Daniela Vanessa Moris

"PERFIL GENÔMICO E SENSIBILIDADE A ANTIFÚNGICOS DE AMOSTRAS SEQUENCIAIS DE *Candida* spp. ISOLADAS DA CAVIDADE ORAL DE INDIVÍDUOS INFECTADOS PELO VÍRUS DA IMUNODEFICIÊNCIA HUMANA".

Tese apresentada ao Programa de Pós Graduação em Doenças Tropicais da Faculdade de Medicina de Botucatu UNESP, para a obtenção do título de doutor.

Orientador: Prof. Dr. Rinaldo Poncio Mendes
Co-Orientadora: Profª Drª Márcia de Souza Carvalho Mellhem
Co-Orientadora: Pesquisadora Marilena dos Anjos Martins

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Palavras-chave: *Candida* spp; Complexo *C psilosis*; *Candida dubliniensis*; Infecção pelo vírus da imunodeficiência humana (HIV).

Dedicatória

Dedico este trabalho

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Sumário

Capítulo I - Introdução

1. Fungos do gênero *Candida*

Várias espécies de leveduras do gênero *Candida,* assim como alguns outros microrganismos, podem colonizar mucosas, pele e trato digestivo, de modo transitório ou durante longos períodos. No entanto, quando estão presentes em diferentes sítios e quando o mesmo microrganismo for isolado repetidamente da mesma amostra biológica, deve-se sugerir alteração da microbiota. O desenvolvimento excessivo de *Candida* spp., nestes sítios de colonização, pode facilitar sua invasão tecidual, principalmente em hospedeiros com condições predisponentes [1].

O trato gastrintestinal, colonizado por *Candida* spp. em até 70% da população normal, pode ser origem de fungemia, por alterações da microbiota residente e pela translocação deste patógeno. Métodos de genotipagem mostraram similaridade entre amostras colonizadoras e infectantes, sugerindo sua origem endógena [2,3]. Qualquer variável que provoque desequilíbrio da microbiota ou lesão da mucosa pode ser agente facilitador da translocação de *Candida* spp. até os capilares mesentéricos. Fatores que aumentam a colonização intestinal por *Candida* spp., tais como uso de antibióticos e oclusão intestinal, ou que determinem atrofia ou lesão da mucosa, como jejum prolongado, nutrição parenteral total, hipotensão arterial e quimioterapia, podem potencializar o fenômeno de translocação no tubo gastrintestinal [4,5].

O número de infecções causadas por espécies de *Candida* não-*albicans* vem aumentando nos últimos anos. Em 1963, *C. albicans, C. parapsilosis, C.*

tropicalis, C. stellatoidea e *C. guilliermondii* eram as cinco espécies que causavam doença no homem. Atualmente são conhecidas cerca de 17 espécies causadoras de infecções em seres humanos [6].

C. albicans, C. parapsilosis, C. tropicalis, C. glabrata, C. krusei, C. guilliermondii e C. lusitaniae são as principais espécies de interesse médico. Porém, casos de doenças superficiais e invasivas relacionadas a espécies emergentes de *Candida* têm sido descritos, tais como *C. kefyr, C. rugosa, C. famata, C. utilis, C. lipolytica, C. norvegensis, C. inconspícua, C. dubliniensis* [7] e *C.pelliculosa (Pichia anomala)* [8].

C. albicans é a espécie isolada com maior freqüência como colonizadora ou causadora de infecções superficiais e invasivas em diferentes sítios anatômicos, em todo o mundo. Possui potencial patogênico bastante conhecido, apresentando como principais fatores de patogenicidade e virulência a capacidade de aderência a diferentes mucosas e epitélios, o dimorfismo, com produção de pseudo-hifas que auxiliam na invasão tissular, a termotolerância e a produção de exoenzimas, como proteinases e fosfolipases [6]. Esta espécie apresenta sensibilidade natural a todas as drogas de uso sistêmico, porém casos de resistência adquirida a azólicos têm sido descritos, principalmente em indivíduos que foram expostos por tempo prolongado a estes antifúngicos. Ao contrário, a resistência à anfotericina B é rara [9].

C. dubliniensis, descrita na Irlanda em 1995 por Sullivan et al.[10], revela características bioquímicas e morfológicas muito semelhantes às de *C. albicans,* sendo necessária a utilização de métodos moleculares para

diferenciá-las. Esta nova espécie foi isolada da cavidade oral de pacientes infectados com o HIV e com aids, entre os quais 17 a 35% foram colonizados ou infectados por esta espécie [11]. Os fatores de risco para a presença desta espécie ainda não estão bem estabelecidos, mas a virulência parece ser semelhante à da *C. albicans.*

Sabe-se que *C. dubliniensis* apresenta maior atividade de proteinase que *C. albicans* e revela maior aderência à mucosa oral. No entanto, a formação de hifas é mais lenta, sugerindo menor poder de invasão [11]. Esta espécie emergente parece ser menos patogênica que *C. albicans,* porém tem maior facilidade de desenvolver resistência aos azólicos[12]. É provável que esta nova espécie de *Candida* já estivesse presente na comunidade há muito tempo e que era identificada incorretamente como *C. albicans.*

Chavasco et al.[13] realizaram a técnica de reação em cadeia do polimerase (PCR) em 37 amostras isoladas de candidíase oral eritematosa, provenientes de pacientes HIV positivos e de HIV negativos, antes identificadas, por método clássico, como *C. albicans, e* armazenadas no Laboratório de Leveduras Patogênicas, da Universidade de São Paulo. Os autores encontraram duas amostras de *C. dubliniensis* (5,4%) e registraram que a técnica de PCR mostrou-se útil, prática e capaz de identificação taxonômica mais acurada.

No Brasil, *C. dubliniensis* foi relatada pela primeira vez por Milan et al. [(14)], em 1999, em amostra isolada de um paciente com aids e candidíase oral, no Estado de São Paulo. Posteriormente, Sano et al.[15], em 2000, isolaram a

espécie de uma criança com três anos de idade, infectada pelo HIV e com candidíase oral, no mesmo Estado. Alves et al.[16], em 2001, descreveram os três primeiros casos de isolamento de *C. dubliniensis* de pacientes com aids, no Estado do Rio Grande do Sul. Ainda são raros os casos de doença sistêmica relacionada a esta nova espécie, a maior parte dos quais se encontra associada à infecção de mucosa oral [12].

Candida spp. faz parte da microbiota normal da boca de 25% a 50% dos indivíduos saudáveis [17], sendo a *C. albicans* a espécie mais prevalente entre as colonizadoras, embora espécies de *Candida* não-*albicans* também tenham sido isoladas[18].

2. Infecção e, ou, colonização da cavidade oral por *Candida* spp. em pacientes infectados pelo vírus da imunodeficiência humana (HIV).

Em indivíduos infectados pelo HIV são frequentes as infecções oportunísticas[19]. O vírus se liga ao receptor $CD4^+$ expresso na superfície dos linfócitos T auxiliares [20,21], levando à destruição dos órgãos linfóides e comprometendo a reposição dos linfócitos T com marcador $CD4^+$ [22]. A deficiência desses linfócitos leva a um grande desarranjo na resposta imune, pois eles modulam as principais funções do sistema imunológico[23]. Essa imunossupressão se manifesta por sintomatologia bastante variada e as vezes tem a boca como sitio de lesões. A infecção pelo HIV está associada ao aumento da proporção de indivíduos colonizados por *Candida* spp., antes

mesmo da instalação de imunossupressão evidente. A freqüência de isolamento de *Candida* spp da cavidade oral desses pacientes aumenta com a progressão do comprometimento imune celular[24]. A transformação da colonização, assintomática, para doença, sintomática, ocorre na vigência dessas condições imunossupressoras [17].

As alterações bucais em pacientes com aids são muito diversificadas, compreendendo vários tipos de lesões, que inúmeras vezes constituem as principais manifestações da doença [25,26]. Entre as manifestações orais em infectados pelo HIV ou com aids, a candidíase oral foi a manifestação mais prevalente, sendo a forma clínica pseudomembranosa a mais observada, seguida da eritematosa, hiperplásica e queilite angular [27,28].

Indivíduos infectados pelo HIV e com baixas contagens de linfócitos T com marcador $CD4^+$ apresentaram maior risco de colonização e infecção por *C. albicans* na cavidade oral do que aqueles com maiores contagens de seus linfócitos[29].

A introdução terapia anti-retroviral altamente potente e efetiva (HAART) levou à mudança do perfil epidemiológico das infecções oportunísticas[30], devido à estabilidade imunológica, à restauração parcial do sistema imune e ao atraso na progressão da aids [30,31]. Assim, observou-se grande redução da candidíase oral, em especial da forma pseudomembranosa [32-33]. No entanto, a redução da incidência da candidíase pode estar relacionada ao uso de inibidores de protease, que demonstraram, *in vivo* e *in vitro,* atividade anti-candida, especialmente o ritonavir e o indinavir [34-36].

A freqüência de isolamento de *C. albicans* e a recorrência clínica da candidíase aumentam com a progressão da infecção pelo HIV [37], provavelmente devido ao comprometimento da resposta imune e à administração prolongada de agentes antifúngicos, que proporcionam a seleção de amostras resistentes [38-40]. A colonização simultânea por diferentes subclones de *C. albicans* poderia explicar a recorrência das infecções [41], o que teria importantes implicações terapêuticas, uma vez que poderiam apresentar diferentes perfis de suscetibilidade aos agentes antifúngicos [37,42,43].

A ação direta do HIV sobre *C. albicans* foi revelada por Gruber et al.[44], em 1997, ao demonstrarem que glicoproteínas do HIV aumentam a virulência deste fungo. Por fim, a ação direta de inibidores de protease sobre a proteinase aspártica de *Candida* spp., que propicia a invasão de mucosa, foi demonstrada por Cassone et al.[34] e por Blanco et al.[35]. Vários estudos demonstraram que os inibidores de protease são dirigidos não somente contra a protease do HIV, mas também contra a produção de proteinase aspártica por *C. albicans*, que pertence à mesma classe da protease viral [34-36,45-47].

Hoegh et al[47] sugeriram que os inibidores de protease do HIV reduziriam a prevalência de candidíase oral em pacientes infectados pelo HIV, pela ação direta desses inibidores sobre a secreção de proteinase aspártica por *Candida* spp. Assim, o sucesso da terapia anti-retroviral poderia ser atribuído a vários fatores, isto é, à inibição de um ou mais fatores de virulência da *C. albicans* e à restauração do estado imunológico dos pacientes infectados [34,46-50].

A secreção de proteinase por *C. albicans* foi avaliada em 18 amostras isoladas de indivíduos infectados pelo HIV, com valores de linfócitos T com marcador $CD4^+$ inferiores a 400 células/mm^3 e em outras 18 procedentes de indivíduos não infectados pelo HIV, que constituíram o grupo controle. Observou-se que todas as amostras de *C. albicans* isoladas de pacientes HIV-positivos secretaram proteinase e apenas 56% das isoladas do grupo controle. A atividade proteolítica foi maior nas amostras obtidas no grupo de pacientes infectados pelo HIV. Os autores também verificaram correlação direta entre atividade de proteinase e resistência a antifúngicos. Amostras de *C. albicans,* causadoras de candidíase oral e produtoras de elevados níveis de proteinase, foram menos sensíveis a antifúngicos azólicos do que as amostras de *C. albicans* isoladas de indivíduos não infectados pelo HIV

Os autores também avaliaram o efeito inibitório de níveis abaixo da concentração inibitória mínima - CIM (1/4 e 1/16) de antifúngicos poliênicos e imidazólicos sobre a produção de proteinase em sete amostras de cada grupo. Observou-se menor efeito inibitório em amostras de *C. albicans,* causadoras de candidíase oral em infectados pelo HIV e produtoras de elevados níveis de proteinase, quando comparado às isoladas do grupo controle e, de maneira geral, um efeito dose-dependente [51].

Barchiesi et al.[52] estudaram a colonização em função de carga viral, quantificação de linfócitos T com marcador $CD4^+$, esquema anti-retrovial utilizado e antecedente de candidíase de orofaringe. O único fator que influenciou a presença de colonização foi o antecedente de candidíase de orofaringe ($p=0,009$). Os autores também demonstraram que 93% das

amostras eram sensíveis e que 7% foram classificados como sensibilidade dependente da dose de fluconazol.

