*C. parapsilosiss.s*	
30C	*C. orthopsilosis*	KX682407	ortho/para*	group A
32C	*C. orthopsilosis*	KX682408	ortho/para*	group A
38C	*C. parapsilosiss.s.*	KX682409	*C. parapsilosiss.s*	
39C	*C. parapsilosiss.s.*	KX682410	*C. parapsilosiss.s*	
40C	*C. orthopsilosis*	KX682411	*C. orthopsilosis*	group A
44C	*C. parapsilosiss.s.*	KX682412	*C. parapsilosiss.s*	
50C	*C. orthopsilosis*	KX682413	Inconclusive	Not done
55C	*C. parapsilosiss.s.*	KX682414	*C. parapsilosiss.s*	
59C	*C. parapsilosiss.s.*	KX682415	*C. parapsilosiss.s*	
60C	*C. parapsilosiss.s.*	KX682416	*C. parapsilosiss.s*	
64C	*C. parapsilosiss.s.*	KX682417	*C. parapsilosiss.s*	
77C	*C. orthopsilosis*	KX682418	*C. orthopsilosis*	group A
83C	*C. parapsilosiss.s.*	KX682419	*C. parapsilosiss.s*	
84C	*C. parapsilosiss.s.*	KX682420	*C. parapsilosiss.s*	
85C	*C. parapsilosiss.s.*	KX682421	*C. parapsilosiss.s*	
86C	*C. parapsilosiss.s.*	KX682422	*C. parapsilosiss.s*	
87C	*C. parapsilosiss.s.*	KX682423	*C. parapsilosiss.s*	
90C	*C. orthopsilosis*	KX682424	*C. orthopsilosis*	group B
91C	*C. parapsilosiss.s.*	KX688042	*C. parapsilosiss.s*	
94C	*C. parapsilosiss.s.*	KX714079	*C. parapsilosiss.s*	
99C	*C. parapsilosiss.s.*	KX714080	*C. parapsilosiss.s*	
100C	*C. parapsilosiss.s.*	KX714081	*C. parapsilosiss.s*	
107C	*C. parapsilosiss.s.*	KX714082	*C. parapsilosiss.s*	
109C	*C. parapsilosiss.s.*	KX714083	*C. parapsilosiss.s*	
113C	*C. parapsilosiss.s.*	KX714084	*C. parapsilosiss.s*	
117C	*C. parapsilosiss.s.*	KX714085	*C. parapsilosiss.s*	
118C	*C. parapsilosiss.s.*	KX714086	*C. parapsilosiss.s*	
120C	*C. parapsilosiss.s.*	KX714087	*C. parapsilosiss.s*	
126C	*C. parapsilosiss.s.*	KX714088	*C. parapsilosiss.s*	
130C	*C. orthopsilosis*	KX714089	ortho/para*	group B
134C	*C. parapsilosiss.s.*	KX714090	*C. parapsilosiss.s*	
138C	*C. parapsilosiss.s.*	KX714091	*C. parapsilosiss.s*	
154C	*C. parapsilosiss.s.*	KX714092	*C. parapsilosiss.s*	
155C	*C. parapsilosiss.s.*	KX714093	*C. parapsilosiss.s*	
158C	*C. parapsilosiss.s.*	KX714094	*C. parapsilosiss.s*	
165C	*C. parapsilosiss.s.*	KX714095	*C. parapsilosiss.s*	
174C	*C. parapsilosiss.s.*	KX714096	*C. parapsilosiss.s*	
2 (H-6)C	*C. parapsilosiss.s.*	KX781709	*C. parapsilosiss.s*	
6E	*C. parapsilosiss.s.*	KX682398	*C. parapsilosiss.s*	
10E	*C. parapsilosiss.s.*	KX682399	*C. parapsilosiss.s*	
21E	*C. parapsilosiss.s.*	KX714097	*C. parapsilosiss.s*	
24E	*C. parapsilosiss.s.*	KX714098	*C. parapsilosiss.s*	
27E	*C. parapsilosiss.s.*	KX714099	*C. parapsilosiss.s*	
29E	*C. parapsilosiss.s.*	KX714100	*C. parapsilosiss.s*	
32E	*C. parapsilosiss.s.*	KX714101	*C. parapsilosiss.s*	
34E	*C. parapsilosiss.s.*	KX714103	*C. parapsilosiss.s*	
37E	*C. parapsilosiss.s.*	KX714104	*C. parapsilosiss.s*	
38E	*C. parapsilosiss.s.*	KX714105	*C. parapsilosiss.s*	
47E	*C. parapsilosiss.s.*	KX714106	*C. parapsilosiss.s*	
56E	*C. parapsilosiss.s.*	KX714107	*C. parapsilosiss.s*	
63E	*C. parapsilosiss.s.*	KX714108	*C. parapsilosiss.s*	
67E	*C. parapsilosiss.s.*	KX714109	*C. parapsilosiss.s*	

C: clinical isolates (blood culture isolate); *C. parapsilosis s.s.*: *Candida parapsilosis sensu stricto*; E: environmental isolates. * It presents a hybrid pattern of both species.

