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Distribuição, fatores de virulência e suscetibilidade a antifúngicos de isolados clínicos do complexo *Candida parapsilosis* em Uberaba – MG.

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Dissertação apresentada ao curso de Pós-Graduação em Ciências Fisiológicas da Universidade Federal do Triângulo Mineiro, como requisito parcial para a obtenção do título de mestre em Ciências Fisiológicas.
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RESUMO

Nos últimos anos tem havido um aumento na incidência de infecções humanas causadas por membros do complexo *Candida parapsilosis*. Este complexo é formado por três espécies distintas, mas intimamente relacionadas: *C. parapsilosis sensu stricto*, *C. orthopsilosis* e *C. metapsilosis*. Estas espécies são fisiologicamente e morfologicamente indistinguíveis e por isso são discriminadas apenas por meio de técnicas moleculares. Considerando o pouco conhecimento sobre a distribuição, propriedades de virulência e perfil de suscetibilidade a antifúngicos das espécies do complexo *C. parapsilosis*, nossos objetivos foram: 1) determinar pela técnica de PCR-RFLP a ocorrência de *C. parapsilosis sensu stricto*, *C. orthopsilosis* e *C. metapsilosis* entre 81 isolados clínicos primariamente identificados como *C. parapsilosis* por meio de métodos fenotípicos; 2) avaliar a capacidade dos isolados formarem biofilmes e produzirem proteases e fosfolipases; e 3) comparar o perfil de suscetibilidade entre estes isolados crescidos como culturas planctônicas ou como biofilmes formados *in vitro*, aos antifúngicos anfotericina B, fluconazol, voriconazol e caspofungina, seguindo as normas do CLSI (Clinical and Laboratory Standards Institute). Setenta e sete isolados (95%) foram identificados como *C. parapsilosis sensu stricto*, 2 (2,5%) como *C. orthopsilosis* e os outros 2 (2,5%) como *C. metapsilosis.* Atividade de proteases foi detectada em 37,7% das amostras de *C. parapsilosis sensu stricto*, enquanto somente 9,1% apresentaram atividade de fosfolipases. Nenhuma das amostras de *C. orthopsilosis* e *C. metapsilosis* foi capaz de produzir proteases ou fosfolipases. A capacidade de produzir biofilmes foi detectada em 43,2% das amostras, entre as quais 33 eram *C. parapsilosis sensu stricto* e 2 eram *C. orthopsilosis*. Isolados com perfil de resistência a antifúngicos foram incomuns: apenas um *C. metapsilosis* apresentou resistência ao fluconazol. Este mesmo isolado foi o único a apresentar suscetibilidade dose dependente ao voriconazol. Além disso, foi detectado um *C. parapsilosis sensu stricto* com suscetibilidade dose dependente ao fluconazol. Em suma, confirmamos que *C. parapsilosis sensu stricto* é a espécie do complexo *C. parapsilosis* mais comumente associada a infecções em humanos. Além disso, esta espécie foi a única que expressou todos os fatores de virulência analisados. Ao contrário, nenhum destes fatores foi detectado em *C. metapsilosis*. De modo geral, as espécies do complexo *C. parapsilosis* foram suscetíveis à anfotericina B, ao voriconazol e à caspofungina, e apresentaram baixo nível de resistência ao fluconazol. Contudo, os isolados produtores de biofilme mostraram acentuada resistência a estes antifúngicos, particularmente ao voriconazol.

Palavras-chave: Complexo *Candida parapsilosis*. Fatores de virulência. Formação de biofilme. Produção de proteinases e fosfolipases. Perfil de suscetibilidade a antifúngicos.

LISTA DE ABREVIATURAS E SIGLAS

AMB - Anfotericina B

CAPD - Diálise Peritoneal Ambulatorial Contínua

CAS - Caspofungina

CIM - Concentração inibitória mínima

CLSI - Clinical and Laboratory Standards Institute

CNA - *Candida* não-*albicans*

CVV - Candidíase vulvovaginal

FLC - Fluconazol

PCR - Polymerase Chain Reaction

RFLP - Restriction Fragment Length Plymorphisms

SADH - Desidrogenase alcoólica secundaria

SAP – Proteinase aspártica secretada

VRZ – Voriconazol

XTT- 2,3-bis (2-metoxi-4nitro-5-sulfofenil) – 5 – (fenilamnocarbonil-tetrazolium)

SUMARIO

1. CONSIDERAÇÕES GERAIS

Nas últimas três décadas observou-se um aumento da incidência de infecções fúngicas. Estes microrganismos podem causar desde infecções superficiais, afetando a pele, cabelo, unhas e membranas mucosas, até infecções sistêmicas, envolvendo a maioria dos órgãos do corpo. A severidade das infecções é influenciada por vários fatores, como a utilização de imunossupressores, cateteres e próteses (Lass-Flör, 2009; Ruping et al., 2008).

Entre os fungos, o gênero *Candida* pertence ao grupo das leveduras, que são microrganismos unicelulares e pleomórficos. Este gênero possui cerca de 150 espécies, das quais 65% são incapazes de crescer à temperatura de 37°C, o que limita a capacidade patogênica destas espécies em seres humanos ou mesmo a possibilidade de serem comensais (Silva et al., 2012). Por outro lado, algumas espécies são componentes da microbiota da pele, do trato gastrintestinal, do trato urinário, do trato respiratório e também das cavidades oral e vulvovaginal em indivíduos saudáveis (Hossain et al.,2003).

Entre as espécies de *Candida* com potencial patogênico, *C. albicans* tem sido historicamente a espécie mais comumente recuperada de amostras clínicas, embora o número de infecções por espécies de *Candida* não-*albicans* (CNA), principalmente *Candida glabrata*, *Candida parapsilosis* e *Candida tropicalis*, tenha aumentado dramaticamente nos últimos anos (Fidel et al.,1999; Pfaller,Diekima 2007;Trofa et al.,2008; van Asbeck et al.,2009;).

O aparente aumento do envolvimento de espécies de CNA em candidíases humanas poderia ser relacionado, pelo menos em parte, ao melhoramento dos métodos diagnósticos, tais como o uso de ágar para isolamento primário com a habilidade de diferenciar as espécies de *Candida* e a introdução de técnicas de diagnóstico molecular na rotina (Liguori. et al. 2009). Outros fatores poderiam ser implicados no aumento da prevalência de espécies de *Candida*, incluindo a introdução e difusão de certas práticas médicas, tais como terapia imunossupressora, o uso de antibióticos de amplo espectro, e o aumento no número de procedimentos cirúrgicos invasivos, como transplantes de órgãos (Kojic and Darouichee, 2004). Além disso, o aumento do número de espécies de *Candida* em candidíases poderia ser um reflexo da seleção de espécies na presença de certos antifúngicos, visto o maior nível de resistência demonstrada para várias espécies de CNA (Gravina et al. 2007).

Em 1928, a *C. parapsilosis* foi isolada em um paciente em Porto Rico com diarreia. Naquela época foi classificada por *Monilia parapsilosis*, sendo assim considerada do gênero *Monilia*, devido a sua incapacidade de fermentar maltose (Ashford, 1928). A espécie foi

nomeada *Monilia parapsilosis* para distinguir do isolado mais comum, *Monilia psilosis*, conhecida hoje como *C. albicans* (Trofa et al.,2008).

Tradicionalmente, essa espécie era separada em três grupos, I, II e III, de acordo com dados fenotípicos e moleculares (Lott et al.,1993), até que em 2005 Tavanti e colaboradores, com base em estudos genéticos moleculares, propuseram a separação dos grupos em distintas espécies intimamente relacionadas. Os grupos II e III foram transformados em *Candida orthopsilosis* e *Candida metapsilosis*, respectivamente, enquanto o grupo I permaneceu como *C. parapsilosis sensu stricto*. Os autores demonstraram que tal separação pode ser baseada na análise de polimorfismo de restrição do gene que codifica a desidrogenase alcoólica secundária (SADH), o qual é comum para todas as três espécies (Tavanti et al., 2005).

Quanto à sua morfologia, as células do complexo *C. parapsilosis* se apresentam de diversas formas, ovais, redondas e cilíndricas, sendo as colônias lisas ou rugosas. Quando cultivadas em ágar sabouraud dextrose, as colônias são brancas, cremosas, brilhantes e lisas ou enrugadas. Essa espécie não produz hifas verdadeiras, mas pode apresentar pseudo-hifas que são caracteristicamente grandes e curvas, sendo consideradas como células gigantes (Trofa et al., 2008).