Ribeiro et al.[53] estudaram a prevalência de *Candida* spp. em mucosa oral de 332 mulheres infectadas ou colonizadas por esse fungo, 127 das quais eram também infectadas pelo HIV (grupo I), enquanto as outras 205 eram soronegativas em relação a este vírus (grupo II). Os autores observaram que 681 das mulheres infectadas pelo HIV se apresentavam colonizadas por diferentes espécies de *Candida* spp. e que foi possível isolar este fungo em todos os casos (100%) em que havia candidíase de mucosa oral. Entre as mulheres não infectadas pelo HIV, esses valores foram iguais a 32% e 80%, respectivamente. Houve grande predomínio de *C. albicans* entre as espécies isoladas de mucosa oral, nos dois grupos: 79% nas HIV-negativas e 78% nas HIV-positivas. A seguir, em escala decrescente de freqüência, no grupo I observaram-se *C. tropicalis* e *C. parapsilosis*, enquanto no grupo II detectaram-se *C. tropicalis*, *C. parapsilosis* e *C. guilliermondii*. Amostras isoladas de pacientes infectadas pelo HIV apresentavam maior atividade enzimática que aquelas provenientes de não-infectadas pelo HIV. Pacientes sob tratamento anti-retroviral combinado potente, com inibidor de protease, apresentaram amostras de *C. albicans* com menor atividade enzimática, equivalente à de amostras isoladas de mulheres não infectadas pelo HIV.

Moris [54] avaliou a prevalência de colonização por *Candida* spp. em indivíduos infectados pelo HIV em função da espécie, da contagem de linfócitos T $CD4^+$ e T $CD8^+$, da carga viral plasmática do HIV, do uso de inibidores de protease viral (IPs) no tratamento anti-retroviral e da atividade enzimática das

amostras de *C. albicans*. Foi realizado um estudo de prevalência-período em 156 indivíduos infectados pelo HIV e em 92 indivíduos controles saudáveis. A prevalência de colonização foi maior em indivíduos infectados pelo HIV (84,0%) do que nos indivíduos saudáveis (28,25%). *C. albicans* foi mais prevalente do que *Candida* não-*albicans* tanto nos indivíduos infectados pelo HIV (82.1% vs 17.9%) quanto nos saudáveis (85.2% vs 14.8%, todas identificadas como *C. parapsilosis*). A prevalência de colonização por *Candida* spp. Em função da contagem de linfócitos T $CD4^+$, independentemente da fase da infecção pelo HIV, tendeu a ser maior nos pacientes com menos de 200 linfócitos $TCD4^+/mm^3$ [$0.05<p<0.10$]. As amostras de *C. albicans* com atividade proteolítica baixa foram prevalentes especialmente em indivíduos infectados pelo HIV e não-aids, sem o uso de IPs. A prevalência de colonização por *Candida* spp. mostrou uma associação direta com carga viral plasmática do HIV, principalmente para *C. albicans*. As amostras de *C. albicans* com atividade proteolítica baixa foram mais prevalentes nos pacientes com aids e com carga viral indetectável [54].

Costa et al.[55] observaram que 62,6% dos 99 pacientes infectados pelo HIV, eram colonizados por *Candida* spp. *C. albicans* apresentou a maior freqüência (50%) enquanto as espécies de *Candida* não-*albicans* foram representadas por *C. tropicalis* (20,9%), *C. parapsilosis* (19,3%), *C. guilliermondii* (4,8%), *C. lusitaniae* (1,6%), *C. krusei* (1,6%) e *C. kefyr* (1,6%). Não se observou associação entre espécie de *Candida* e uso da terapia anti-retroviral. A resistência ao fluconazol foi observada em 8,1% das amostras, 8,2% dos quais foram obtidos de 49 doentes em uso de ARV e foi mais

freqüente que ao voriconazol. Nenhuma das amostras revelou-se resistente à anfoterecina B.

Blignaut [56] observou colonização e infecção por *Candida* spp. na mucosa oral de 87 crianças de orfanatos e 330 adultos infectados pelo HIV, aids e não-aids não-hospitalizados em Gauteng (África do Sul). A prevalência de colonização e, ou, infecção foi de 41,7% entre as crianças, predominando as espécies de *Candida* não-*albicans*, e de 91,5% entre os adultos com predomínio de *C. albicans*. A prevalência de *C. dubliniensis* foi considerada elevada, porém com identificação baseada apenas em métodos fenotípicos. Nenhum dos pacientes recebia tratamento anti-retroviral, no momento do estudo.

Sánchez-Vargas et al.[57,58] estudaram 111 pacientes HIV-positivos, 51 dos quais eram adultos e 60 eram crianças e 201 controles saudáveis - 109 adultos e 92 crianças. As amostras foram coletadas através de *swabs* de lesões orais, da mucosa bucal e da superfície dorsal da língua. O isolamento de *Candida* spp. não diferiu entre adultos com e sem infecção pelo HIV (74,5% e 61,5%, respectivamente) e crianças infectadas pelo vírus HIV ou não (60,0% e 40,2%,respectivamente). A presença ou ausência de colonização oral em pacientes HIV positivos não se associou com a contagem de linfócitos TCD4 $^{+}$ (623/mm^3 nos colonizados e 643/mm^3 em indivíduos não-colonizados), e nem com a carga viral (52275 cópias / mL nos colonizadas e 55173 cópias / mL em indivíduos não-colonizados). Além disso, a candidíase oral também não foi associada à contagem de linfócitos TCD4 $^{+}$, 775/mm^3 em pacientes com candidíase oral e 643/mm^3 naqueles sem candidíse oral, ou da carga viral

(60.995 e 55.173 cópias / mL em pacientes com e sem candidíase, respectivamente). O esquema anti-retroviral não influenciou a alteração do estado de colonização ou infecção - 69,4% dos pacientes submetidos à HAART e 59,5% dos pacientes sem tratamento anti-retroviral [57, 58]. A candidíase foi menos prevalente em pacientes submetidos à HAART (36,1%) do que em doentes sem tramento anti-retroviral (45,9%). Achado semelhante foi também observado em uma coorte de crianças com contagem de células TCD4 $^{+}$ inferior a 500 /mm^3. *C. albicans* foi a espécie mais prevalente nos pacientes infectados e colonizados, bem como em indivíduos sadios; o sorotipo A predominou em todos os quatro grupos. Espécies de *Candida* não-*albicans*, principalmente *C. glabrata* e *C. tropicalis*, foram isoladas de 16,5% pacientes colonizados e 38,5% de pacientes com candidíase. O isolamento simultâneo de múltiplas espécies foi observado em apenas nove episódios de infecção ou de colonização, sete dos quais incluíam *C. albicans* e *Candida* não-*albicans*, e dois apresentavam associação de duas espécies de *Candida* não-*albicans*. As porcentagens de resistência e suscetibilidade intermediária para os antifúngicos estudados, analisando-se as amostras em conjunto, foram respectivamente: itraconazol (ITZ) - 10,7% e 28,9%; cetoconazol (CTZ) - 10,2% e 20,8%; miconazol (MCZ) - 2,7% e 17,6%; fluconazol (FCZ) - 3,2% e 11,2%; anfotericina B (AMB) - 0,0 e 2,1%; 5-fluorcitosina (5-FC) - 0,0 e 0,0%. A maioria das amostras resistentes eram de *C. glabrata* isoladas de indivíduos HIV-negativos e de *C. albicans* originadas de pacientes HIV-positivos, com candidíase pseudomembranosa. Os resultados das avaliações de resistência e suscetibilidade intermediária das amostras de *C. albicans*, foram,

respectivamente, 10,3% e 28,7% para CTZ; 8,1% e 21,3% para ITZ; 0,7% e 28,7% para MCZ; e 2,2% e 5,9% para FCZ [57,58].

A genotipagam é útil na avaliação da distribuição espacial e temporal de subtipos de *C. albicans* nas infecções humanas. Mesmo assim, pouco se conhece sobre a variação clonal da *C. albicans* em diferentes episódios de candidiase oral recorrente ou durante o período de colonização da cavidade bucal ao longo da infecção pelo HIV [59]. Apesar de a maioria dos episódios de recidiva da doença ser creditada à permanência da amostra infectante original, poucos trabalhos avaliam quantos episódios são causados por re-infecção com novas e diferentes amostras de *Candida* spp.[60].

A genotipagem de leveduras isoladas durante os episódios de recidiva da infecção permite, além da análise genômica de similaridade entre as amostras, a possibilidade de se comparar o perfil de sensibilidade dessas leveduras, uma vez que diferentes subtipos das espécies de *Candida* podem apresentar variados graus de suscetibilidade aos antifúngicos nas amostras seqüenciais. Assim, a genotipagem também é útil como marcador epidemiológico para os estudos de resistência aos antifúngicos [60].

Em estudo prospectivo para avaliar a variação genotípica de amostras de *C. albicans* isoladas da cavidade oral de 16 indivíduos infectados pelo HIV, com ou sem candidíase, foram estudadas cinco colônias isoladas, de cada um dos indivíduos; 443 amostras foram identificadas como *C. albicans* por métodos tradicionais. Quando as amostras foram submetidas à técnica de amplificação aleatória de DNA polimórfico (RAPD) para análise genética de

similaridade, verificou-se a prevalência de até 14 genótipos diferentes de *C. albicans* por indivíduo nos momentos seqüenciais [59]. Esse estudo também revelou que leveduras isoladas de coletas seqüenciais de indivíduos com candidíase oral demonstravam maior nível de similaridade quando comparadas com as isoladas dos indivíduos apenas colonizados por *C. albicans. O*s autores sugeriram que as amostras de *C. albicans* responsáveis por candidíase recorrente são mais resistentes devido ao uso prolongado de antifúngicos e, portanto, mais estáveis.

Utilizando-se técnicas para subtipagem de DNA de amostras de *C. albicans* de pacientes com recidiva e sob terapia prolongada ou intermitente com antifúngicos, observou-se aumento gradual de resistência a essas drogas nessas amostras e introdução ou seleção de amostras diferentes da original, com menor sensibilidade aos antifúngicos utilizadas[61]. A intensidade da colonização aumenta com o tempo, até que se observe o primeiro episódio de candidíase oral. Observaram-se variações genéticas e até substituição de uma espécie por outra de *Candida* sp, durante esses episódios [29].

Desde o início da década de 80 foram observadas amostras de *Candida* sp resistentes ao cetoconazol, primeiro azólico utilizado no tratamento da candidiase oral em pacientes com aids. A introdução dos triazólicos iniciou uma nova era no tratamento das infecções fúngicas. O fluconazol tem demonstrado ser eficaz e bem tolerado [62] e apresenta ampla distribuição tecidual, com maior concentração em fígado, pulmões e rins [63]. Apesar da eficácia do fluconazol no tratamento de pacientes com aids e candidíase oral, as recidivas são freqüentes, motivo pelo qual muitos doentes têm recebido fluconazol por tempo

prolongado, o que levou à seleção de fungos resistentes e ao relato de falência do tratamento [64].

C. dubliniensis tem sido objeto de ampla investigação, em decorrência de sua identidade fenotípica e similaridade genotípica à *C. albicans* e da identificação de amostras resistentes a drogas antifúngicas, como o fluconazol [65-68].

Características cariotípicas de *C. dubliniensis* e *C. albicans* foram estudadas por Magee et al. [68], utilizando a técnica de eletroforese em campo pulsátil (PFGE), eles observaram que em determinadas regiões cromossômicas do genoma de *C. dubliniensis* havia maior freqüência de translocações, quando comparadas com regiões homólogas da *C. albicans*, sugerindo maior instabilidade genômica de *C. dubliniensis.* Este achado poderia estar relacionado à maior virulência desta espécie que a de *C. albicans.*

A resistência de cepas de *C. dubliniensis* a drogas antifúngicas azólicas pode ser induzida *in vitro* e foi observada em amostras clínicas. Em geral, os mecanismos moleculares pelos quais *C. dubliniensis* adquire resistência a compostos azólicos são decorrentes da atividade aumentada das bombas de efluxo, modificações da enzima-alvo da ação das drogas antifúngicas e alterações na via de biossíntese do ergosterol, importante componente da membrana citoplasmática de leveduras [69].