Table S2. Collective data of the patients, virulence factors (phospholipase, proteinase and biofilm) and antifungal susceptibility of the *C. parapsilosis* complex isolates, obtained from the University Hospital of Botucatu Medical School, São Paulo, Brazil.

Patient/ Isolate	Sex	Age	Clinical Ward	Relapse	Proteinase activity*	Phospholipase activity*	Biofilm production*	Antifungal Suscepibility** (A1/A2/A3/A4/A5)	Species	Clinical Outcome
1C		27 days	Neonatal ICU	N	Negative	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Death
2C	M	44 years	Emergency Room	N	Negative	Negative	Low	S/S/S/ S/S	*C. parapsilosis s.s.*	Death
4C	M	74 years	Urology	N	Negative	Negative	Low	S/R/S/ S/S	*C. parapsilosis s.s.*	Death
17C	M	15 years	Pediatrics	Y	Negative	Negative	Low	S/S/S/ S/S	*C. parapsilosis s.s.*	Cure
21C			Neonatal ICU	Y	Negative	Negative	Medium	S/S/S/S/S	*C. parapsilosis s.s.*	Cure
22C	M	71 years	Emergency Room ICU	N	Negative	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Death
26C	M	52 years	Emergency Room	N	Negative	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Death
30C	F			N	Negative	Negative	Low	S/S/S/S/S	*C. orthopsilosis*	Cure
32C	M	1 year	Pediatrics	Y	Negative	Negative	Low	S/S/S/S/S	*C. orthopsilosis*	Cure
38C	F	4 months	Pediatrics	N	Negative	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Cure
39C	M	42 years	Gastroclinic	N	Strongly Positive	Negative	Medium	S/S/S/S/S	*C. parapsilosis s.s.*	Death
40C	F	74 years	Urology	N	Negative	Negative	Low	S/S/S/S/S	*C. orthopsilosis*	
44C	M	37 years	Central ICU	N	Positive	Negative	Low	S/S/S/S/S	*C. parapsilosi s.s.s*	Cure
50C	F	60 years	Urology	N	Negative	Negative	Medium	S/S/S/S/S	*C. orthopsilosis*	Death
55C	F	34years	Ginecology	Y	Strongly Positive	Negative	Low	S/DDS/S/S/S	*C. parapsilosis s.s.*	Death
59C	M	7 months	Pediatrics	N	Positive	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	
60C		15 days	Neonatal ICU	N	Negative	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Death
64C	M	72 years	Neurosurgery	N	Strongly Positive	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Death
77C	M	10 years	Pediatrics	N	Negative	Negative	Low	S/S/S/S/S	*C. orthopsilosis*	
83C	M	59 years	Emergency Room ICU	N	Positive	Negative	Low	S/DDS/S/S/S	*C. parapsilosis s.s.*	
84C	F			N	Negative	Negative	Low	S/DDS/S/S/S	*C. parapsilosis s.s.*	Cure
85C	F	30 years	Central ICU	N	Negative	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	
86C	F			N	Negative	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Cure
87C	F	9 days	Neonatal ICU	N	Negative	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Cure
90C		28 days	Neonatal ICU	N	Negative	Negative	Medium	S/S/S/S/S	*C. orthopsilosis*	Death
91C	F	61 years	Hematology	N	Positive	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Death
94C	F	28 years	Ginecology	N	Positive	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	
99C	F	86 years	Central ICU	N	Positive	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Death
100C	F			N	Positive	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Cure
107C	F	71 years	Cardiac Surgery	N	Strongly Positive	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Cure
109C	M	4 months	Pediatrics	Y	Negative	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Death
113C	F	57 years	Emergency Room	N	Strongly Positive	Negative	Low	S/R/S/S/S	*C. parapsilosis s.s.*	
117C		27 days	Neonatal ICU	N	Strongly Positive	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Death
118C	F	16 years	Ginecology		Positive	Negative	Low	S/DDS/S/S/S	*C. parapsilosis s.s.*	
120C	F	72 years	Cardiac Surgery	N	Strongly Positive	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Death
126C	M	11 months	Pediatrics	Y	Negative	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	
130C	M	4 months	Pediatrics	Y	Negative	Negative	High	S/S/S/S/S	*C. orthopsilosis*	Cure
134C	F	40 years	Emergency Room	N	Strongly Positive	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Cure
138C	F			N	Strongly Positive	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Cure
154C	F	36 years	Central ICU	N	Strongly Positive	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	
155C	M	48 years	Urology	N	Strongly Positive	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Cure
158C	M	48 years	Urology	N	Positive	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Cure
165C	F	78 years	Emergency Room	N	Negative	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Death
174C	F	6 months	Pediatrics	N	Strongly Positive	Negative	Low	S/S/S/S/S	*C. parapsilosis s.s.*	Cure
2 (H-6)C	F	57 years	Central ICU	N	Negative	Negative	Low	S/DDS/S/DDS/DDS	*C. parapsilosis s.s.*	Death
Fisher Test* **	NS	**P=0.04**	NS	NS	<u>P = 0.03</u>	NS	<u>P = 0.02</u>	NS	NS	