2. EPIDEMIOLOGIA

C. parapsilosis é encontrada na natureza, não sendo considerada como um patógeno humano obrigatório, tendo sido isolada em animais domésticos, solo e em ambientes marinhos (Fell et al., 1967; Bernardis et al., 1999; Kuhn et al., 2004; Tosun et al., 2013; Weems et al., 1992).

Dentre as CNA, é um dos mais importantes agentes causadores de infecções fúngicas, e sua prevalência vem aumentando significativamente a cada ano (Silva et al., 2011; Trofa et al., 2008; Silva et al., 2014). É comum em ambientes hospitalares, sendo a segunda espécie de *Candida* mais isolada em amostras de hemocultura (Almirante et al 2006; Pfaller e Diekema, 2007; Trofa et al., 2008). É um patógeno oportunista, principalmente entre os recém-nascidos e pacientes imunodeprimidos (Almirante et al 2006; Pfaller e Diekema, 2007; Roilides et al.,2004; Safdar et al., 2002; Trofa et al.,2008).

A população com o maior risco de infecções hospitalares são os neonatos de baixo peso, uma vez que estes possuem integridade da pele comprometida e são mais suscetíveis por utilizarem cateteres por longos períodos (Benjamin et al., 2004; Bonassoli et al.,2005).

Estudos mostram que a *C. parapsilosis* vem desempenhando um papel importante em micoses superficiais, em particular nas onicomicoses, sendo inclusive algumas vezes implicada como a espécie de *Candida* mais comumente associada a esta condição. Segundo Ataides et al. (2012), em um estudo realizado em Goiânia, GO, a espécie mais frequente foi a *C. parapsilosis*, representando 53,4% das leveduras isoladas de onicomicoses. Um estudo recentemente publicado pelo nosso grupo mostrou resultados semelhantes, ou seja, uma alta prevalência de *C. parapsilosis* entre os agentes etiológicos de dermatomicoses, particularmente onicomicoses, em pacientes de Uberaba, MG (Silva et al., 2014).

Um estudo realizado entre 2004 e 2008 apontou a *C. parapsilosis* como a terceira causa de infecções invasivas, sendo que 34,8% dos casos foram associados à fungemia, e a *C. parapsilosis* foi mais prevalente em pacientes jovens com idade media de 47,8 anos (Pfaller et al.,2012).

Apesar do reconhecimento do complexo *C. parapsilosis*, até o momento pouco se sabe sobre a epidemiologia das duas espécies novas do complexo, *C. orthopsilosis* e *C. metapsilosis* (Lockhart et al., 2008). Apenas dois surtos foram notificados em que a *C. orthopsilosis* (*C. parapsilosis* grupo II) estava envolvida. O primeiro deles foi notificado em um hospital na cidade de San Antonio do estado americano do Texas relatado por Lin et al.(1995), onde estudos moleculares demostraram que a maioria dos isolados estavam relacionados com o grupo II e o outro por Zancopé-Oliveira et al. (2000), que descreveram um pequeno surto dessa espécie em um hospital no Brasil.

Um estudo realizado por Tosun et al. (2013) demostraram que os isolados de *C. metapsilosis* e *C. orthopsilosis* estavam associados a pacientes mais velhos. Estes resultados estão parcialmente de acordo com estudos anteriores que mostraram forte associação apenas de *C. orthopsilosis* com pacientes idosos (≥ 60 anos) (Lockhart et al., 2008; Catón et al., 2011).

De acordo com vários estudos, dentre as espécies do complexo *C. parapsilosis,* a *C. parapsilosis sensu stricto* é a mais prevalente em infecções humanas. Borghi et al. (2011) demostraram taxas de prevalência de 95%, 3,6% e 1,4% para *C. parapsilosis sensu stricto*, *C. orthopsilosis* e *C. metapsilosis*, respectivamente, em infecções fúngicas invasivas. Feng et al. (2012) descreveram resultados semelhantes, porém para candidíase superficial, onde a *C. parapsilosis sensu stricto* representou 96,5% dos isolados, seguida pela *C. metapsilosis* (2.5%) e *C. orthopsilosis* (1.0%). No mesmo ano Tosun et al. (2013) avaliaram a distribuição das espécies do complexo em amostras clínicas oriundas tanto infecções superficiais quanto de infecções profundas, e demostraram que *C. orthopsilosis* e *C. metapsilosis* juntas

representavam menos de 10% dos agentes etiológicos destas infecções. Isto também foi demostrado por Trevino-rangel et al. (2012), com as seguintes frequências de isolamento: *C. parapsilosis sensu stricto* (90,4%), *C. othopsilosis* (8,4%) e *C. metapsilosis* (1,2%).

Apesar destes dados, a caracterização do complexo *C.parapsilosis* ainda não está concluída, por isso são necessários mais estudos para compreender melhor as características, principalmente a distribuição de cada membro do complexo, especialmente *C. orthopsilosis* e *C. metapsilosis*.

3. MANIFESTAÇÕES CLÍNICAS

As espécies do complexo *C. parapsilois* são responsáveis por uma ampla variedade de manifestações clinicas sendo consideradas agentes patogênicos oportunistas. As infecções causadas por estas espécies vão desde doenças invasivas, como fungemia, endocardite e peritonite que geralmente ocorrem em associação com procedimentos invasivos ou dispositivos médicos, a infecções superficiais como a onicomicose. Diversos estudos mostram estas espécies causando candidemias (Cantón et al., 2011; Garcia-Effron et al., 2012; Levy et al., 1998; Miranda et al., 2012; Romeo et al., 2012; Ruiz et al., 2013; van Asbeck et al., 2007), as quais resultam em uma taxa de mortalidade entre 4% a 45% (Trofa et al., 2008; van Asbeck et al., 2009).

A incidência de endocardites fúngicas vem aumentando ao logo das duas últimas décadas, sendo responsáveis por cerca 1,3% a 6% dos casos de endocardite infecciosa. Isso pode estar relacionado com a melhora de métodos diagnósticos e com o aumento da intensidade de terapias médicas que predispõem os pacientes às infecções fúngicas (Garzoni et al.,2007; Pierrotti et al.,2002)

As espécies de *Candida* são os agentes mais comuns envolvidos na endocardite fúngica, seguidas pelas espécies de *Aspergillus* (Abgueguen et al., 2002) e a *C. parapsilosis* é identificada em 17% dos casos, tornando-se a segunda espécie mais comum após *C. albicans* (Garzoni et al., 2007). Em relação à peritonite causada por fungo, embora incomum, está associada a pacientes submetidos à diálise peritoneal ambulatorial contínua (CAPD), tendo uma taxa de mortalidade entre 5% a 25% dos casos (Wang et al., 2000). Vários trabalhos tem relatado que a *C. parapsilosis* é a espécie predominante nestes casos (Chen et al., 2006; Wang et al., 2000; Yinnon et al., 1999).

As otomicoses constituem infecções relativamente raras que causam otite média ou externa. No entanto, quando isso ocorre, *C. parapsilosis*, é a primeira ou segunda espécie a ser isolada (Vennewald et al., 2003).

Esta espécie era raramente mencionada como um agente causador de lesões patológicas das unhas, mas nos últimos anos ganhou reconhecimento como agente etiológico mais comum causando onicomicose, devido a sua capacidade de decompor queratina (Ataides et al.,2012; Figueiredo et al., 2007; McGinley et al.,1988; Silva et al., 2014; Vermelho et al., 2010). Outros fatores podem estar relacionados com o isolamento de *C. parapsilosis* em infecções de unhas: distrofia traumática anterior e atividade de jardinagem, uma vez que esta levedura pode frequentemente ser isolada a partir do solo (Gautret et al., 2000).

Nas meningites causadas por fungos a *C. parapsilosis* é uma espécie rara, principalmente em adultos (Trofa et al., 2008; Bagheri et al., 2010). A artrite fúngica ocorre com pouca frequência e está mais relacionada com as espécies de *Candida*. Um estudo realizado em 1992 demostrou oito casos de artrites causadas por *C. parapsilosis*, dos quais sete sofreram intervenção cirúrgica, com colocação de uma prótese articular (Weems et al.,1992).

A candidíase vulvovaginal (CVV) é uma infecção comum, afetando 70-75% das mulheres em idade fértil, podendo ser recorrente (Foxman et al.,1998). Ultimamente, CVV causada por espécies não-*albicans*, como a *C. parapsilosis*, *C. krusei* e *C. tropicalis* é cada vez mais comum devido ao uso excessivo ou até mesmo indevido de antifúngicos (Zhang et al.,2014). Além disso, existe uma grande preocupação em relação ao aumento da incidência de CVV causada por espécies de *Candida* não-*albicans*, uma vez que estas espécies são mais difíceis de ser tratadas, pois tendem a apresentar menor suscetibilidade aos azóis ou até mesmo resistência a essa classe de antifúngicos. Entre as espécies não-*albicans* a *C. parapsilosis* apresenta maior suscetibilidade aos azóis, quando comparadas com as outras espécies (Yang et al., 2005).