C. parapsilosis, desde a década de 80, se apresenta como importante causa de fungemia, sendo responsável por 7% a 15% das candidemias nos

Estados Unidos da América [70] e na Europa [71]. Em países da América Latina, a *C. parapsilosis* tem sido reconhecida como a segunda principal causa de infecção invasiva [72-74].

C. parapsilosis prolifera-se em soluções contendo glicose, produz biofilme e com freqüência coloniza pele e mucosas, tem ampla distribuição na natureza, sendo isoladas de animais domésticos, solo e ambiente marinho [75].

A fungemia por *C. parapsilosis* está associada à utilização de cateter venoso[70]. Amostras clínicas desta espécie são sensíveis à anfotericina B e aos triazólicos [76].

Baseado-se em técnicas moleculares, esta espécie foi dividida em três grupos (I, II e III). Estudos moleculares recentes, demonstraram que não se trata de subrupos e, sim de, três espécies distintas: *C. parapsilosis* (grupo I), *C. orthopsilosis* e *C. metapsilosis,* substituindo respectivamente os grupos II e III [77-80].

Moris et al. [81], em revisão sobre colonização por *Candida* spp. em indivíduos infectados pelo vírus da imunodeficiência humana, sugeriram que os resultados da análise de fatores envolvidos na colonização e infecção são muitas vezes contraditórios, o que demonstra o quanto a patogenia da candidíase ainda é desconhecida. Esses autores também concluíram que os trabalhos que avaliaram colonização e infecção por *Candida* spp. da cavidade bucal de pacientes infectados pelo HIV indicam a necessidade de novos estudos para melhor entender o comportamento dessas leveduras em pacientes infectados pelo vírus da imunodeficiência humana.

3. Objetivos

Com base nas considerações apresentadas, os objetivos do presente estudo foram:

- Caracterizar, fenotipicamente, amostras de *Candida* spp. isoladas da cavidade bucal de pacientes infectados pelo vírus HIV, aids e não-aids;
- Identificar a prevalência de leveduras do complexo *Candida psilosis* na cavidade oral de indivíduos infectados pelo HIV; determinar o perfil de sensibilidade a fluconazol, itraconazol, cetoconazol, voriconazol, caspofungina e anfotericina B dessas leveduras;
- Identificar a prevalência de *Candida dubliniensis* na cavidade oral de indivíduos infectados pelo HIV; determinar o perfil de sensibilidade a fluconazol, itraconazol, cetoconazol, e anfotericina B dessas leveduras;
- Determinar o perfil de sensibilidade a fluconazol, itraconazol, cetoconazol e anfotericina B das amostras de *Candida* spp. isoladas de da cavidade bucal de indivíduos infectados pelo HIV;

- Analisar a similaridade genética das amostras de *C. albicans* coletadas em episódios seqüenciais de colonização e infecção da cavidade bucal; comparar o perfil de sensibilidade a fluconazol, itraconazol, cetoconazol e anfotericina B das leveduras isoladas em amostras seqüenciais do mesmo paciente, cujos genótipos, foram determinados.

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Capítulo II - Resultados

Os resultados serão apresentados sob forma de quatro trabalhos, segundo as normas de apresentação do periódico o qual serão submetidos, o *Journal of Clinical Microbiology*.

1. Prevalence and antifungal susceptibility of *Candida psilosis* complex isolated from the oral cavity of HIV-infected individuals.

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Running title: Characteristcs of *C. psilosis* isolated from HIV - infected individuals

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KEY WORDS: *C psilosis* complex, human immunodeficiency virus-infected individuals, oral cavity.

ABSTRACT

At present few data are available on prevalence and susceptibilities of *Candida psilosis complex* which contains 3 species *C. parapsilosis, C. metapsilosis* and *C. orthopsilosis*, in HIV-infected individuals. In our study isolates of *Candida* spp. were obtained from oral cavitiy of 15 HIV – infected individuals, and these isolates were identified as *C. psilosis* complex by means of polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). Prevalence of the C. *psilosis* complex was 4.7%, with 2.2% being *C. parapsilosis* and 2.5% *C. metapsilosis,* while no *C. orthopsilosis* was isolated.. *In vitro* studies demonstrated that all isolates were susceptible to amphotericin B (AMB), fluconazole (FLC), ketoconazole (KTC), itraconazole (ITC), voriconazole (VRC) and caspofungin (CASPO). Studies with *C. parapsilosis* and *C. metapsilosis* showed minimum inhibitory concentration MIC_{50} (mg/L) and MIC_{90} (mg/L), were similar for all drugs tested. There were no marked differences in the MICs for *C. parapsilosis* and *C. metapsilosis* isolates for all antifungal compounds tested, except for FLC, which was significantly higher for *C. metapsilosis* than *C. parapsilosis*. Based upon the frequency of candidiasis and that certain isolates of *C. psilosis* complex respond differently to FLC therapy, our data may be of therapeutic relevance with respect to susceptibility to specific antifungal agents and the potential for acquirement of drug resistance.

INTRODUCTION

Candida parapsilosis was first isolated by Ashford, in 1928, from the feces of a patient with diarrhea in Puerto Rico (2). This species was considered non-pathogenic until 1940, when Joachim and Polayes (13) attributed the first fatal case of endocarditis to *C. parapsilosis* in a patient using illicit drugs (13). Although less common than *Candida albicans*, *C*. *parapsilosis* produces several infections in the bloodstream and urinary tract, as well as vagina, peritoneum, oral cavity, joints and endocardium (4, 16, 34, 36). Further, *C. parapsilosis* is also an emergent and opportunistic nosocomial pathogen known to produce superficial and systemic candidiasis (18, 35, 36), but severe, invasive disseminations have also been reported (16, 34,35).

It is of interest that in approximately 50% of the healthy adult human population *Candida* spp is present in the oral cavity (1), and the mucosal surfaces are considered to serve as the main reservoir. In addition, *Candida* spp. is found in dental biofilm, co-aggregated with bacterial species present in the oral cavity, or even directly adhering to the salivary pellicle (3). Oropharyngeal *Candida* infections occur frequently in patients infected with HIV who have less than 400 $CD4^+$ lymphocyte counts. It should be noted that the presence of oral *Candida* is considered a biomarker associated with a deterioration in immunodeficiency disorders and is indicative of a progressive disease (24, 33).

Several predisposing conditions are associated with development of candidiasis including asymptomatic colonization or clinical conditions such as

specific immune deficiencies, use of total prostheses, endocrine disorders, soft tissue lesions, medications including antibiotics and corticosteroids, as well as smoking (1, 7, 18, 33, 36).

The presence of *Candida* species is increasing in frequency globally but to a greater extent in tropical regions. Recently *C. parapsilosis* was found to exist in three different species forms classified by genotyping as *C*. *parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* (30). Although *C. psilosis* complex has been recognized for a number of years, little is known about the transmission and infectivity of two rare species present within the complex, *C. orthopsilosis* and *C. metapsilosis*. *C.parapsilosis.* Previous studies reported decreased responsiveness of *C. parapsilosis* to fluconazole (FLC) and caspofungin (CASPO) which suggested that resistance might become a cause for clinical concern (11, 16, 20, 28, 32). It is thus important to determine the distribution of these species in our environment in particular in HIV infected individuals and to correlate genotypic variation in these species with virulence capabilities as well as susceptibility to antifungal drugs.

The growing importance of human fungal infections and increasing number of fungal species involved in these infections led to an improvement in laboratory techniques to identify and characterize fungal infections in an attempt to establish routine, inexpensive methods for therapeutic applications. The existing commercial methods using yeast morphology and biochemistry can not distinguish *C. parapsilosis* from *C. metapsilosis* or *C. orthopsilosis*. Since yeast methodology is not effective in distinguishing the different species of *C. psilosis* complex, studies were conducted using genotypic technology to differentiate

these species of *C. psilosis* complex (5, 17, 19, 30).

The aim of the study was thus to (1) distinguish the different forms of *C. psilosis* complex using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP), (2) characterize the *C.psilosis* complex clusters genotypically using randomly amplified polymorphic DNA (RAPD) and (3) determine the susceptibility of the three different especies of *C. psilosis* complex to various antifungal agents.

MATERIALS AND METHODS

Approval was obtained from the Ethics Committees approval of the Comitê de Etica em Pesquisa, Faculdade de Medicina de Botucatu, São Paulo State University (UNESP) and Comitê de Etica em Pesquisa com seres Humanos, Adolfo Lutz Institute. Informed patient consent was required for this study.

Fungal isolates. The characteristics of the 15 HIV positive individuals from which *C. psilosis* complex was isolated are presented in Table1 .*Candida* spp. were obtained from the oral cavity of these individuals with the use of swabs. The swabs were then placed on plates containing Sabouraud agar (Difco, Detroit, Michigan) supplemented with 0.05% chloramphenicol. The plates were incubated at 30°C for 24h.The isolates were plated on CHROMagar *Candida* (CHROMagar, Paris France) at 35ºC for 48h. Subsequently, the color and morphology of one colony of each isolate was then analyzed to establish

the presence of *Candida* spp. In order to differentiate and identify *Candida* spp. at the microscopic level isolates were incubated in cormeal agar (Oxoid, Ltda, Brasil) supplemented with 1% Tween 80 (Synth, Labsynth, Brasil) to verify the morphology (15) and in API 20C AUX (BioMérieux Marcy l'Etoile, France) according to the manufacturers instructions to determine the biochemical characteristics.

DNA extraction. Each isolate was plated on Sabouraud Dextrose Agar (Difco, Detroit, Michigan) and incubated at 30ºC for 24 hr. The yeast cells were then transferred to a microcentrifuge tube containing 1ml of 50mM EDTA pH8, vortexed and centrifuged at 13,000 x g for 15 min. The supernatant was discarded and the pellet was resuspended in 200µl 50mM EDTA (Sigma Chemical, USA) pH8 containing 40µl lysis enzyme obtained from *Trichoderma harzianum* (10mg/ml) (Sigma Chemical, USA), followed by incubation at 37ºC for 2-3h. Subsequently, this mixture was centrifuged at 13,000 x g for 10 min and the pellet was dissolved in lysis buffer containing 10mM Tris-HCl (Sigma Chemical, USA) pH 8, 10mM EDTA (Sigma Chemical, USA), 0.5% sodium dodecyl sulfate (SDS) (Synth, Labsynth, Brasil), 0.01% N-laurylsacozyl (Sigma Chemical, USA) and 10µg/ml proteinase K (Sigma Chemical, USA). The mixture was then vortexed and incubated at 56ºC for 2-3h. DNA was extracted by the phenol/chloroform/isoamyl alcohol and precipitated with isopropanol according to Sambrook et al (26). After washing the pellet with 70% ethanol for 10min at 10,000g, DNA was dissolved in 50 µl ultra pure water. DNA concentrations were determined using a NanoDrop1000 (Thermo Fisher Scientific, USA) apparatus as described by Sambrook et al (26). For PCR and

RAPD amplification, 1µl of each DNA sample (approximately 100ng) was used. DNA was frozen at -20ºC until use.

Molecular identification of *psilosis* complex

PCR. The protocol used for PCR and PCR-RFLP was previously described by Tavanti and coworkers (30).The secondary alcohol dehydrogenase (SADH) fragment was amplified for *C. parapsilosis* isolates. One pair of primers (Invitrogen, Ltda, Brasil) was chosen: sense (S1F: 5' GTTGATGCTGTTGGATTGT-3') and anti-sense (S1R: 5'-CAATGCCAAATCTCCCAA-3'). The amplification conditions were as follows: a first denaturation cycle at 94ºC for 5min, followed by 30 denaturation cycles at 94ºC for 45s, annealing at 50ºC for 1min and elongation at 72ºC for 45s, with a final extension step at 72ºC for 10min. Amplified DNA products were separated by electrophoresis on a 2% agarose gel in 89 mM Tris/HCl, 89mM boric acid, 2 mM EDTA, (TBE buffer) pH 8 at 100V for 35 min and a 100-bp DNA ladder was used as molecular size marker (Invitrogen, Ltda, Brasil). The gel was stained with ethidium bromide (0.5mg/ml) and DNA bands were visualized by UV transillumination Mini Bis Pro (Bio-Imaging Systems, Ltda, Israel) .