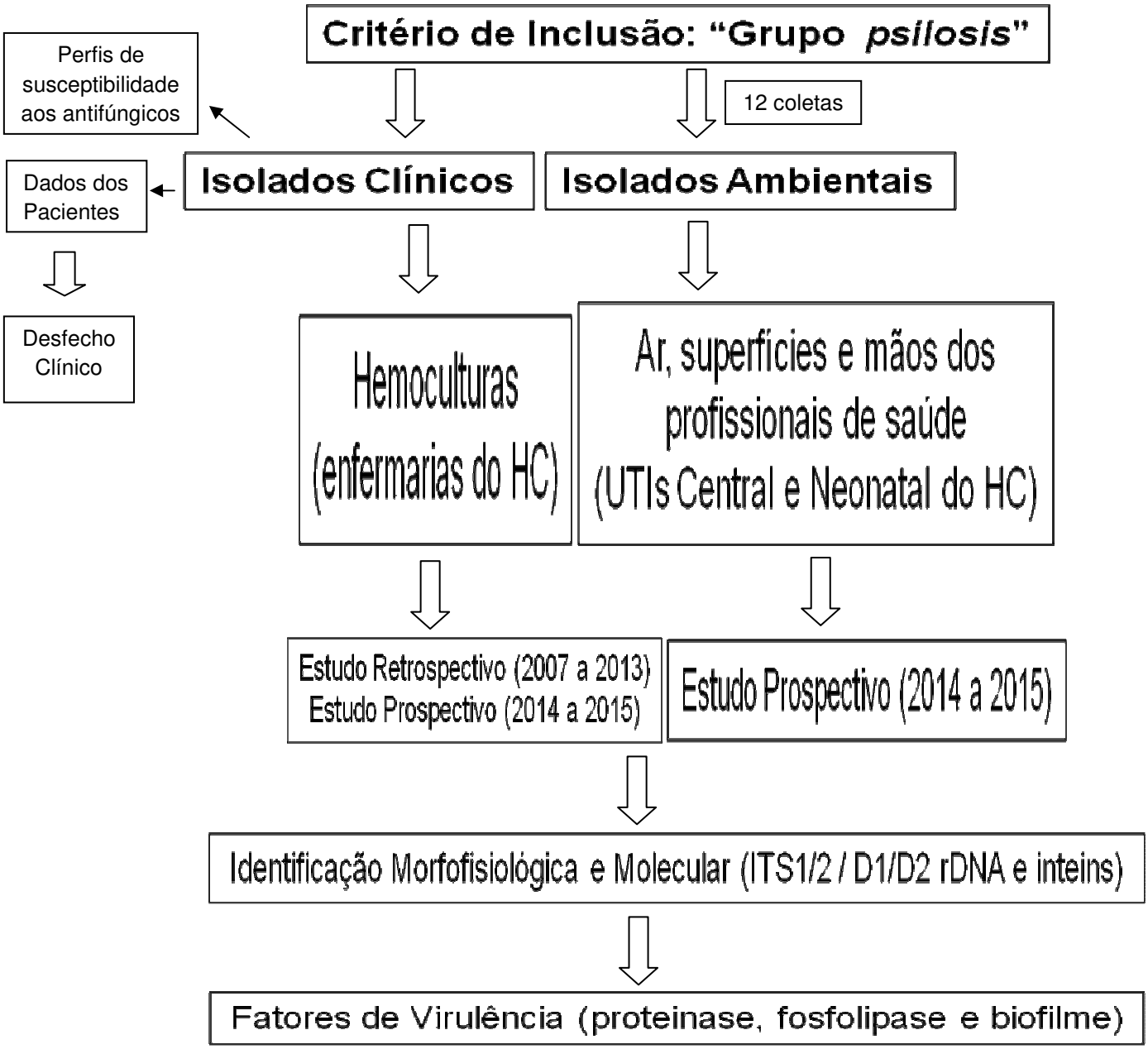
* Proteinase and Phospholipase activity: Pz =1 (negative); Pz ≥ 0,64 < 1 (positive) and Pz < 0,64 (strongly positive); Biofilm values (ODs): values of average ofoptical densities; Biofilm production: high, medium and low production (based on average of optical densities: < 0,176 low production; 0,176 – 0,353 medium production; > 0,353 high production); N.I.: not informed. *C. parapsilosis s. s.* (*Candida parapsilosis sensu stricto*).