Entre as espécies de *Candida*, *C. parapsilosis* não é uma causa frequente de infecção urinária (Trofa et al.,2008).

4. FATORES DE VIRULÊNCIA

A patogênese das candidíases é facilitada por um conjunto de fatores de virulência presentes nas espécies de *Candida*. A despeito de importantes pesquisas objetivando identificar esses fatores de virulência, particularmente em *C. albicans*, relativamente pouco é

conhecido sobre os determinantes de virulência nas espécies de CNA (Silva et al., 2011; Haynes et al., 2001). Além disso, em relação ao complexo *C. parapsilosis*, os poucos dados existentes sugerem que as três espécies podem apresentar diferentes potenciais de virulência. Por exemplo, Orsi et al. (2010) compararam o potencial patogênico entre as espécies do complexo *C. parapsilosis* utilizando um modelo de infecção *in vitro* em micróglia. Esses autores demostraram que a *C. metapsilosis* é menos virulenta, quando comparada com as demais espécies do complexo. Mais recentemente, outro estudo realizado por Németh et al.(2013) utilizando um modelo *in vivo* com larvas de *Galleria mellonella* confirmaram a menor virulência da *C. metapsilosis* em relação às demais espécies do complexo.

Os seguintes fatores, dentre outros, são reconhecidos como determinantes para a virulência das espécies de *Candida*: habilidade de formação de biofilme, produção de proteinases e fosfolipases e resistência aos antifúngicos.

4.1 FORMAÇÕES DO BIOFILME

Biofilmes são agregados de microrganismos associados a uma superfície e incrustados em uma matriz extracelular. Eles são considerados a forma mais prevalente de crescimento microbiano na natureza e frequentemente estão associados com infecções clínicas. É um importante fator de virulência para várias espécies de *Candida* (Tumbarello et al., 2007).

A formação do biofilme inicia-se com a aderência dos microrganismos em tecidos ou dispositivos médicos, sendo o mais comum o cateter venoso central. Em alguns casos o cateter ou o fluido de infusão estão contaminados, mas na maioria das vezes os microrganismos são adentrados por meio da pele do paciente ou pela contaminação das mãos dos profissionais da saúde (Douglas et al., 2003; Tumbarello et al., 2007).

A formação do biofilme proporciona benefícios aos microrganismos que os constitui, são eles: maior concentração de nutrientes, os quais estão presentes na matriz polimérica; proteção contra agentes nocivos, como desidratação e substâncias químicas, como antifúngicos (Ramage et al., 2001); possibilita a troca de material genético e a capacidade de colonizar diferentes nichos ecológicos (Flemming,1993; Mittelman, 1998).

Poucos estudos descrevem a estrutura do biofilme de *C. parapsilosis*. De acordo com Ferreira et al. (2009), este possui uma camada múltipla composta por uma rede de leveduras e pseudo-hifas. Além disso, possui um agrupamento irregular de blastoconídeos numa camada basal. Silva et al. (2009) demostraram que o biofilme possui uma grande quantidade de

carboidratos e uma pequena quantidade de proteínas, sendo que a formação do biofilme por esta espécie é totalmente dependente da estirpe.

Estudos demostram que há diferenças entre as espécies do complexo *C. parapsilosis* quanto à capacidade de formação de biofilme. Tavanti et al. (2010) relataram que 64.5% das amostras de *C. parapsilosis sensu stricto* foram capazes de formar biofilme. Toro et al. (2011) mostraram que 58.5% dos isolados de *C. parapsilosis sensu stricto* foram capazes de formar biofilme, enquanto nenhum dos isolados clínicos de *C. orthopsilosis* e *C. metapsilosis* produziu biofilme *in vitro*. Dados semelhantes foram encontrados por Tavanti et al. (2007) e Tosun et al. (2013). Por outro lado, existem dados que demonstram que as três espécies do complexo são capazes de produzir biofilmes (Melo et al., 2011; Abi-chacra et al., 2013). No entanto, vale ressaltar que vários destes estudos mencionados não são diretamente comparáveis porque eles diferem em aspectos importantes, tais como o processo de formação do biofilme, os métodos para detectar a produção do biofilme (ou seja, coloração com cristal violeta, ensaios de redução de XTT e medida da transmitância ou absorbância sem coloração) e os critérios para considerar um isolado como um produtor de biofilme.

4.2 PROTEINASES

A secreção de aspartil proteinase (Sap) é reconhecida como um importante determinante de virulência para *Candida*, especialmente *C. albicans* (Koga-Ito et al., 2006; Ruchel et al., 1982) Ao contrario da *C. albicans*, a *C. parapsilosis* tem uma baixa atividade de proteinase. Apenas três Saps foram identificados em *C. parapsilosis*, duas das quais permanecem em grande parte descaracterizadas (Merkerová et al., 2006).

A Sapp1p tem sido caracterizada bioquimicamente (Dostál et al., 2005; Fusek et al., 1994). O pseudogene SAPP2P, produz uma proteinase funcional (Sapp2p), que constitui cerca de 20% das Saps isoladas dos sobrenadantes das culturas (Fusek et al.,1993).

Essas proteinases facilitam a colonização e invasão dos tecidos do hospedeiro por meio do rompimento das membranas mucosas do hospedeiro e pela degradação de importantes proteínas de defesa imunológica (Pichova et al., 2001).

A produção de proteinase pode variar de acordo com os isolados de *C. parapsilosis,* e seu papel na patogênese permanece obscuro. Há uma tendência em comparar a produção desta enzima em relação ao local de coleta, uma vez que isolados da pele e vulvovaginal apresentam uma maior atividade de proteinase, quando comparadas com amostras de sangue (Trofa et al., 2008).

Um estudo realizado por Tosun et al.(2013) demostraram que 34,2% (29/77) dos isolados de *C. parapsilosis sensu stricto* expressaram proteinases. Além disso, nenhum dos seus isolados de *C. metapsilosis* ou *C. orthopsilosis* apresentaram atividade para esta exoenzima. No entanto, Trevino-Rangel et al. (2013) relataram que 17% (5/30), 7% (2/30) e 60% (3/5) dos seus isolados de *C. parapsilosis sensu stricto*, *C. orthopsilosis* e *C. metapsilosis,* respectivamente, produziram proteinases.

Outros estudos mostram uma alta prevalência da expressão de proteinases em isolados de *C. parapsilosis sensu stricto* (Abi-chacra et al., 2013; Ge et al., 2011; Németh et al., 2013). De modo similar, existem relatos que encontraram também elevada porcentagem de expressão destas enzimas entre isolados de *C. metapsilosis* e *C. orthopsilosis* (Ge et al., 2011; Németh et al., 2013; Trevino-Rangel et al., 2013). De acordo com Ge et al. (2011), as razões para essa variabilidade entre os estudos são desconhecidas, mas pode ser devido aos meios utilizados para os testes enzimáticos, pois a sensibilidade dos meios utilizados nos ensaios variam; outra razão pode estar relacionada com as diferentes temperaturas de incubação utilizadas nos ensaios.

4.3 FOSFOLIPASE

As fosfolipases são também frequentemente consideradas fatores associados com a patogenicidade de *Candida* (Price et al.,1982). Sua função durante a infecção não está ainda bem compreendida, embora se acredite que elas estão envolvidas na ruptura das membranas do hospedeiro (Kantarcioglu e Yucel, 2002; Ghannoum, 2000). Sua presença poderia contribuir para danificar as membranas das células hospedeiras, o que poderia promover danos celulares e/ou exposição de receptores que facilitariam a aderência de *Candida* (Ghannoum, 2000).

A elevada produção destas enzimas pode estar relacionada a um aumento dos níveis de patogenicidade das leveduras, já que amostras que expressam altas quantidades de fosfolipases apresentam uma maior capacidade de aderência e invasão nas células do hospedeiro (Ibrahim et al., 1995). Abi-chacra et al. (2013), encontraram atividade baixa ou indetectável destas enzimas nas espécies do complexo *C. parapsilosis*. Tay et al. (2011) demostraram que a expressão de fosfolipases é muito mais frequente em isolados de *C. albicans* (53.8 - 73%) em comparação com aqueles de CNA (2-17%), incluindo isolados *C. parapsilosis*, o que sugere que estas enzimas não são, provavelmente, fatores de virulência importantes para espécies de CNA. Por outro lado, Trevino-Rangel et al. (2013) relataram a

expressão destas enzimas em 97% (29/30) dos isolados de *C. orthopsilosis*, 80% (4/5) dos *C. metapsilosis* e 63% (19/30) dos *C. parapsilosis sensu stricto*. Uma alta prevalência na expressão de fosfolipases foi demostrada também em isolados de *C. parapsilosis sensu stricto* 90,5% (19/21) e *C. metapsilosis* 91,7% (11/12) por Ge et al. (2012).