PCR-RFLP: The PCR product of the amplification of SADH fragments from all *C. parapsilosis* clinical isolates and from *C. parapsilosis* ATCC 22019, *C. metapsilosis* ATCC 96143 and *C. orthopsilosis* ATCC 96139 were digested for 90 min with BshNI (Fermentas, Life Sciences, Canada) in a 20µl reaction volume containing 6µl PCR product, 2µl of 10X buffer O (supplied with enzyme)

and 2μl 20U BshNI/uL. Digestion products were loaded onto 2% agarose gel in TBE buffer at 100V for 35 min and a 100-bp DNA ladder was used as molecular size marker (Invitrogen, Ltda, Brasil). The gel was stained with ethidium bromide (0.5mg/ml) and DNA bands were visualized by UV transillumination (Bio-Imaging Systems Ltda, Israel). Type strains of *C. parapsilosis* of the American Type Culture Collection (ATCC) 90019, *C. metapsilosis* ATCC 96143 and *C. orthopsilosis* ATCC 96141 were included.

RAPD analysis. Amplification was carried out with the kit Ready-to-Go-RAPD Analysis Beads (GE Healthcare, United Kingdom). RAPD beads were composed of 1.5 units of Taq DNA polymerase, 10mM Tris-HCl pH 8.3, 30mM KCl, 3 mM $MgCl_2$, 400mM of each dNTP, and stabilizers such as bovine serum albumin. Each reaction was performed by adding each DNA template and 25pmol of the primer to a final volume of 25 μl. The selection of primers was determined after testing six primer sequences for exclusive use in RAPD (GE Healthcare, United Kingdom). The primer p2 (5'-GTTTCGCTCC-3') was selected for *C. parapsilosis* and *C. metapsilosis* isolates. The amplification was performed in an automated thermal cycler (Progene, USA) and consisted of one initial denaturation cycle at 95ºC for 5 min, 45 denaturation cycles at 95ºC for 1 min, annealing at 32ºC for 1 min, and extension at 72ºC for 2 min. The procedure was completed with a final extension cycle for 10min. DNA samples were tested in duplicate. Each amplification run contained a negative control (ultra pure water). After thermal cycles, PCR products were eletrophoresed on polyacrylamide gels in TBE buffer pH 8 and stained with ethidium bromide. The sizes of fragments were based on the comparison with a 100-bp DNA

ladder (Invitrogen, Ltda, Brasil) and visualized by UV transillumination Mini Bis Pro (Bio-Imaging Systems, Ltda, Israel). The band profiles in RAPD for *C. parapsilosis* and *C. metapsilosis* clinical isolates were compared to those for the type strains *C. parapsilosis* ATCC 22019, *C. orthopsilosis* ATCC 96141 and *C. metapsilosis* ATCC 96143.

Analysis of data and dendrogram generation. The different band positions of RAPD fingerprinting patterns for isolates of the C.*psilosis* complex were analyzed by using the program Bionumerics version 5.10 (Applied Maths, Kortrijk, Bélgica) for the analysis of relationships among a number of strains, the coefficient of Dice and similarity coefficients (S_{AB}), which constructs dendrograms by unweighted pair-group method using arithmetic averages (UPGMA) method (1.5% optimization and 2% tolerance), was used. (29)

Antifungal agent susceptibility testing

Standard antifungal susceptibility tests were performed according to a reference microdilution method established by Europen Committee on Antimicrobial Susceptibility Testing (EUCAST) (6). The employed antifungal agents were amphotericin B (AMB), ketoconazole (KTC), fluconazole (FLC), itraconazole (ITC), voriconazole (VRC) and caspofungin (CASPO). Interpretative criteria for CASPO were recently established by Clinical and Laboratory Standards Institute (CLSI) (minutes of the June 2007 meeting of the CLSI Antifungal Subcommittee) as follows: a minimum inhibitory concentration (MIC) of ≤2 mg/L is indicative of susceptibility to CASPO in isolates. Interpretive criteria for AMB have not yet been established; however, for purposes of comparison, isolates with MIC of ≤1 mg/L following AMB exposure were

considered susceptible to this drug (21, 22, 25). Positive control was performed using *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 for each batch of isolates tested.

Statistical analyses. Differences in antifungal MIC distributions were examined using the Mann-Whitney U test. Comparison of frequencies was carried out by Fisher's exact test. The criterion for significance was set at $p < 0.05$ (27, 38).

RESULTS

Molecular identification of the C.*pasilosis* complex.

All isolates displayed a 716bp fragment confirmed by PCR as belonging to *C.parapsilosis*. Of the *C. parapsilosis* isolates from the 15 HIV – positive individual used in this study, 7 (46.7 %) were identified as *C. parapsilosis,* and 8 (53.3%) as *C. metapsilosis.* As shown in Figure 1, for *C. parapsilosis* , a fragment of *SADH* gene (716 bp) was amplified by PCR (Fig. 1, lanes 1, 2, 3 and 4). Isolates were molecularly distinguished as *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* (Fig. 1, lanes 5, 6, 7, 8 and 9), as follows: *C. parapsilosis isolates* possessed one BanI restriction site, which revealed two bands at positions 521 and 196 bp. *C. orthopsilosis isolates* had no restriction sites, which yielded one band at position 716 bp. Finally *C. metapsilosisis* isolates possessed three BanI restriction sites, which yielded four bands at positions 370, 188, 93, and 60 bp.

RAPD analysis. RAPD analysis enabled the verification of intrastrain differences between 7 *C. parapsilosis* and 8 *C. metapsilosis* isolates obtained from the oral cavity of 15 HIV- infected individuals. One isolate did not amplify with the selected primer and was excluded thus leaving 14 isolates that were studied.The dendrogram based on S_{AB} values, generated for *C. parapsilosis* and *C. metapsilosis* isolates is presented in Figure 4. The mean S_{AB} was 85% for *C. parapsilosis* and 91% for *C. metapsilosis*. The Dendron database (29) displayed three clusters for *C. parapsilosis*, and *C. metapsilosis* (Fig 2).

Prevalence of the *psilosis* complex. The prevalence of the C. *psilosis* complex was 4.7% and the entire *C. psilosis* complex colonized in the 15 of 318 isolates from 214 HIV positive individuals. *C. parapsilsois* occurred with a prevalence of 2.2%, while *C. metapsilosis* was 2.5%. No *C. orthopsilosis* was isolated from the oral swabs of HIV – infected individuals.

Antifungal susceptibility testing. All tested organisms grew after 24h incubation. The results of susceptibility tests to AMB, FLC, KTC, ITC, VRC and CASPO for 7 *C. parapsilosis* and 8 *C. metapsilosis* isolates are presented in Table 2 and Figure 3. All isolates were susceptible to the different antifungal agents tested. The only significant difference in susceptibility between species was observed for FLC to *C. parapsilosis*, which showed higher sensitivity than *C. metapsilosis*, MIC_{50} equal to 0.5 and 1 mg/L and MIC_{90} equal to 1 and 2 mg/L, respectively.

DISCUSSION

Phenotypic methodology is not able to differentiate the strains within the *C.psilosis complex.* Tavanti and coworkers (30) carried out a study based upon molecular techniques to differentiate the species within *C.psilosis* complex and suggested that *C. parapsilosis* be reclassified into *3* genotypes : *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosi*s. The prevalent species was *C. parapsilosis* (74.1%) and *C. orthopsilosis* (25.9%). Six samples from the oral vaginal and anal mucosa were evaluated and identified as *C. parapsilosis* (83.3%) and *C. orthopsilosis* (16.7%) (30). The analysis of 400 clinical samples isolated from different sites and previously identified as *C. parapsilosis* demonstrated that the prevalent species was *C. parapsilosis* (91.8%), followed by *C. orthopsilosis* (8.2%) (31)

Few studies evaluated the prevalence of these three strains of *C.psilosis* in cases of infection or colonization, especially in the oral cavity of HIV-infected individuals. In the current study, the prevalence of of *C. parapsilosis,* was 4.7% in HIV – positive individuals. The amplification of gene *SADH* by PCR, followed by analysis of the genetic profile generated by the restriction enzyme "*Ban*I" by PCR-RFLP, indicated that 7 isolates were molecularly distinguished as *C. parapsilosis* (2.2%) and 8 as *C. metapsilosis* (2.5%). It is of interest that *C. metapsilosis* was reported to rarely be present in samples isolated from urine, skin and blood of patients admitted to care units of the different hospitals and worldwide collection (5, 30, 31). As noted in the case of *C. orthopsilosis* and *C. parapsilosis*, there was a lack of virulence with *C. metapsilosis* (9). *C. orthopsilosis*, was obtained from blood, nails, skin, lungs, urine, as well as

indwelling catheters (19, 30, 31, 32)

Lin and coworkers (19) evaluated the occurrence of the C. *psilosis* complex in samples from four states of the United States by means of immunoenzymatic methods and sequencing of the ITS region. In 45 samples isolated from urine, blood, skin, hands, vaginal mucosa, bronchial lavage and suture *C. parapsilosis* was identified in 73.4% samples, *C. orthopsilosis* in 22.2% and *C. metapsilosis* in 4.4%. However, oral cavity samples were not examined in this study. Enger and coworkers (5) identified 89 clinical isolates collected from 15 different sites of patients from Europe, Asia, and USA by means of Cp3-13-specific probe hybridization. *C. parapsilosis* was identified in 78 (89.7%) samples, followed by *C. orthopsilosis*, isolated from 8 samples (9.2%), and *C. metapsilosis* in one sample (1.1%). The evaluation included 14 (15.7%) samples from the oral cavity, which were identified as *C. parapsilosis* (64.3%) and *C. orthopsilosis* (35.7%), but no *C. metapsilosis* was found at this site. However, in one sample isolated from pharyngeal mucosa *C. metapsilosis* was identified. These findings differ from our results, which showed 46.7% *C. parapsilosis* and 53.3% *C. metapsilosis* in the oral cavity isolates. It is noteworthy that all individuals in the current study were HIV positive, whereas there was no information regarding the origin of the isolates studied by Enger and coworkers (5). Kocsube and coworkers (14) determined the genetic variability of strains of *C. psilosis* complex in 20 samples isolated from 6 different sites of patients hospitalized in two Hungarian hospitals using RAPD and sequencing of the ITS region. The prevalent species was *C. parapsilosis* (90%), followed by *C. metapsilosis* (10%). One sample isolated from the throat was identified as *C. metapsilosis*. No *C. orthopsilosis* was detected. Silva and

coworkers (28) studied 169 samples isolated from 9 different anatomical sites of patients hospitalized in a tertiary care hospital in Portugal. *C. parapsilosis* was identified in 160 (94.7%) samples, *C. orthopsilosis* in 5 (2.9%) and *C. metapsilosis* in 4 (2.4%). Of the 69 mucosal samples isolated, 94.2% corresponded to *C. parapsilosis* and 5.8% to *C. metapsilosis.*

Some studies in Brazil reported the occurrence of isolates of the *C. psilosis* complex in patients with candidemia (12, 11, 36). Iida and coworkers (12) analyzed 52 samples of the C.*psilosis* complex isolated from the bloodstream of patients from Brazil and Japan (24 strains from Brazil and the remainder from Japan) by means of phylogenetic analysis of the ITS region. Data indicated that *C. parapsilosis* corresponded to 49 (94.2%) samples and *C. metapsilosis* to three (5.8%). No *C. orthopsilosis* was identified. The above-mentioned studies show a higher occurrence of *C. parapsilosis*, 55.8% to 94.7%, within the *C. psilosis* complex, which was equal to 46.7% in the current study. The clinical relevance of *C. orthopsilosis* and *C. metapsilosis* as etiological factors in candidiasis development can not be assessed , due to the small number of oral cavity samples analyzed (5, 14, 30, 32). However, it is conceivable that, although occurring less frequently, there are infections in which *C. orthopsilosis* and *C. metapsilosis* are present. The current study demonstrated that 53.3% of colonized oral cavity samples from HIV-infected individuals, initially identified as *C. parapsilosis,* were in fact *C. metapsilosis.* Thus it is possible that in previous studies correlating *C.parapsilosis* infectivity with a particular sample may in fact have been mediated by *C. metapsilosis* or *C. orthopsilosis*. This is of clinical relevance especially with respect to potential therapeutic regimes and potential development of resistance.