** Antifungal tested: Amphotericin B (A1); Fluconazole (A2); Voriconazole (A3); Caspofungin (A4); Micafungin (A5). S: sensitive; DDS: dose-dependent susceptibility; R: resistance.

***Significant Fisher's Test values, considering association with the clinical outcomes (P values in bold) or with species (P values underlined).

MATERIAIS E MÉTODOS

FLUXOGRAMA



Análise do padrão dos polimorfismos mediante a técnica de PFGE de fragmentos de restrição em isolados do complexo *C. parapsilosis* de hemoculturas, do ar, superfícies e mãos dos profissionais de saúde.

Diversos protocolos já foram realizados. Foi possível obter a separação dos cromossomos inteiros (sem o uso de enzima de restrição) (Figura A1), mas encontramos dificuldades na fase em que foi necessário o uso das enzimas de restrição (NOTI e BssHII) de forma a proporcionar um maior padrão de bandas discriminatórias.

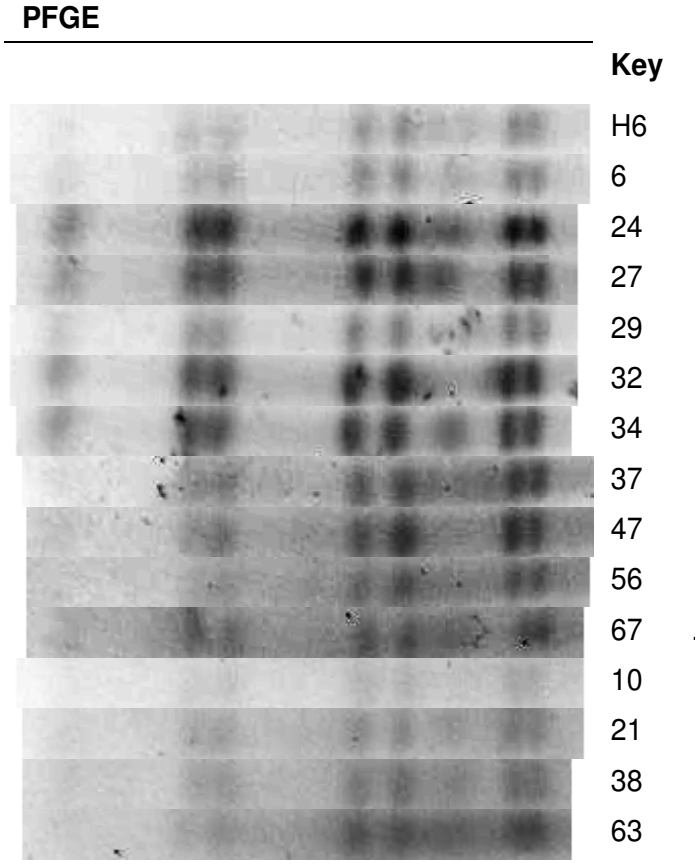


Figura A1. Separação de cromossomos inteiros pela técnica de Pulse-Field-Gel-Electrophoresys (PFGE) em isolados clínicos (H-6) e ambientais (os demais) de *C. parapsilosis sensu stricto.*

frontiers in Microbiology

REVIEW
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Evolution and Application of Inteins in *Candida* species: A Review