A variação na expressão de fosfolipase nas espécies do complexo *C. parapsilosis* pode esta relacionada ao uso de diferentes testes enzimáticos, o tamanho da amostra e/ou variação biológica entre os isolados (Ge et al., 2012).

5. ANTIFÚNGICOS

Apesar do desenvolvimento das pesquisas em relação à terapia antifúngica, poucas são as classes de antimicóticos disponíveis para o tratamento de infecções fúngicas. Além disso, as células fúngicas quando comparadas com as células humanas têm uma similaridade bioquímica e fisiológica, com isso o tratamento torna-se limitado.

A maioria dos antifúngicos como os derivados azólicos (fluconazol e voriconazol) e os derivados poliênicos (anfotericina B) agem na membrana celular fúngica, que possui o ergosterol que tem uma estrutura semelhante ao colesterol das células humanas. Enquanto que as equinocandinas (caspofungina) são drogas que agem na parede celular fúngica representando uma ótima escolha (Odds et al., 2003).

C. parapsilosis não é considerada uma espécie propensa ao desenvolvimento de resistência a antifúngicos (Pfaller e Diekema, 2004; Pfaller e Diekema, 2008; Kuhn et al., 2004; van Asbeck et al., 2008). Todavia, estudos recentes sugerem que a sua menor suscetibilidade aos azóis e equinocandinas pode tornar-se um motivo de preocupação clínica (Cantón et al., 2011; Moudgal et al., 2005; Sarvikivi et al., 2005).

O surgimento de cepas de *C. parapsilosis* com a sensibilidade diminuída ao fluconazol (FLC), tem sido relacionado ao uso extensivo de FLC e à má higienização das mãos, possibilitando que estas cepas sejam transmitidas por todo o ambiente hospitalar (Almirante et al., 2006).

Um estudo recente relatou sobre o padrão de resistência antifúngica dos isolados de *Candida* na corrente sanguínea, mostrando quatorze amostras de *C. parapsilosis* resistentes ao FLC (Pfaller et al.,2011).

Historicamente, a anfotericina B (AMB) é o medicamento mais utilizado em infecções por *C. parapsilosis*, embora apresente alguns problemas quando utilizado na terapia

convencional, como sua baixa solubilidade aquosa e elevada toxicidade. Uma alternativa para substituir a AMB poderia ser o FLC (Ostrosky-Zeichner et al., 2003; Kuhn et al., 2004).

Quanto à caspofungina (CAS), a mais nova classe de agentes antifúngicos, trabalhos têm demonstrado valores de CIM (Concentração Inibitória Mínima) elevados para *C. parapsilosis*, quando comparado com outras espécies de *Candida* (Ostrosky-Zeichner et al., 2003; Melo et al.,2007).

Poucos estudos abordaram os perfis de suscetibilidade antifúngica entre as espécies do complexo *C. parapsilosis* (Weems et al., 1992; Melo et al., 2011; Pfaller e Diekema et al., 2008). Chen et al., 2010 demonstraram que a maioria dos isolados do complexo *C. parapsilosis* foi suscetível aos azóis, AMB e equinocandinas. No entanto, isolados de *C. metapsilosis* tiveram valores de CIM significativamente mais elevados para FLC e voriconazol (VRC), quando comparado com as outras espécies do complexo. Já em outro estudo, Gomez- Lopes et al., 2008 mostraram CIMs de AMB mais elevado para *C. parapsilosis sensu strictu*, o que está de acordo com os resultados relatados por Miranda-Zapico et al., 2011.

Vários estudos demonstram a resistência dos antifúngicos ao biofilme de *Candida*. (Trofa et al., 2008; Chandra et al., 2001; Silva et al., 2012). Apesar da estrutura do biofilme de *C. parapsilosis* ser menos complexa, a resistência a anfotericina B e aos compostos azólicos é semelhante ao biofilme de *C. albicans* (Ruzicka et al., 2007; Katragkou et al., 2008). Toro et al. (2011) demostraram que o VRZ é altamente eficaz contra células planctônicas e ineficaz contra o biofilme de *C. parapsilosis sensu stricto*. No entanto, os níveis terapêuticos de equinocandinas podem inibir a atividade metabólica dos biofilmes de *C. parapsilosis* (Kuhn et al., 2002; Katragkou et al., 2008). Em relação à anfotericina B, estudos demostram que os biofilmes de *C. parapsilosis* são mais suscetíveis a este antifúngico (Melo et al., 2011; Tay et al., 2011).

O desenvolvimento de resistência aos agentes antifúngicos é considerado uma importante causa de falha do tratamento das infecções fúngicas. Portanto, se pudermos escolher de forma inteligente um antifúngico ótimo de acordo com o agente etiológico, a eficácia da terapia contra as micoses poderia ser melhorada (Gupta & Kohli, 2003). Para isso, é de fundamental importância conhecer o perfil de suscetibilidade dos fungos patogênicos aos agentes antifúngicos.

6.CONSIDERAÇÕES FINAIS

O aumento da incidência de infecções humanas causadas por leveduras do complexo *C. parapsilosis* tem incentivado o desenvolvimento de pesquisas buscando elucidar as características destes microrganismos. Até o momento está claro o protagonismo da *C. parapsilosis sensu stricto* nas infecções humanas, tanto superficiais quanto invasivas. Este patógeno tem uma elevada afinidade por nutrição parenteral, coloniza as mãos dos profissionais de saúde, e tem a capacidade de formar biofilmes em superfícies de próteses, cateteres venosos centrais e dispositivos de longa permanência.

Contudo, novos estudos ainda são necessários para melhor conhecimento das propriedades de virulência, padrão de resistência aos antifúngicos e distribuição de cada membro do complexo, especialmente das duas espécies mais raramente isoladas: *C. orthopsilosis* e *C. metapsilosis*. Estes conhecimentos serão importantes para definir se a discriminação de rotina entre os membros do complexo *C. parapsilosis* é necessária para os laboratórios clínicos. A constatação de diferenças importantes nas propriedades de virulência e suscetibilidade aos antifúngicos entre as espécies desse complexo podem certamente influenciar as decisões terapêuticas.

Por fim, é importante mencionar que a prevalência das leveduras do complexo *C. parapsilosis* varia dependendo de diferenças regionais, e até o momento não havia estudos sobre a distribuição e características de virulência das espécies desse complexo na região de Uberaba.

Participação dos co-autores

O manuscrito apresentado a seguir foi formatado de acordo com as instruções aos autores da revista **European Journal of Clinical Microbiology & Infectious Diseases**.

Em atendimento ao regulamento do CPGCF, listamos abaixo a participação de cada co-autor, fora a mestranda e o orientador.

Larissa Beatriz Silva: Identificação fenotípica dos fungos e avaliação da produção de biofilmes; realização de testes de suscetibilidade dos biofilmes aos antifúngicos.

Diego Batista Carneiro de Oliveira: Coleta, isolamento e identificação fenotípica dos fungos; realização de testes de suscetibilidade aos antifúngicos.

Paulo Roberto da Silva; Coleta, isolamento e identificação fenotípica dos fungos.

Kennio Ferreira Paim: Coleta e isolamento dos fungos, avaliação da expressão de fatores de virulência (produção de proteinases e fosfolipases).

Leonardo Euripedes de A. e Silva: Coleta e isolamento dos fungos, identificação molecular das espécies do complexo *Candida parapsilosis*.

Mario León Silva-Vergara: Discussão e interpretação dos resultados.

Comprovante de Submissão

EJCM: Submission Confirmation for Distribution, virulence factors and antifungal susceptibility of *Candida parapsilosis* complex clinical isolates from Uberaba, Minas Gerais, Brazil.

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Distribution, virulence factors and antifungal susceptibility of *Candida parapsilosis* complex clinical isolates from Uberaba, Minas Gerais, Brazil.