Susceptibility testing

The development of methods for treatment of patients with candidiasis has routinely used susceptibile yeasts for testing to select appropriate antifungal compounds. The finding that resistant strains of *C. albicans* isolates from AIDS patients with oral and esophageal candidiasis developed resistance to azole suggested that fungal strains may become unresponsive to certain drug treatment. (8). Thus, it was important to determine the sensitivity of newly recognized *C. psilosis* complex isolates from HIV positive individuals to established an effective drug therapy.

Sensitivity to AMB was confirmed in previous studies to be at a MIC ≤1µg/mL (10, 11, 14, 19, 30, 31).It is noteworthy that, Silva and coworkers (28) reported two cases out of 160 isolates of *C. parapsilosis* which developed resistance to AMB, and Lockhart and coworkers (20) reported the MICs of *C. orthopsilosis* and *C. metapsilosis* to amphotericin B were lower than those of *C. parapsilosis.* The evaluation of sensitivity to FLC revealed all isolates were susceptible, although *C. metapsilosis* required a higher MICs than *C. parapsilosis*, MIC_{50} of 1.0 and 0.5 mg/L, respectively and MIC_{90} equal to 1.0 and 2.0 mg/L, respectively. These findings are in agreement with those of Gonçalves and coworkers (11), who also found higher MICs for *C. metapsilosis* than *C. parapsilosis*. Our results with KTZ confirmed those of Lin and coworkers (19), showing all isolates susceptible to this antifungal compound. Several authors reported sensitivity of *C. psilosis* complex isolates to ITC (10, 11, 14), as noted in our study. The exceptions are 15 of 33 *C. orthopsilosis* isolates SD-D to ITC, but 13 of the samples were isolated from the same patient (30), and

one of four *C. metapsilosis* also SD-D to this azole compound (11).The majority of the isolates were susceptible to VRC, as found in our study (10, 11, 14, 30, 31). However, Silva and coworkers (28) noted resistance to VRC of 8 out of 160 *C. parapsilosis* isolates. Sensitivity to CASPO was previously reported (10, 11, 20, 28, 31). However, Silva and coworkers (28) demonstrated 61 of 160 *C. parapsilosis* isolates were resistant to CASPO. Lockhart and coworkers (20) and van Asbeck and coworkers (32) also observed that a higher MICs were required for CASPO for *C. parapsilosis* than for *C. orthopsilosis* or *C. metapsilosis*. In our study, there were no marked differences in MICs revealed strains.

However, resistance of *C. parapsilosis* to VRC and CASPO, was reported by Silva and coworkers (28), but this may be related to a geographical regional pattern. The sensitivity of the different species that constitute the *C. psilosis* complex to antifungal compounds need to be elucidated to better understand geographical differences in the prevalence the 3 species of *C.psilosis* complex and the impact of treatment, especially for immunosuppressed patients. In addition, distinction of species within the complex may be important to determine if MIC differences noted are associated with differences in clinical outcomes.

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TABLES AND FIGURES

TABLE 1 - Characteristics of the 15 HIV- positive individuals from which *C. psilosis* complex was isolated.

INDIVIDUALS		ISOLATES no. (%)		TOTAL	
Characteristic	no.	*C. parapsilosis*	*C. metapsilosis*	no. (%)	*p Value*
GENDER	**15**				**0.31**
Male		03 (20.0)	06 (40.0)	09 (60.0)	
Female		04 (26.7)	02 (13.3)	06 (40.0)	
STAGE	**15**				**0.62**
AIDS		04 (26.7)	03 (20.0)	07 (46.7)	
non-AIDS		03 (20.0)	05 (33.)3	08 (53.3)	
$CD4^+$ T cells (n^o/mm^3)	**15**				**0.57**
< 200		01 (6.7)	03 (20.0)	04 (26.7)	
≥ 200		06 (40.0)	05 (33.3)	11 (73.3)	
Viral load(copies/ml)	**13**				**0.18**
Undetectable		01 (7.7)	03 (23.1)	04 (30.8)	
< 10,000		04 (30.8)	01 (7.7)	05 (38.5)	
≥ 10,000		01 (7.7)	03 (23.0)	04 (30.7)	

Fisher's exact test

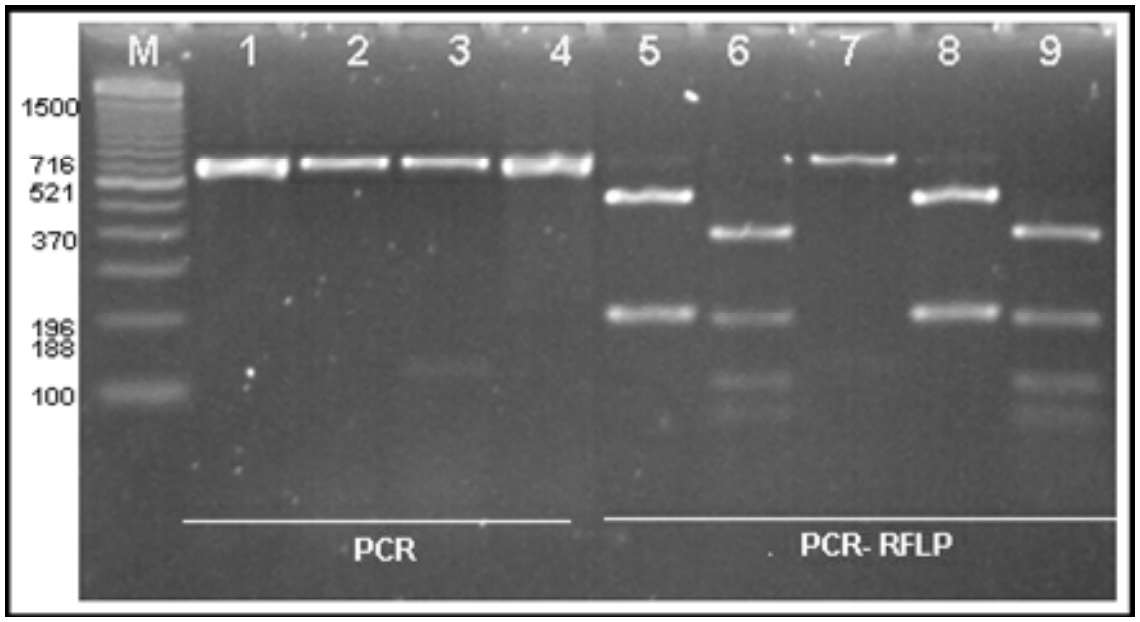

FIGURE 1 – Discrimination of *C. psilosis* complex isolates, according to their *SADH* gene profiles and *Ban*I restriction digestion of *SADH* gene. 100-bp ladder (lane M); *SADH* fragment amplification (PCR product of 716bp): lane 1, *C. parapsilosis* ATCC 22019; lane 2, *C. metapsilosis* ATCC 96143; lane 3, *C. orthopsilosis* ATCC 96141; lane 4, isolates from the oral cavity of HIV patients. *Ban*I restriction digestion of *SADH*-PCR product: lane 5, *C. parapsilosis* ATCC 22019; lane 6, *C. metapsilosis* ATCC 96143; lane 7, *C. orthopsilosis* ATCC 96141; lanes 8 and 9, *C. parapsilosis* and *C. metapsilosis* isolates from the oral cavity of HIV patients.

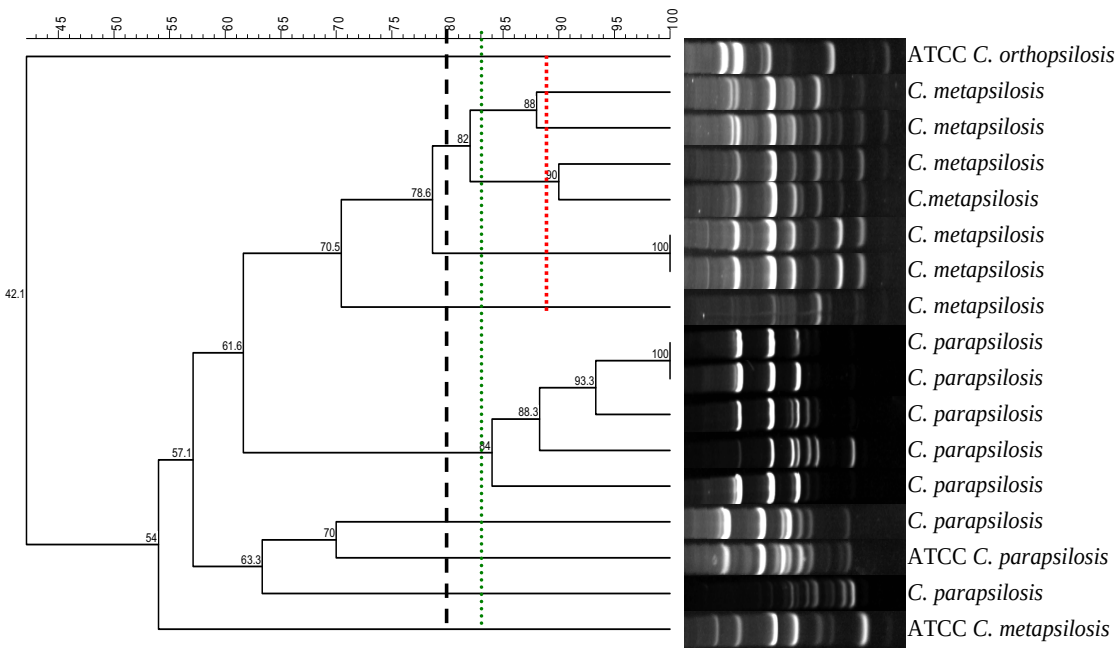

FIGURE 2 – Dendrograms generated for oral isolates of *C. parapsilosis* (n=7) e *C. metapsilosis* (n=8) from HIV-infected patients with asymptomatic Candida carriage. Vertical dashed lines mark the positions of S_{AB} values of 85.0% and 91.0%. Heavy vertical lines to the right of each dendrogram mark the position of clusters.

TABLE 2 – Geometric means (GM) and median of minimum inhibitory concentration (MIC), and MIC ranges of amphotericin B (AMB), fluconazole (FCL), ketoconaloze (KTC), itraconazole (ITC), voriconazole (VRC) and caspofungin (CASPO) for 15 isolates of the *C. parapsilosis* and *C. metapsilosis* tested by EUCAST procedure.

Antifungal agent	Species (no. of isolates)										*p* value
	C. parapsilosis (7)					*C. metapsilosis* (8)					
	GM (mg/L)	Median (mg/L)	MIC range	MIC_{50}	MIC_{90}	GM (mg/L)	Median (mg/L)	MIC range	MIC_{50}	MIC_{90}	
AMB	0.30	0.250	0.125-0.50	0.25	0.5	0.150	0.185	0.015-0.5	0.125	0.5	0.40
FLC	0.45	0.500	0.25-1.0	0.50	1.0	1.41	1.500	1.0-2.0	1.00	2.00	0.001
KTC	0.02	0.015	0.015-0.03	0.015	0.03	0.02	0.023	0.015-0.03	0.015	0.03	0.28
ITC	0.02	0.015	0.015-0.03	0.015	0.03	0.03	0.030	0.015-0.06	0.03	0.06	0.09
VRC	0.02	0.015	0.015-0.015	0.015	0.015	0.02	0.015	0.015-0.03	0.015	0.03	0.23
CASPO	0.37	0.500	0.125-1.0	0.5	1.0	0.23	0.250	0.125- 0.25	0.25	0.25	0.28