*José A. L. Fernandes[1†], Tâmara H. R. Prandini[2†], Maria da Conceição A. Castro[1], Thales D. Arantes[1,3], Juliana Giacobino[2], Eduardo Bagagli[2] and Raquel C. Theodoro[1]**

[1] Institute of Tropical Medicine of Rio Grande do Norte, Universidade Federal do Rio Grande do Norte, Natal, Brazil, [2] Department of Microbiology and Immunology, Institute of Biosciences, Universidade Estadual Paulista Julio de Mesquita Filho, Botucatu, Brazil, [3] Post-graduation Program in Biochemistry, Universidade Federal do Rio Grande do Norte, Natal, Brazil*

Inteins are invasive intervening sequences that perform an autocatalytic splicing from their host proteins. Among eukaryotes, these elements are present in many fungal species, including those considered opportunistic or primary pathogens, such as *Candida* spp. Here we reviewed and updated the list of *Candida* species containing inteins in the genes *VMA*, *THRRS* and *GLT1* and pointed out the importance of these

Participação co-autoria em **"Evolution and Application of Inteins in *Candida* species: a Review".**

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Molecular identification and phylogenetical analysis of dermatophyte fungi from Latin America.

Garcia Garces H[1], Hrycyk MF[1], Giacobino J[1], Capela Machado G[1], Domingos Arantes T[2], Theodoro RC[2], Bosco SM[1], Bagagli E[1].

⊕ Author information

Abstract

Dermatophytes constitute a complex group of fungi, comprised of by the genera Trichophyton, Epidermophyton and Microsporum. They have the ability to degrade keratin and cause human and animal infections. Molecular techniques have made their identification faster and more accurate, and allowed important advances in phylogenetic studies. We aim to identify molecularly and to determine the phylogenetic relationships in dermatophyte fungi from Brazil and other Latin American countries, using DNA sequencing of the nuclear ribosome regions ITS1-5.8S-ITS2 and D1/D2. DNA of 45 dermatophytes was extracted and amplified by PCR for identification at the species level by sequencing of those ribosomal regions. The software mega 6.0 was used to establish the phylogenetic relationships via the Maximum Likelihood method. Out of 45 strains, 43 were identified by ITS (95.5%) and 100% by D1/D2 sequencing. Two strains could not be identified by ITS. Phylogenetic analyses separated the genera Trichophyton and Microsporum, which presented an uncertain relationship with Epidermophyton floccosum, depending on the ribosomal marker. Both regions can provide efficient identification of dermatophytes, whereas phylogenetic analysis revealed complex relations among dermatophyte fungi.

KEYWORDS: Arthrodermataceae; Dermatophytes; molecular identification; phylogeny

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Fungal peritonitis in patients undergoing peritoneal dialysis (PD) in Brazil: molecular identification, biofilm production and antifungal susceptibility of the agents.

Giacobino J[1], Montelli AC[1], Barretti P[2], Bruder-Nascimento A[1], Caramori JT[2], Barbosa L[3], Bagagli E[1].

⊕ **Author information**

Abstract

This paper presents data on fungal peritonitis (FP) in patients undergoing peritoneal dialysis (PD) at the University Hospital of Botucatu Medical School, São Paulo, Brazil. In a total of 422 patients, 30 developed FP, from which the medical records and the fungal isolates of 23 patient cases were studied. All patients presented abdominal pain, cloudy peritoneal effluent, needed hospitalization, had the catheter removed and were treated with fluconazole or fluconazole plus 5-flucitosine; six of them died due to FP. Concerning the agents, it was observed that Candida parapsilosis was the leading species (9/23), followed by Candida albicans (5/23), Candida orthopsilosis (4/23), Candida tropicalis (3/23), Candida guilliermondii (1/23), and Kodamaea ohmeri (1/23). All the isolates were susceptible to amphotericin B, voriconazole and caspofungin whereas C. albicans isolates were susceptible to all antifungals tested. Resistance to fluconazole was observed in three isolates of C. orthopsilosis, and dose-dependent susceptibility to this antifungal was observed in two isolates of C. parapsilosis and in the K. ohmeri isolate. Biofilm production estimates were high or moderate in most isolates, especially in C. albicans species, and low in C. parapsilosis species, with a marked variation among the isolates. This Brazilian study reinforces that FP in PD is caused by a diverse group of yeasts, most prevalently C. parapsilosis sensu stricto species. In addition, they present significant variation in susceptibility to antifungals and biofilm production, thus contributing to the complexity and severity of the clinical features.

KEYWORDS: Candida spp.; antifungal susceptibility; biofilm; peritoneal dialysis; peritonitis

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