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Abstract

The purposes of this study were (i) to determine by RFLP-*Ban* I assay the occurrence of *Candida parapsilosis* sensu stricto, *C. orthopsilosis* and *C. metapsilosis* among 81 clinical isolates primarily identified as *C. parapsilosis*; (ii) to evaluate their *in vitro* production of virulence factors; and (iii) to compare their susceptibility profiles, grown as planktonic cells and biofilms, against amphotericin B, fluconazole, voriconazole, and caspofungin by following the Clinical and Laboratory Standards Institute (CLSI) guidelines. Seventy seven isolates (95%) were identified as *C. parapsilosis* sensu stricto, 2 (2.5%) as *C. orthopsilosis*, and 2 (2.5%) as *C. metapsilosis*. Protease activity was detected in 37.7% of the isolates of *C. parapsilosis* sensu stricto, whereas only 9.1% exhibited phospholipase activity. None of the *C. metapsilosis* or *C. orthopsilosis* was able to produce protease or phospholipase. Biofilm production was detected in 43.2% of the isolates, among of which 33 were *C. parapsilosis* sensu stricto and 2 were *C. orthopsilosis.* Antifungal resistance was uncommon; only one *C. metapsilosis* was fluconazole resistant. In conclusion, we confirm that among the members of *C. parapsilosis* complex, *C. parapsilosis* sensu stricto is the most common species associated with human infections. Moreover, this species was the only one to express all the assayed virulence factors. On the contrary, none of these factors was detected in *C. metapsilosis*. Overall, *C. parapsilosis* complex was susceptible to amphotericin B, voriconazole and caspofungin, and had very low levels of fluconazole resistance. However, biofilm-producing isolates showed a marked resistance to these antifungal agents, particularly to voriconazole.

Keywords: *Candida parapsilosis* complex, virulence factors, proteases, phospholipases, biofim production, antifungal susceptibility.

Introduction

Over the past years, there has been an increase in human infections caused by yeasts from the genus *Candida*. Although *C. albicans* has historically been the most common species recovered from clinical samples, the number of infections due to non-*albicans Candida* (NAC) species has increased dramatically [1, 2]. Indeed, several studies have documented a decline in the proportion of infections caused by *C. albicans* and a corresponding increase in infections due to NAC [3-5]. Among NAC, *Candida parapsilosis* has gained recognition not only as agent of invasive fungal infections, where it is considered the second most frequent agent of candidemia in Latin America, Asia and Europe [1], especially in newborns [6], but also as important etiological agent of superficial mycoses, such as onychomycoses [4, 5].

Based on genetic evidence, in 2005 *C. parapsilosis* was recognized as a complex composed of three distinct but closely related species, namely *Candida parapsilosis* sensu stricto, *Candida orthopsilosis,* and *Candida metapsilosis* [7]. These species are physiologically and morphologically indistinguishable. Thus, DNA-based techniques are needed in order to differentiate the species within the complex. One of such techniques, proposed by Tavanti et al [7], is the restriction fragment length polymorphism (RFLP) analysis with BanI digestion of polymerase chain reaction (PCR) products of the secondary alcohol dehydrogenase (SADH) gene. This molecular approach has proved useful and has been readily adopted by several researchers [8-13].

Candida pathogenicity, as with other microorganisms, is determined by the expression of a number of virulence factors. While much is known about these factors produced by *C. albicans*, relatively little on this subject has been studied to NAC [14, 15]. With respect to *C. parapsilosis*, the knowledge of its virulence determinants is also not complete, but formation of biofilm, secretion of extracellular enzymes (such as proteases and phospholipases), and resistance to drugs seems to play an important role in its pathogenesis [16]. Since new species of the *C. parapsilosis* complex were recognized, differences in the degrees of antifungal susceptibility and virulence have been found among them [9, 11, 17-19]. However, as there are not commercially available assays to differentiate among the three species of the *C. parapsilosis* complex, few published studies have made this discrimination [9]. Thus, the unambiguous differentiation of these species is of fundamental importance in order to define precisely their virulence properties and antifungal susceptibility profiles. This knowledge could have clinical relevance, as it may be useful in guiding therapeutic decisions [9, 18].

The purposes of this study were (i) to determine by RFLP-*Ban* I assay the occurrence of *C. orthopsilosis*, *C. metapsilosis* or *C. parapsilosis* sensu stricto among 81 clinical isolates primarily

identified as *C. parapsilosis* by conventional methods; (ii) to evaluate their ability to form biofilm and to produce proteases and phospholipases; and (iii) to compare their *in vitro* susceptibility profiles, grown as planktonic cells and biofilms, against amphotericin B, fluconazole, voriconazole, and caspofungin by following the Clinical and Laboratory Standards Institute (CLSI) guidelines.

Materials and methods

Clinical isolates of Candida parapsilosis

A total of 81 isolates originally identified as *C. parapsilosis* through the use of standard morphological and biochemical methods were included in the study. Among these isolates, 52 were originated from our previous study [5]. The samples were isolated from different clinical specimens, including nails, skin (hands, feet, face and body), blood, urine, catheter tip, and secretions. Clinical specimens were obtained from patients who were seen at the Hospital de Clínicas da Universidade Federal do Triângulo Mineiro (UFTM) and other medical centers and practices in Uberaba, MG, Brazil. Only one isolate per patient was included. This study was approved by the ethics committee of UFTM (protocol 1361/2010).

Identification as *C. parapsilosis* was performed based on the following standard methods, according to de Hoog et al. [20]: carbon and nitrogen assimilation assays, sugar fermentation, germ tube test, urease activity, microscopic morphology on corn-meal agar with tween 80 and growth in chromogenic medium CHROMagar Candida® (Difco Laboratories, Detroit, MI, USA).

Molecular identification of C. parapsilosis species complex

Molecular identification of *C. parapsilosis* species complex was performed according to Tavanti et al. [7]. Briefly, yeast genomic DNA extracted as described by Ferrer et al. [21] was used as template for PCR amplification of a 716-bp fragment from the SADH gene. The amplification conditions were 94ºC for 7 min, followed by 35 cycles of denaturation at 95ºC for 30 s, annealing at 55ºC for 1 min and elongation at 72ºC for 90 s, with a final extension step of 5 min at 72ºC. The PCR products were then digested with BanI restriction enzyme (New England Biolabs, Ipswich, MA, USA) and the digestion patterns were used to differentiate among the three related species as follows: *C. orthopsilosis* has no restriction site, *C. metapsilosis* has three BanI restriction sites and *C. parapsilosis* sensu stricto has one BanI restriction site.

The reference strains *Candida parapsilosis* (ATCC 22019), *Candida orthopsilosis* (ATCC 96141) and *Candida metapsilosis* (ATCC 96143) were used as controls in all experiments.

Phospholipase Production

The isolates were screened for the phospholipase activity by measuring the size of the zone of precipitation after growth on egg yolk agar as described by Price et al. [22], with some modifications. The test medium consisted of Sabouraud Dextrose Agar (Himedia Laboratories, Mumbai, India), 1 mol/L NaCl, 0.005 mol/L CaCl2 and 8% sterile egg yolk Emulsion (Sigma-Aldrich, St. Louis, MO, USA). Ten microlitre of a suspension of 10^7 yeast cells per ml of each isolate were inoculated onto the surface of the test medium in duplicate. The plates were incubated at 37ºC for 7 days, after which the diameter of the precipitation zone around the colony was measured. Phospholipase activity (Pz) was expressed as the ratio of the diameter of the colony to the diameter of the colony plus the precipitation zone. The phospholipase activity was classified as negative (Pz = 1), very low (Pz = 0.90-0.99), low (Pz = 0.80-0.89), high (Pz = 0.70-0.79) and very high (Pz < 0.69), as previously reported [23]. Each experiment was performed twice.

Protease production

Determination of protease production was performed based on Ruchel et al. [24] using bovine serum albumin (BSA) agar [1.17% yeast carbon base, 0.25% Protovit® (Roche AS, São Paulo, SP, Brazil) and 0.2% BSA (Fraction V, Sigma-Aldrich) adjusted to pH 5.0, sterilized by filtration, and added to an autoclaved cooled solution of 2% agar]. Protease activity was detected by inoculating 10 µl of a yeast suspension of 10^7 cells per ml onto a BSA plate. The plates were incubated at 37ºC for 7 days. Protease activity (Pz) was measured and scored according to phospholipase testing, as mentioned above.