Mann-Whitney test

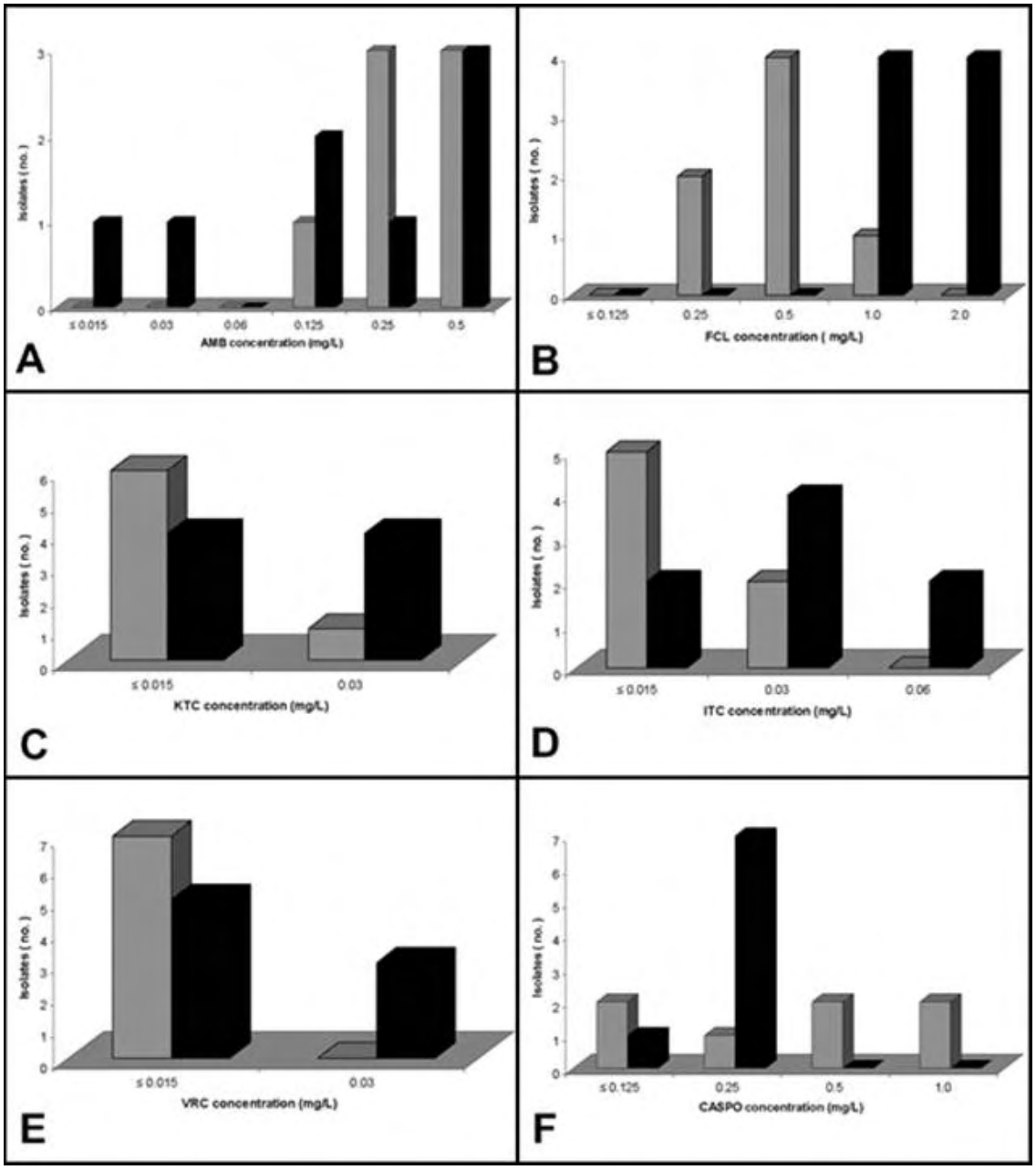

FIGURE 3 – Distribution of amphotericin B (AMB), fluconazole (FCL), ketoconaloze (KTC), itraconazole (ITC), voriconazole (VRC) and caspofungin (CASPO) minimum inhibitory concentration (MIC) for 15 isolates of the *C. psilosis* complex tested by EUCAST procedure. Gray bars indicate *C. parapsilosis* isolates, black bars indicate *C. metapsilosis* isolates.

2 - Prevalence and antifungal susceptibility profile of *Candida dubliniensis* isolated from the oral cavity of HIV-infected individuals.

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Running title: *Candida dubliniensis* isolated from HIV - infected individuals

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KEY WORDS: *Candida dubliniensis*, human immunodeficiency virus-infected individuals, oral cavity.

ABSTRACT

Candida dubliniensis is a recently described species of yeast. This emerging oral pathogen shares many phenotypic and biochemical characteristics with *Candida albicans* and molecular methods are needed to distinguish both species. In our study, 318 *Candida* spp. isolates were obtained from oral cavitiy of HIV infected individuals, and 270 of them were identified as *C. albicans* by classical methods. All the *C. albicans* isolates were submitted to DNA extraction and PCR identification. The isolates identified as *C. dubliniensis* were also tested for determination of the minimal inhibitory concentration to amphotericin B, fluconazole, ketoconazole, and itraconazole using the microdilution method as to AFST/EUCAST. Only three isolates were identified as *C. dubliniensis*. Thus, its prevalence was 0.9% in relation to the 318 *Candida* spp isolates and 1.1% in relation to the 270 isolates phenotypically identified as *C. albicans*. All the *C. dubliniensis* isolates were susceptible to the antifungal compounds tested. This study reinforces the importance of screening germ-tube-positive yeasts by molecular methods to properly identify the *C. dubliniensis* isolates, shows its low prevalence among HIV positive individuals in our region, and its high susceptibility to antifungal compounds.

INTRODUCTION

Sullivan and coworkers (49) described the species *Candida dubliniensis* associated with oral candidiasis in human immunodeficiency virus (HIV)-infected and acquired immunodeficiency syndrome (AIDS) patients. Oral candidiasis caused by *C. dubliniensis* in HIV-positive patients has also been reported by other authors (3,4, 6, 17, 20, 32). *C. dubliniensis* shares many phenotypic characteristics with *Candida albicans* isolates and molecular methods are needed to distinguish between both species (4,11, 25, 49, 50). In addition, *C. dubliniensis* is known to have higher proteinase activity than *C. albicans* and higher adherence to the oral mucosa. However, hypha formation is slower, suggesting lower invading capacity (21, 27, 46). Although less common than *C. albicans, C. dubliniensis* have been isolated from bloodstream of patients in intensive care units (2, 31, 52), bronchoalveolar lavage specimens (15), urinary tract, as well as vagina (14), endocardium (8) and oral cavity (3, 9, 29, 34, 36) of HIV-negative patients.

Studies carried out in the North America and Ireland have shown that the prevalence of *C. dubliniensis* in the oral cavity of HIV-positive individuals ranged from 11.1 to 17.5% (20, 30) and from 18.0 to 32.0% (10, 50), rather higher than that obtained in South American countries, which varied between 0.0 and 3.9% (1, 3, 11, 32, 45). The reasons for such a difference remain unknown.

Susceptible C. *dubliniensis* isolates rapidly develop a stable fluconazole-resistant phenotype during *in vitro* exposure to this antifungal agent (33),

suggesting that this species could develop resistance to this azole compound within a short time (35, 39, 42, 48).

The phenotypic identity between *C. dubliniensis* and *C. albicans,* the high frequency of candidiasis in immunosuppressed patients, and the common use of fluconazole in the treatment of such infections have led to the assessment of the sensitivity profile of this new species to different antifungal agents; its dose-dependent resistance (SSD) or fluconazole resistance (FLC) ranged from 16 to ≥ 64mg/L (17, 19, 54).

The aims of this study were thus (1) to distinguish *C. dubliniensis* from presumptive *C. albicans* isolates from the oral cavity of HIV-positive individuals by using polymerase chain reaction (PCR), (2) to evaluate its prevalence, and (3) to determine the susceptibility of this species to various antifungal agents.

MATERIALS AND METHODS

Approval was obtained from the Research Ethics Committees, Botucatu Medical School, São Paulo State University (UNESP), and Human Research Ethics Committee, Adolfo Lutz Institute. Informed patient consent was required for this study.

Fungal isolates. *Candida* spp. were isolated from the oral cavity of 214 HIV individuals, and 270 of the 318 isolates were initially identified as *C. albicans* by conventional morphological and physiological methods (23) and

subjected to molecular typing. Collection was done by means of swabs, which were then placed on plates containing Sabouraud agar (Difco, Detroit, Michigan) supplemented with 0.05% chloramphenicol. The plates were incubated at 30°C for 24h. The isolates were plated on CHROMagar *Candida* (CHROMagar, Paris France) at 35ºC for 48h. Subsequently, the color and the morphology of one colony of each isolate were analyzed to establish the presence of *C. albicans*. To differentiate and identify *Candida* spp. at microscopic level, isolates were incubated in cormeal agar (Oxoid Brasil Ltda) supplemented with 1% Tween 80 (Synth, Labsynth, Brasil) to verify their morphology (23) and in API 20C AUX (BioMérieux Marcy l'Etoile, France) to determine their biochemical characteristics according to the manufacturers' instructions.

DNA extraction. Each isolate was plated on Sabouraud Dextrose Agar (Difco, Detroit, Michigan) and incubated at 30ºC for 24 h. The yeast cells were then transferred to a microcentrifuge tube containing 1ml of 50mM EDTA pH8, vortexed and centrifuged at 13,000 x g for 15 min. The supernatant was discarded and the pellet was resuspended in 200µl of 50mM EDTA (Sigma Chemical, USA) pH8 containing 40µl lysis enzyme obtained from *Trichoderma harzianum* (10mg/ml) (Sigma Chemical, USA), followed by incubation at 37ºC for 2-3h. Subsequently, this mixture was centrifuged at 13,000 x g for 10 min and the pellet was dissolved in lysis buffer containing 10mM Tris-HCl (Sigma Chemical, USA) pH 8, 10mM EDTA (Sigma Chemical, USA), 0.5% sodium dodecyl sulfate (SDS) (Synth, Labsynth, Brasil), 0.01% N-laurylsacozyl (Sigma Chemical, USA) and 10µg/ml proteinase K (Sigma Chemical, USA). The

mixture was then vortexed and incubated at 56ºC for 2-3h. DNA was extracted by phenol/chloroform/isoamyl alcohol and precipitated with isopropanol according to Sambrook et al (43). After washing the pellet with 70% ethanol for 10min at 10,000g, DNA was dissolved in 50 µl ultra pure water. DNA concentrations were determined by using a NanoDrop1000 (Thermo Fisher Scientific, USA). For PCR and RAPD amplification, 1µl of each DNA sample (approximately 100ng) was used. DNA was frozen at -20ºC until use.

Molecular identification of *C. dubliniensis*

PCR. The protocol used for PCR was previously described by Mannarelli and Kurtzman (28). Two pairs of primers (Invitrogen Brasil Ltda) were chosen, for *C. albicans*: sense (CAL5- 5´TGTTGCTCTCTCGGGGGCGGCCG-3´) and anti-sense (NL4CAL– 5´AAGATCATTATGCCAACATCCTAGGTAAA3´), and for *C. dubliniensis:* sense (- 5´AGTTACTCTTTCGGGGGTGGCCT-3´) and anti-sense (NL4CAL– 5´AAGATCATTATGCCAACATCCTAGGTAAA3´). The following amplification conditions were adopted: a first denaturation cycle at 94ºC for 5min, followed by 35 denaturation cycles at 94ºC for 30s, annealing at 65ºC for 45s and elongation at 72ºC for 30s, with a final extension step at 72ºC for 10min. Amplified DNA products were separated by electrophoresis on 2% agarose gel in TBE (89 mM Tris/HCl, 89mM boric acid, 2 mM EDTA, pH 8) buffer at 100V for 35 min and a 100-bp DNA ladder was used as molecular size marker (Invitrogen, Brasil, Ltda). The gel was stained with ethidium bromide (0.5mg/ml) and DNA bands were visualized by UV transillumination Mini Bis Pro (Bio-Imaging). The band profiles in PCR for *C. albicans* and *C. dubliniensis* clinical isolates were compared to those for the type strains *C. albicans* of the

American Type Culture Collection (ATCC) 76615 and *C. dubliniensis* of the Centraalbureau voor Schimmelcultures (CBS) 9768.

Antifungal agent susceptibility testing

Standard antifungal susceptibility tests were performed according to a reference microdilution method established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (13). The employed antifungal agents were amphotericin B (AMB), ketoconazole (KTC), fluconazole (FLC) and itraconazole (ITC). Interpretive criteria for AMB have not yet been established; however, for purposes of comparison, isolates with MIC of ≤1 mg/L following AMB exposure were considered susceptible to this drug (35,38,42). Positive control was performed using *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 for each batch of isolates tested.