Biofilm formation

The *in vitro* biofilm formation of *Candida parapsilosis* complex isolates was determined as described by Jin et al. [25] with some modifications. Briefly, after overnight growth in Sabouraud dextrose broth (Himedia), yeast cells were harvested and resuspended in RPMI 1640 medium (Himedia) with L-glutamine but without bicarbonate and buffered to pH 7.0 with 0.165 mol/L morpholinepropanesulphonic acid (MOPS; Sigma-Aldrich, St. Louis, MO, USA) to a density of 1.0×10^7 cells/ml. A hundred microliters of this standardized cell suspension were added to each well of a flat-bottomed 96-well microtiter plate. Each strain was inoculated in replicate in five wells. As a negative control, test medium without cells was added to three wells of each plate. The plates

were incubated for 1.5 h at 37ºC without agitation to allow cells to attach to the bottom of the wells. After the adhesion stage, non-adherent cells were removed by washing the wells twice with 150µl of sterile phosphate buffered saline (PBS). Fresh RPMI 1640 medium (100µl) was then added to the wells, and the plates were further incubated for 48 h at 37ºC.

Quantification of biofilm

A semiquantitative measure of biofilm formation was performed using the 2,3-Bis(2-Methoxy-4-Nitro-5-Sulfophenyl)-5-[(Phenylamino)Carbonyl]-2H-Tetrazolium Hydroxide (XTT) reduction assay, essentially as described by Melo et al. [26]. The biofilms were first washed twice with 200 µl of PBS, and then 200 µl of PBS and 12 µl of the XTT-menadione solution were added to each of the prewashed biofilm and to control wells (for the measurement of background XTT-reduction levels). The XTT-menadione solution was prepared fresh on each day of testing by adding 1.5 ml of XTT (1 mg/ml in sterile saline; Sigma-Aldrich) to 300 µl menadione solution (0.4 mM in acetone; Sigma-Aldrich). The plates were then incubated in the dark for 2h at 37°C. Following incubation, 100 µl of solution was transferred to new wells and the color change in the solution was measured with a microtiter plate reader (TP-Reader Basic, ThermoPlate) at 490 nm. The biofilm production was measured as optical densities (OD) higher than 0.200 [9]. The absorbance values for the controls (containing no cells) were subtracted from the values for the test wells to eliminate spurious results due to background interference. Data were recorded as arithmetic mean of absorbance values.

Antifungal susceptibility testing

The *in vitro* minimum inhibitory concentrations (MICs) of amphotericin B (AMB) (Pfizer), Caspofungin (CAS) (Merck-EUA), fluconazole (FLZ) (Pfizer, Guarulhos, SP, Brazil) and voriconazole (VRZ) (Pfizer) on planktonic cells were determined using broth microdilution method in accordance with CLSI documents M27-A3 [27] and M27-S4 [28]. Stock solutions were prepared in sterile water (CAS and FLZ) or dimethyl sulphoxide (DMSO; Sigma-Aldrich) (AMB and VRZ). Antifungal agents were then diluted with RPMI-1640 medium buffered to pH 7.0 with MOPS. AMB and VRZ were tested at concentrations from 0.03 to 16 µg/ml. CAS concentrations ranged from 0.015 to 8 µg/ml, while FLZ was tested at concentrations ranging from 0.015 to 64 µg/ml. The MICs were determined by visual inspection following incubation in a humid atmosphere at 35°C for 24 to 48h. For azoles, MIC endpoints were defined as the lowest antifungal concentration that resulted in a prominent decrease (≥50% inhibition) in growth compared to the growth in the control

well (antifungal-free medium). The lowest concentration inhibiting any visible growth was used as the MIC for AMB and CAS. The MIC_{50} and MIC_{90} were the minimum concentrations of antifungal agents required to inhibit 50% and 90% of isolates tested, respectively.

The MICs for sessile cells (biofilm) were determined with the previously described microtiter-based assay [29] with some modifications. After biofilm formation in 96-well microtiter plates for 48 h as described above, the medium was aspirated, and non-adherent cells were removed by thoroughly washing the biofilms twice with PBS. Residual PBS was removed by blotting with paper towels before the addition of antifungal agents. AMB, CAS, FLZ and VRZ were then added to the biofilms at the same concentrations used in the planktonic cell susceptibility assay and the plates were incubated for 48h at 37°C. Sessile MICs (SMICs) were determined at 50% metabolic inhibition compared to control (antifungal-free) by using the XTT reduction assay described above. Testing of these isolates was performed in triplicate.

Statistical analysis

Associations among the virulence factors were analyzed using the Mann–Whitney or the chi-square test as appropriate. Differences between the SMIC values and their MICs were tested using McNemar's test. *P* values of <0.05 were considered to be statistically significant. All statistical analyses were performed with Statistica 7.0 (Statsoft, Inc. Tulsa, USA) software.

Results

Of the 81 *C. parapsilosis* complex isolates, 77 (95%) were identified as *C. parapsilosis* sensu stricto, 2 (2.5%) as *C. orthopsilosis*, and the remaining 2 (2.5%) proved to be *C. metapsilosis*. The distribution of these three species within different age groups, gender and sites of isolation is detailed in Table 1. The three species of the *C. parapsilosis* complex were found in both genders. While *C. parapsilosis* sensu stricto was isolated from patients of all age groups, *C. metapsilosis* and *C. orthopsilosis* were obtained only from older patients (ages ranging from 50 to 76 years). *C. parapsilosis* sensu stricto was isolated from all kinds of clinical specimens, while *C. orthopsilosis* was recovered from superficial candidiasis (toenail and skin) and *C. metapsilosis* from invasive candidiasis (urine and wound secretion).

Regarding the production of hydrolytic enzymes, none of the *C. metapsilosis* or *C. orthopsilosis* isolates was able to produce protease or phospholipase (Table 1). Protease activity was detected in 37.7% (29/77) of the isolates of *C. parapsilosis* sensu stricto, whereas only seven strains (9.1%) exhibited phospholipase activity. Most protease-producing strains (18/29) had high or very

high enzymatic activity with Pz values ranging from 0.43 to 0.78. Among the phospholipase positive isolates, 71.4% (5/7) possessed enzymatic activity high or very high (Pz ranging from 0.48 to 0.77). We noted also that only two strains secreted both phospholipase and protease.

Biofilm production was detected in 35 (43.2%) of the *C. parapsilosis* complex isolates, among of which 33 were *C. parapsilosis* sensu stricto and 2 were *C. orthopsilosis* (Table 1). The mean A_{490} value for the 35 biofilm-producing strains was 0.264 (± 0.056, SD) with a range of 0.204-0.398, a 1.95-fold difference between the highest and lowest biofilm-producing strains.

There was no statistically significant association between biofilm-forming ability and the clinical origin of the isolates ($p = 0.300$). Similarly, biofilm production in *C. parapsilosis* sensu stricto isolates was not correlated to secretion of proteinase ($p = 0.658$) or phospholipase ($p = 0.828$).

The distribution of MICs and SMICs for planktonic and biofilm-grown *C. parapsilosis* complex isolates, respectively, is shown in Table 2, whereas Table 3 summarizes the antifungal susceptibility data for these isolates, including range of the MICs and SMICs, MIC_{50}, $SMIC_{50}$, MIC_{90} and geometric mean – GM. Since the number of *C. metapsilosis* and *C. orthopsilosis* strains was very small, its MIC_{50} and MIC_{90} were not determined for any of the antifungal agents tested. According to the new species-specific interpretive breakpoints [28] or old CLSI breakpoints [27], all isolates were susceptible to amphotericin B and caspofungin. Likewise, all isolates except one *C. metapsilosis* (whose fluconazole MIC was 16 μg/ml) were sensitive to fluconazole. This same fluconazole-resistant *C. metapsilosis* isolate was the only one to display dose-dependent susceptibility to voriconazole (MIC = 0.5 μg/ml). In addition, we detected one *C. parapsilosis* sensu stricto isolate with dose-dependent susceptibility to fluconazole (MIC = 4 μg/ml). In contrast, most of the isolates were resistant to all antifungals tested after they were grown as biofilms. There was a statistically significant increase ($p<0.001$) in the SMICs for all antifungal agents as compared to their planktonic MICs. Amphotericin B, caspofungin and fluconazole had some activity only against biofilms of *C. parapsilosis* sensu stricto (when SMICs were ≤ 1 μg/ml, ≤ 2 μg/ml and ≤ 2 μg/ml, respectively). In contrast, biofilm of all the isolates evaluated were resistant to voriconazole (SMIC ≥ 1μg/ml) (Table 2).

Discussion

Since 2005, when *C. parapsilosis* was recognized as a complex composed by *C. parapsilosis* sensu stricto, *C. orthopsilosis* and *C. metapsilosis*, surveillance data about the distribution, antifungal resistance and virulence factors of these three species are continually collected and analyzed by a number of authors [8-13, 17, 19, 30-33]. Moreover, an increasing prevalence of

human infections caused by *C. parapsilosis* complex species has been reported in recent years [1, 3]. Despite of these facts, the characterization of the *psilosis* group is not completed yet, so further studies are needed to better understand the characteristics, including putative virulence traits, drug resistance trends and distribution of each member of the complex and especially of the two rarely isolated species, *C. orthopsilosis* and *C. metapsilosis* [34, 35].

After molecular analysis we found that *C. orthopsilosis* and *C. metapsilosis* accounted for 5% of all isolates. These results agree with the findings of several other studies which reported that, within the *C. parapsilosis* complex, *C. parapsilosis* sensu stricto is the most prevalent species associated with human infections, while *C. orthopsilosis* and *C. metapsilosis* together represent fewer than 10% of the etiological agents of these infections [8-13, 18, 31, 32]. It is important to note that our results are more closely related to those which examined strains isolated from different body sites and tissues [8, 9, 11, 13, 31] than to those which were based solely on isolates from superficial [12] or invasive fungal infections [10, 18, 32].

In this study, most of the isolates (69/81) were obtained from superficial *Candida* infections. Nevertheless, it is interesting to observe that *C. metapsilosis* strains were only recovered from invasive infections. In the literature there is no relationship between *C. metapsilosis* and invasive infections. In fact, this species together with the other two species of the *psilosis* group have been reported as cause of both superficial and invasive infections in humans [8-12, 32]. Thus, our finding seems not to have clinical relevance and may simply be related to the limited number of *C. metapsilosis* isolates that was found in this panel.

Of note, all *C. orthopsilosis* and *C. metapsilosis* strains were isolated from older patients. These findings are similar to those recently reported by Tosun et al. [13] and are partially in accordance with previous studies that showed strong association between *C. orthopsilosis* and elderly patients (≥60 years) [8, 10]. Regarding *C. metapsilosis*, to the best of our knowledge, our work is then the second study to demonstrate isolation of this species exclusively from elderly patients and in our study both isolates were from patients over 70 years old. Although the exact age of the patients was not mentioned in the study by Tosun et al. [13], we hypothesized that aging would be associated with an increased susceptibility to infections caused by *C. metapsilosis*. Indeed, it is widely accepted that aging is associated with a decline in immune function, a process termed immune senescence, which makes an individual more susceptible to infections [36], including opportunistic fungal infections [37]**.** The assumption that aging is a risk factor for infection with *C. metapsilosis* could be in line with previous work showing that this yeast is the least virulent species of the group [34, 38]. However, to date there are few reports with information about patients' age, thus further studies with a greater number of isolates and extensive demographic information are needed to confirm the association between aging and infection by *C. metapsilosis*.

As aforementioned, *C. metapsilosis* has been reported as a less virulent member of the *C. parapsilosis* complex in an *in vitro* infection model using microglial cells [34] and also more recently in an *in vivo* model system using *Galleria mellonella* larvae [38]. Indeed, some studies have been shown that there are significant differences among the species that comprise the *C. parapsilosis* complex relative to the expression of virulence factors, including the production of extracellular hydrolytic enzymes and the ability to form biofilms [9, 13, 19, 26, 38].

Secretion of hydrolytic enzymes is thought to play an important role in the pathogenesis of disease caused by *C. parapsilosis*, facilitating its adherence and tissue invasion, or damaging cells of the host immune system to avoid antimicrobial attack [38]. Among these enzymes, there have been contradictory findings in terms of protease and phospholipase activity within the members of the *psilosis* group. Tosun et al. [13] reported that 34.2% of *C. parapsilosis* sensu stricto isolates (13/38) were protease positive. In addition, none of their isolates of *C. metapsilosis* (3 strains) or *C. orthopsilosis* (1 strain) exhibited protease activity. Our data are similar to these results given that we detected protease activity only in isolates of *C. parapsilosis* sensu stricto with a positivity rate of 37.7% (29/77). However, variable protease activity has been found by other authors among *C. parapsilosis* isolates. For instance, Treviño-Rangel et al. [19] reported that 17% (5/30), 7% (2/30) and 60% (3/5) of *C. parapsilosis* sensu stricto, *C. orthopsilosis* and *C. metapsilosis* isolates, respectively, were significant producers of protease. On the other hand, a number of studies identified a high proportion of protease-producing *C. parapsilosis* sensu stricto isolates, ranging from 66.1% to 100% [33, 38-40]. Similarly, recent reports found also a high proportion of isolates of both *C. metapsilosis* and *C. orthopsilosis* exhibiting protease activity [19, 33, 38]. According to Ge et al. [33], the reasons for this variability among studies are unknown, but it may be due to the media used for enzymatic tests, as sensitivity of different media varies; another potential reason might be related to the different incubation temperatures used in the assays (30 or 37ºC).

In terms of phospholipase, our findings concur with the results of previous studies which found low or undetectable phospholipase activity among *C. parapsilosis* isolates [40, 41]. Phospholipase activity has been detected in higher percentages (53.8-73%) in *C. albicans* isolates as compared to those of non-*albicans Candida* isolates (2-17%), including *C. parapsilosis* isolates, suggesting that this enzyme is probably not a significant virulence factor for non-*albicans Candida* species [41]. However, as for the protease activity, there have been contradictory findings in terms of phospholipase activity in *C. parapsilosis*. Treviño-Rangel et al. [19] showed that 97% (29/30) of *C. orthopsilosis*, 80% (4/5) of *C. metapsilosis* and 63% (19/30) of *C. parapsilosis* sensu stricto possessed secreted phospholipase activity. A study by Ge et al. [33] also reported a remarkably high proportion of both *C. parapsilosis* sensu stricto and *C. metapsilosis* isolates producing phospholipase *in vitro* [90.5% (19/21) and 91.7% (11/12), respectively]. Some reasons have been

proposed to explain the wide variation in phospholipase activity of *C. parapsilosis*, including the use of different media for enzymatic test, small sample size, and/or inherent biological variation among isolates [33].

Our results show that there is variable ability of members of the *psilosis* complex to form biofilms *in vitro*, since that biofilm formation was detected in 42.8% (33/77) of *C. parapsilosis* sensu stricto and in 100% (2/2) of *C. orthopsilosis* isolates, whilst it was not detected in *C. metapsilosis* isolates. In contrast with our data, some studies have shown that *C. parapsilosis* sensu stricto is the only species of the complex to form biofilms [9, 13]. In line with these works, Tavanti et al. [42], evaluating a total of 33 *C. orthopsilosis* clinical isolates, showed that none of them was biofilm-positive. On the other hand, there are other data demonstrating that all three species of the *psilosis* group are able to produce biofilm [26, 40]. Nevertheless, it is noteworthy that several of these mentioned studies are not directly comparable because they differ in important aspects, such as the biofilm formation process, the methods to evaluate biofilm production (i.e., crystal violet staining, XTT-reduction assays or measured transmittance or absorbance without staining) and the criteria for considering an isolate as a biofilm producer. In this respect, as far as we know, there are no widely accepted criteria which allow us to categorize a *Candida* strain as a biofilm producer. We adopted the criterion suggested by Toro et al. [9] where isolates showing XTT OD readings higher than 0.200 were considered biofilm producers. According to Melo et al. [26] an isolate exhibiting XTT OD readings ≥0.200 is considered as high biofilm producer. Thus, the adoption of this criterion should ensure that all isolates categorized as biofim-positive are truly biofilm producers.

Regarding the antifungal susceptibility profile, most of the isolates exhibited low *in vitro* MICs and were susceptible to the antifungal agents tested (amphotericin B, caspofungin, fluconazole and voriconazole). When applying the breakpoints proposed for *C. parapsilosis* for interpretation of the MIC results of *C. metapsilosis* and *C. orthopsilosis* [28], antifungal resistance was found to be restricted to only one *C. metapsilosis* isolate, which showed resistance to fluconazole. This isolate was also the only one to display dose-dependent susceptibility to voriconazole. In addition, one fluconazole dose-dependent susceptible strain of *C. parapsilosis sensu stricto* was observed. In agreement with these results, a number of previous investigations have shown that *C. parapsilosis* complex isolates are usually susceptible to amphotericin B, voriconazole and caspofungin, and have low levels of fluconazole resistance [8, 11, 13, 18, 30, 31]. Likewise, fluconazole resistance among *C. metapsilosis* isolates has also been reported by other authors [13, 18, 30-32]. In turn, Chen et al. [30] showed that *C. metapsilosis* strains had significantly higher MIC values to voriconazole than those of *C. parapsilosis* sensu stricto and *C. orthopsilosis*, although their *C. metapsilosis* strains were neither resistant nor dose-dependent susceptible to voriconazole when antifungal susceptibility test was determined at 24h (MIC ranged from 0.03 to 0.125 μg/ml).