RESULTS

Molecular identification of C. *dubliniensis.*

The results obtained in the amplification of fragments using primers CAL5 and NL4CAL (*C. albicans*), and CDU2 and NL4CAL (*C. dubliniensis*) showed that of the 270 *C. albicans* isolates used in this study, 267 (98.9 %) were identified as *C. albicans,* and three (1.1%) as *C. dubliniensis*. The characteristics of the three HIV-positive individuals from which *C. dubliniensis* was isolated are presented in Table 1. As shown in Figure 1, the samples that

produced fragments with primers CAL5 and NL4CAL were identified as *C. albicans* (Fig 1 lanes 1 and 3) and those producing fragments with primers CDU2 and NL4CAL were identified as *C. dubliniensis* (Fig. 1, lanes 6 and 7). The size of the fragment amplified by primers CAL5 and NL4CAL, and by primers CDU2 and NL4CAL, 175 bp, corresponds to those reported by Mannarelli and Kurtzman (27), allowing the identification of the two respective species. It must be highlighted that one of the three *C. dubliniensis* isolates was identified in mixed culture with *C. albicans.*

Prevalence of *C. dubliniensis*. The prevalence of *C*. *dubliniensis* was 0.9% relative to the 318 *Candida* spp isolates and 1.1% relative to the 270 *C. albicans* isolates from the oral swabs of HIV-infected individuals.

Antifungal susceptibility testing. All tested organisms grew after 24h incubation. The results of susceptibility tests to AMB, FLC, KTC and ITC for three *C. dubliniensis* isolates are shown in Table 2 and Figure 2. All isolates were susceptible to the different antifungal agents tested.

DISCUSSION

C. dubliniensis, first described in Ireland, shares phenotypic features with *C. albicans* and has been more frequently isolated from the oral cavity of HIV-positive patients (49). It is of interest, however, that several studies have reported finding *C. dubliniensis* in other body sites, including the respiratory tract, blood, central nervous system, vagina, urine, skin, and feces of both HIV-

positive and HIV-negative patients (14,36,40). In many cases, *C. dubliniensis* and *C. albicans* were isolated from the same plate. As there is phenotypic identity and the identification is based on only one colony, *C. dubliniensis* prevalence could have been underestimated (50).

Previous studies comparing phenotypic methods for the identification of *C. dubliniensis* have found no method was reliable to differentiate this species from *C. albicans*. Culture media used to differentiate between *C. albicans* and *C. dubliniensis* were useful for phenotypic screening, but definitive identification still requires genotyping techniques (11,12,16). Consequently, in this study we used a PCR method to identify *C. dubliniensis* (28); this methodology has been used in other studies (4, 9).

Several studies have evaluated *C. dubliniensis* prevalence in cases of infection or colonization, especially in the oral cavity of HIV-infected individuals. In the current study, *C. dubliniensis* prevalence was 0.9% in HIV-positive individuals and three out of 270 samples (1.1%), initially identified as *C. albicans* which colonizes the oral cavity of HIV-infected individuals, were in fact *C. dubliniensis.*

Higher prevalence rates for *C. dubliniensis* were obtained in studies conducted in Ireland, where this species was recovered from the oral cavity of 32 and 25% HIV-positive individuals with and without clinical symptoms of oral candidiasis, respectively (10). In subsequent epidemiological studies investigating *C. dubliniensis* prevalence in the oral cavity of HIV-infected populations, the prevalence rates ranged from 0 to 48% (3,18).

Our results are in marked contrast to those obtained in Ireland (10) and Turkey, where the prevalence was 11.4% (53), as well as in USA, where a frequency of 11.0 to 48.0% was obtained (20, 18), and in Argentina, where the frequency was 12.9 to 20.2% (5,26) in the oral cavity of HIV-positive individuals. It is noteworthy that all isolates studied by Jabra-Rizk and coworkers (18) were from subgingival periodontal lesions, showing a 48.0% prevalence of *C. dubliniensis* in subgingival sites of HIV-positive individuals.

Conversely, our results agreed with those of some Brazilian studies (3,9, 11,29,32) that reported lower prevalence of *C. dubliniensis* in HIV-positive patients. Milan and coworkers (32) evaluated 108 Brazilian AIDS patients with oropharyngeal candidiasis and reported that three (2.7%) of them were positive to *C. dubliniensis*. Mariano and coworkers (29) found a low prevalence rate of *C. dubliniensis* (2.0%) among 548 yeast isolates obtained from a stock culture collection previously identified as *C. albicans.* All *C. dubliniensis* isolates were obtained from oropharyngeal samples and nine of them were from HIV-positive patients. Delgado and coworkers (11) evaluated 161 *Candida* spp. isolates recovered from the oral cavity of 147 colonized HIV-positive individuals; *C. dubliniensis* was identified in two (1.2%) of the 161 samples. Back-Brito and coworkers (3) evaluated *C. dubliniensis* prevalence among oral isolates from Brazilian HIV-positive and HIV-negative individuals; *C. dubliniensis* was identified only when isolated from HIV-negative individuals (1.9%).

C. dubliniensis isolation from the oral cavity of control individuals varied widely from 0 to 13.3% (6,7,9,34,44). Interestingly, Chavasco and coworkers (9) evaluated the presence of *C. dubliniensis* in samples isolated from HIV-positive

and HIV-negative individuals with oral erythematous candidiasis in São Paulo City, Brazil; 4.5% and 5.1% of the samples previously identified as *C. albicans* were actually *C. dubliniensis* in HIV-positive and HIV-negative individuals, respectively. These findings differ from our results, which showed 1.1% of *C. dubliniensis* isolates in the oral cavity. It is noteworthy that isolates in the current study were colonizing, whereas those studied by Chavasco and coworkers (9) were the etiological agents of oral erythematous candidiasis.

Based on the potential risk for the development of resistance to fluconazole (33), we suggested the evaluation of antifungal susceptibility of *C. dubliniensis* isolates. Our results showing susceptibility to AMB confirmed previous studies (4,11,29,36,53). *C. dubliniensis* isolates were also susceptible to FLC, as observed by several authors (3, 11, 26, 29). It is noteworthy that resistance to FLC was demonstrated for 25% of the isolates (17) and susceptible dose-dependence (SD-D) for 13.3% (18) and 25.0% (53). The results of susceptibility to ITC are similar to those observed for FLC. Most studies, including ours, have shown susceptibility (11,29,36), except concerning some isolates revealing SD-D, observed for 5.5% (26) and 50.0% (4). Nevertheless, the small number of tested isolates should be considered. Our results showing susceptibility to KTZ confirmed previous studies (11,29,36).

This study reinforces the importance of screening germ tube-positive yeasts by molecular methods to properly identify *C. dubliniensis* isolates; it also shows the low prevalence of this species among HIV-positive individuals in our region and its high susceptibility to antifungal compounds.

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TABLES AND FIGURES

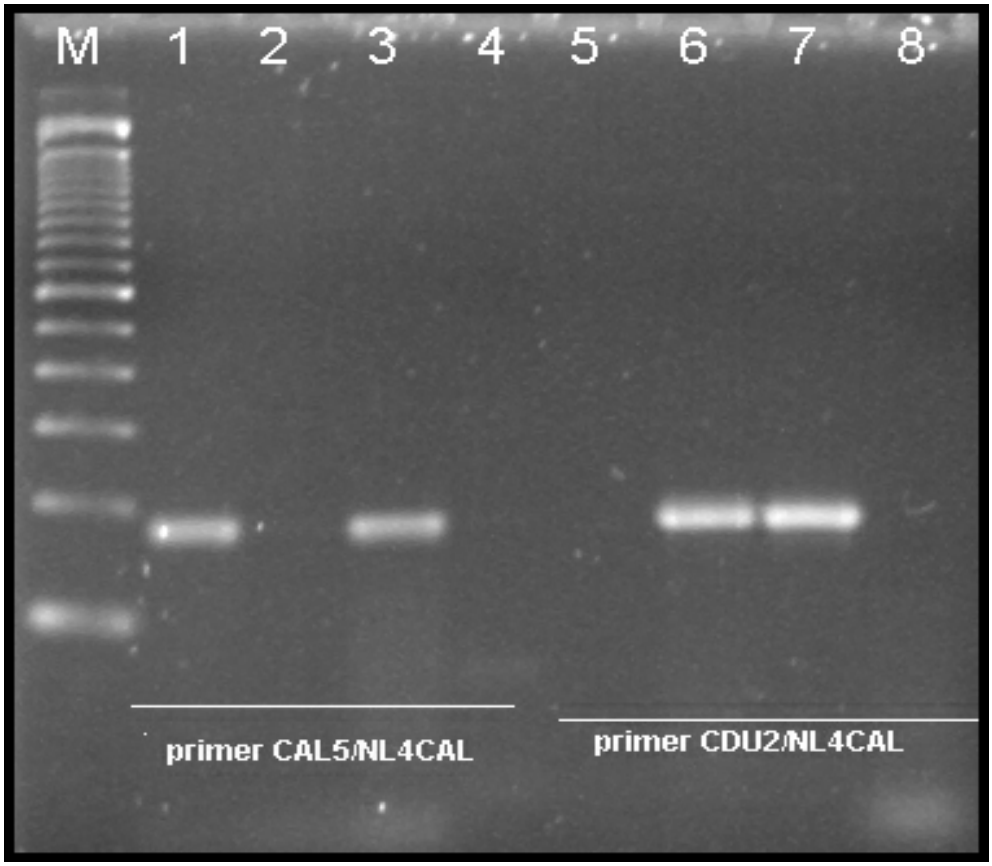

FIGURE 1 – Discrimination of *C.albicans* and *C. dubliniensis* isolates, according to fragment amplification (PCR product of 175bp).100-bp ladder (lane M); lines 1 to 4 fragment amplification with primer CAL5/NL4CAL: lane 1, *C.albicans* (ATCC 76615); lane 2, *C. dubliniensis* (CBS 9768); lane 3 and 4, isolates from the oral cavity of HIV patients, *C. albicans* and *C. dubliniensis* respectively. Lines 5 to 8 fragment amplification with primer CDU2/NL4CAL: lane 5, *C.albicans* (ATCC 76615); lane 6, *C. dubliniensis* (CBS 9768); lane 7 and 8, isolates from the oral cavity of HIV patients, *C. dubliniensis* and *C. albicans* respectively.

TABLE 1 – Characteristics epidemiological, clinical and laboratorial of three HIV positive individuals with *C. dubliniensis* in the oral cavity.

	PATIENTS		
	1	**2**	**3**
Gender	Female	Male	Male
Stage/ AIDS	Non-AIDS	AIDS	AIDS
Oral cavity	Candidiasis*	Colonization	Colonization
$CD4^+$ T cells (no./mm^3)	455	41	294
Viral load(copies/ml)	< 10,000	≥ 10,000	Undetectable

* mixed culture with *C. albicans*

TABLE 2 – Geometric mean (GM), median and range of minimum inhibitory concentration (MIC), of amphotericin B (AMB), fluconazole (FCL), ketoconaloze (KTC), itraconazole (ITC) for three *C. dubliniensis* isolates tested by EUCAST procedure.