The interest in a better characterization of the *psilosis* group relies, besides the emergence of *C. parapsilosis* as a significant nosocomial pathogen, also on the different antifungal susceptibility profile that has been observed for the cryptic species *C. orthopsilosis* and *C. metapsilosis* [32]. As pointed out by Cantón et al. [10], different *in vitro* antifungal susceptibility patterns have been reported, with *C. parapsilosis* sensu stricto being less susceptible to amphotericin B, echinocandins, and fluconazole than *C. metapsilosis* or *C. orthopsilosis*. Our data are inadequate to make a proper assessment in this regard because of the low number of *C. metapsilosis* and *C. orthopsilosis* isolates included in the study. Anyway, in the present study we observed that MICs for *C. parapsilosis* complex isolates were in general within the MIC ranges reported by others [9-11, 13, 31].

There is an increasing concern about biofilm production by *Candida* species, because it is known that biofilm-associated cells have enhanced resistance to host defense mechanisms and are also substantially more resistant to antifungal therapy [1, 3]. Considering specifically the *C. parapsilosis* complex, our results agree with previous studies showing that, regardless of species, SMICs were commonly several-fold higher than their planktonic MICs [9, 26, 41]. In addition, our biofilm MIC results confirmed the findings of Toro et al. [9] that, although voriconazole is highly effective against planktonic cells, it is ineffective against the biofilm of *C. parapsilosis* sensu stricto. Finally, we found that amphotericin B was moderately active against biofilm-associated *C. parapsilosis* sensu stricto. This observation is in accordance with recently published reports where the authors also observed *C. parapsilosis* biofilms susceptible to amphotericin B [26, 41].

In conclusion, we confirm that among the members of *C. parapsilosis* complex, *C. parapsilosis* sensu stricto is the most common species associated with both superficial and invasive human infections. Moreover, this species was the only one to express all the assayed *in vitro* virulence factors. On the contrary, none of these factors was detected in *C. metapsilosis* isolates. Overall, *C. parapsilosis* complex isolates were susceptible to amphotericin B, voriconazole and caspofungin, and had very low levels of fluconazole resistance. However, biofilm-producing isolates showed a marked resistance to these antifungal agents, particularly to voriconazole.

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Table 1: Distribution of *C. parapsilosis* complex species within different age groups, gender and sites of isolation, and their virulence properties.

	***C. parapsilosis* complex species**			
	C. parapsilosis sensu stricto **(n=77)** **95.0%**	***C. metapsilosis*** **(n=2)** **2.5%**	***C. orthopsilosis*** **(n=2)** **2.5%**	**Total (n=81) 100.0%**
Age (years)				
0-15	8(100.0)	0(0)	0(0)	8(9.9)
16-29	8(100.0)	0(0)	0(0)	8(9.9)
30-49	29(100.0)	0(0)	0(0)	29(35.8)
≥50	32(88.8)	2(5.6)	2(5.6)	36(44.4)
Gender				
Male	25(92.6)	1(3.7)	1(3.7)	27(33.3)
Female	52(96.2)	1(1.9)	1(1.9)	54(66.7)
Site of isolation				
Fingernail	11(100.0)	0(0)	0(0)	11(13.6)
Toenail	29(96.6)	0(0)	1(3.4)	30(37.0)
Skin	28(100.0)	0(0)	0(0)	28(34.5)
Urine	1(50.0)	1(50.0)	0(0)	2(2.5)
Blood	6(100.0)	0(0)	0(0)	6(7.4)
Wound secretion	1(50.0)	1(50.0)	0(0)	2(2.5)
Catheter tip	1(50.0)	0(0)	1(50.0)	2(2.5)
Virulence factors				
Protease				
Very high	6(100.0)	0(0)	0(0)	6(7.3)
High	12(100.0)	0(0)	0(0)	12(14.7)
Low	9(100.0)	0(0)	0(0)	9(11.1)
Very low	2(100.0)	0(0)	0(0)	2(2.5)
Negative	48(92.4)	2(3.8)	2(3.8)	52(64.4)
Phospholipase				
Very high	2(100.0)	0(0)	0(0)	2(2.5)
High	3(100.0)	0(0)	0(0)	3(3.7)
Low	2(100.0)	0(0)	0(0)	2(2.5)
Very low	0(0)	0(0)	0(0)	0(0)
Negative	70(94.6)	2(2.7)	2(2.7)	74(91.3)
Biofilm formation				
Positive	33(94.3)	0(0)	2(5.7)	35(43.2)
Negative	44(95.7)	2(4.3)	0(0)	46(56.8)

Table 2: Minimum inhibitory concentration (MIC) distribution of antifungal drugs for planktonic and sessile (biofilm) cells of *Candida parapsilosis* complex species.

Species	Antifungal agent	Type of MIC* (nº of isolates)	No. of isolates for which the MIC (μg/ml) was:											
			≤0,03	0,06	0,12	0,25	0,5	1	2	4	≥8	≥16	32	≥64
C. parapsilosis sensu stricto	Amphotericin B[c]	Planktonic MIC[a] (77)			2	25	43	7						
		Biofilm SMIC[b] (33)						4	4	3	3	19		
	Caspofungin[d]	Planktonic MIC (77)	1			2	14	49	11					
		Biofilm SMIC (33)						3			30			
	Fluconazole[e]	Planktonic MIC (77)				14	26	26	10	1				
		Biofilm SMIC (33)				1		1	3		1	1	1	25
	Voriconazole[c]	Planktonic MIC (77)	66	9	1	1								
		Biofilm SMIC (33)								2	2	29		
C. orthopsilosis	Amphotericin B	Planktonic MIC (2)						2						
		Biofilm SMIC (2)										2		
	Caspofungin	Planktonic MIC (2)					1	1						
		Biofilm SMIC (2)									2			
	Fluconazole	Planktonic MIC (2)						1	1					
		Biofilm SMIC (2)												2
	Voriconazole	Planktonic MIC (2)	1		1									
		Biofilm SMIC (2)										2		
C. metapsilosis	Amphotericin B	Planktonic MIC (2)				1	1							
		Biofilm SMIC (0)												
	Caspofungin	Planktonic MIC (2)				1	1							
		Biofilm SMIC (0)												
	Fluconazole	Planktonic MIC (2)							1			1		
		Biofilm SMIC (0)												
	Voriconazole	Planktonic MIC (2)		1			1							
		Biofilm SMIC (0)												

[a]Planktonic MICs were determined by the CLSI broth microdilution method, whereas [b]biofilm SMICs were measured by the XTT reduction assay.
[c]Amphotericin B and Voriconazole were tested at concentrations from 0.03 to 16 μg/ml. [d]Caspofungin concentrations ranged from 0.015 to 8 μg/ml, while [e]Fluconazole was tested at concentrations ranging from 0.015 to 64 μg/ml.

Table 3: Antifungal susceptibility data (expressed in µg/ml) for *Candida parapsilosis* complex species growing in planktonic and sessile states.

Species (no. of isolates)	Antifungal agent	Plankctonic cells				Sessile cells	
		MIC[a] range	MIC_{50}[b]	MIC_{90}[b]	GM[c]	SMIC[d] range	$SMIC_{50}$[e]
C. parapsilosis sensu stricto (77)	Amphotericin B	0.12- 1	0.5	0.5	0.35	1 - ≥16	≥16
	Caspofungin	0.03 - 2	1	1	0.37	1 - ≥8	≥8
	Fluconazole	0.25 - 16	0.5	1	1.00	0.25 - ≥64	≥64
	Voriconazole	≤0.03 – 0.25	≤0.03	≤0.03	0.06	4 - ≥16	≥16
C. orthopsilosis (2)	Amphotericin B	1	-	-	-	≥16	-
	Caspofungin	0.25 – 0.5	-	-	-	≥8	-
	Fluconazole	1 - 2	-	-	-	≥64	-
	Voriconazole	≤0.03 – 0.12	-	-	-	≥16	-
C. metapsilosis (2)	Amphotericin B	0.25 – 0.5	-	-	-	-	-
	Caspofungin	0.5 - 1	-	-	-	-	-
	Fluconazole	2 - 16	-	-	-	-	-
	Voriconazole	0.06 – 0.5	-	-	-	-	-

[a]MIC: Minimum inhibitory concentration
[b]MIC_{50} and MIC_{90}: MIC at which 50% and 90% of the isolates were inhibited.
[c]Geometric means
[d]SMIC: Minimum concentration of antifungal agents needed to reduce metabolic activity of the biofilms by 50%.
[e]SMIC50: SMIC at which 50% of the biofilms were inhibited.

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