Antifungal agent	**MIC (mg/L)**				
	GM	**Median**	**range**	**MIC_{50}**	**MIC_{90}**
AMB	0.08	0.06	0.06-0.125	0.06	0.125
FLC	0.125	0.125	0.125	0.125	0.125
KTC	0.015	0.015	0.015	0.015	0.015
ITC	0.02	0.015	0.015-0.03	0.015	0.03

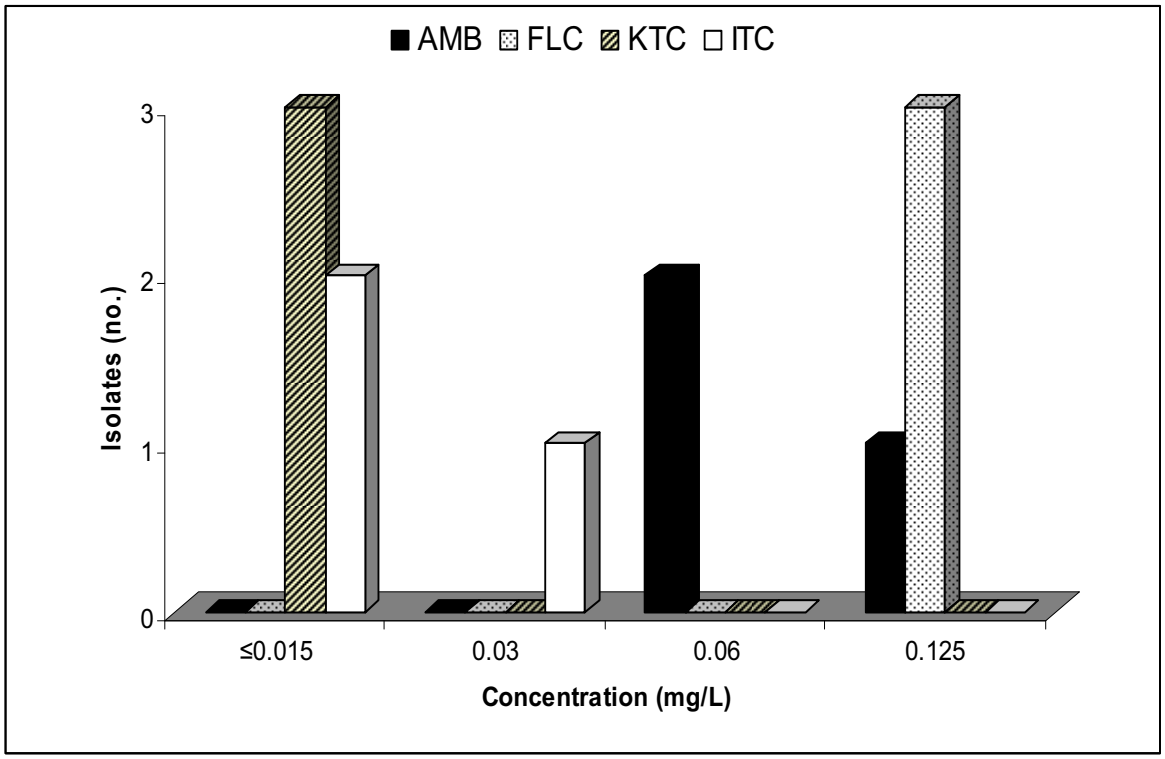

FIGURE 2 – Distribution of amphotericin B (AMB), fluconazole (FCL), ketoconaloze (KTC) and itraconazole (ITC) minimum inhibitory concentration (MIC) for three *C. dubliniensis* isolates tested by EUCAST procedure.

3. Species distribution and antifungal susceptibility profile of oral *Candida* isolates from HIV-infected individuals.

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Running title: *Candida* spp isolated from HIV - infected individuals

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KEY WORDS: *Candida* spp, antifungal susceptibility profile, human immunodeficiency virus-infected individuals, oral cavity.

ABSTRACT

Oropharyngeal candidiasis continues to be a common opportunistic infection in the oropharynge of patients infected by the human immunodeficiency virus. Although *Candida albicans* remains the most common causative agent, other non-*albicans* species have been also identified. The aims of this study were to evaluate the prevalence of the different *Candida* spp in the oral cavity of HIV infected individuals and to determine the minimal inhibitory concentration to four antifungal compounds. A total of 300 samples were isolated and evaluated as to susceptibility to four antifungal compounds using the microdilution method as to AFST/EUCAST. *C. albicans* isolates were phenotypically and genotypically identified. methods; for *Candida* non-*albicans* species only phenotical methods were used. Comparison of frequencies was carried out by the qui-square test and significance was set at $p<0.05$. Prevalence of *C. albicans* was 89.0%, *C. glabrata* 6.0%, *C. tropicalis* 4.3% and *C. krusei* 0.7%. Prevalence of resistance and susceptibility dose-dependent, taken together, to fluconazole was 0.75% in *C. albicans*, 50.0% in *C. glabrata*, 0.0% in *C. tropicalis* and 100.0% in *C. krusei* [*C.glabrata*>(*C. albicans*=*C. tropicalis*)]; as to ketoconazole, it was 0.75% in *C. albicans*, 0.0% in *C. tropicalis*, 0.0% in *C. glabrata*, and 50% in *C. krusei* [*C. albicans*=*C. glabrata*= *C. tropicalis*]; as to itraconazole, 1.9% in *C. albicans*, 72.2% in *C. glabrata*, 0.0% in *C. tropicalis*, and 50.0% in *C. krusei* [higher incidence of resistance in *C. glabrata*]; as to amphotericin B, all the isolates were susceptible. Cross-resistance to the three azole compounds was observed in two *C. albicans* and one *C. krusei*. Cross-resistance to two azole compounds

was showed by one *C. krusei* and nine *C. glabrata* isolates. Our results show predominance of *C. albicans* in the oral cavity of HIV positive individuals, the presence of cross-resistance to azoles compounds, and a prevalence of resistance that suggests the evaluation of antifungal susceptibility testing whenever antifungal treatment with azoles is planned.

INTRODUCTION

Candida yeasts can be the agents of local or systemic opportunistic infections, mainly in hospitalized patients, especially those under intensive treatment (1). Infections caused by opportunistic agents including *Candida* spp. are frequent in diverse pathological states that induce immunodeficiency such as neutropenia, neoplasia, decompensated *diabetes mellitus*, malnutrition, organ transplantation, and AIDS (1,25).

The Th1 profile of cell-mediated immune response is considered fundamental for the defense against fungal infections, including those caused by *Candida* spp. Thus, when the number of CD4+ T lymphocytes is below a protective level, like in AIDS patients, other defense mechanisms are needed to protect against oral candidiasis. In addition, Imiam *et al*. (21) showed severe vaginal candidiasis in HIV-infected patients with normal or mildly decreased $CD4^+$ T lymphocytes counts or $CD4^+$ T/$CD8^+$ T ratio. When immunosuppression increases, oral candidiasis can be observed, and in more immunocompromised patients, esophageal candidiasis is noticed. These findings led those authors to

suggest a hierarchy of mucosal infections caused by *Candida* spp. in HIV-infected patients.

After the introduction of highly active antiretroviral therapy (HAART), a reduction in the occurrence of oropharyngeal candidiasis was observed (6). But, for patients with late diagnosis or those who do not respond appropriately to antiretroviral therapy, oropharyngeal candidiasis is frequent (4,15,19,49,57) Topical antifungal therapy may be used to treat mild episodes of oropharyngeal candidiasis (22, 35), but systemic antifungal therapy is needed for the treatment of extensive and recurrent episodes of this infection (9, 44, 46).

The development of methods for routine susceptibility tests of yeasts has been useful to select the appropriate antifungal compound for the treatment of candidiasis-patients. Azole-resistant strains of *C. albicans* have been isolated from AIDS patients with oropharyngeal and esophageal candidiasis (9,16,50,55).

Thus, the aims of this study were to investigate the prevalence of the different species of the genus *Candida* and the antifungal susceptibility profiles of these isolates from the oral cavity of HIV-positive individuals.

MATERIALS AND METHODS

Samples were collected from the oral cavity of HIV-positive individuals from June 2002 to July 2006. The patients were assisted at the Special

Outpatient Clinic for Infectious and Parasitic Diseases or at the Ward of Tropical Diseases of Botucatu Medical School, São Paulo State University (UNESP). Approval was obtained from the Research Ethics Committee, Faculdade de Medicina de Botucatu, UNESP, and from the Ethics Committee on Human Research, Adolfo Lutz Institute (IAL).Written informed consent was obtained from every patient for this study.

Fungal isolates. Material was collected by swabbing the oral mucosa of patients with a sterile cotton swab. The swabs were then placed on plates containing Sabouraud agar (Difco, Detroit, Michigan) supplemented with 0.05% chloramphenicol. The plates were incubated at 30°C for 24h.The isolates were plated on CHROMagar *Candida* (CHROMagar, Paris France) at 35ºC for 48h. Subsequently, the color and the morphology of one colony of each isolate were analyzed to establish the presence of a pure colony of *Candida* spp. To differentiate and identify *Candida* spp. at the microscopic level, isolates were incubated in cormeal agar (Oxoid, Ltda, Brasil) supplemented with 1% Tween 80 (Synth, Labsynth, Brasil) to verify the morphology (24) and in API20C AUX (BioMérieux Marcy l'Etoile, France), according to the manufacturer's instructions, to determine the biochemical characteristics.

Molecular differentiation between *C. albicans* and *C. dubliniensis*

C. albicans and *C. dubliniensis* were differentiated by polymerase chain reaction (PCR), as previously described by Mannarelli and Kurtzman (28).

DNA extraction. Each isolate was plated on Sabouraud Dextrose Agar (Difco, Detroit, Michigan) and incubated at 30ºC for 24h. The yeast cells were then transferred to a microcentrifuge tube containing 1ml of 50mM EDTA pH8,

vortexed and centrifuged at 13,000 x g for 15 min. The supernatant was discarded and the pellet was resuspended in 200µl of 50mM EDTA (Sigma Chemical, USA) pH8 containing 40µl lysis enzyme obtained from *Trichoderma harzianum* (10mg/ml) (Sigma Chemical, USA), followed by incubation at 37ºC for 2-3h. Subsequently, this mixture was centrifuged at 13,000 x g for 10 min and the pellet was dissolved in lysis buffer containing 10mM Tris-HCl (Sigma Chemical, USA) pH 8, 10mM EDTA (Sigma Chemical, USA), 0.5% sodium dodecyl sulfate (SDS) (Synth, Labsynth, Brasil), 0.01% N-laurylsacozyl (Sigma Chemical, USA), and 10µg/ml proteinase K (Sigma Chemical, USA). The mixture was then vortexed and incubated at 56ºC for 2-3h. DNA was extracted by phenol/chloroform/isoamyl alcohol and precipitated with isopropanol according to Sambrook and coworkers (48). After washing the pellet with 70% ethanol for 10min at 10,000 x g, DNA was dissolved in 50 µl ultra pure water. DNA concentrations were determined by using a NanoDrop1000 (Thermo Fisher Scientific, USA). For PCR and RAPD amplification, 1µl of each DNA sample (approximately 100ng) was used. DNA was frozen at -20ºC until use (48).

PCR. The protocol used for PCR was previously described by Mannarelli and Kurtzman (28). Two pairs of primers (Invitrogen Brasil Ltda) were chosen, for *C. albicans*: sense (CAL5- 5´TGTTGCTCTCTCGGGGGCGGCCG-3´) and anti-sense (NL4CAL– 5´AAGATCATTATGCCAACATCCTAGGTAAA3´), and for *C. dubliniensis:* sense (- 5´AGTTACTCTTTCGGGGGTGGCCT-3´) and anti-sense (NL4CAL– 5´AAGATCATTATGCCAACATCCTAGGTAAA3´). The following amplification conditions were adopted: a first denaturation cycle at 94ºC for 5min, followed by 35 denaturation cycles at 94ºC for 30s, annealing at

65ºC for 45s and elongation at 72ºC for 30s, with a final extension step at 72ºC for 10min. Amplified DNA products were separated by electrophoresis on 2% agarose gel in TBE (89 mM Tris/HCl, 89mM boric acid, 2 mM EDTA, pH 8) buffer at 100V for 35 min and a 100-bp DNA ladder was used as molecular size marker (Invitrogen, Brasil, Ltda). The gel was stained with ethidium bromide (0.5mg/ml) and DNA bands were visualized by UV transillumination Mini Bis Pro (Bio-Imaging). The band profiles in PCR for *C. albicans* and *C. dubliniensis* clinical isolates were compared to those for the type strains *C. albicans* of the American Type Culture Collection (ATCC) 76615 and *C. dubliniensis* of the Centraalbureau voor Schimmelcultures (CBS) 9768.

Antifungal agent susceptibility testing

Standard antifungal susceptibility tests were carried out according to a reference microdilution method established by the Europen Committee on Antimicrobial Susceptibility Testing (EUCAST) (14). Amphotericin B (AMB), ketoconazole (KTC), fluconazole (FLC), and itraconazole (ITC) were the antifungal compounds employed. These drugs were obtained as reagent-grade powders from Sigma-Aldrich Brasil Ltda.

As regards susceptibility to FLC, the isolates were classified as susceptible (S), intermediate (I) and resistant (R), according to EUCAST (14). Considering the degree of susceptibility to KTC and ITC, the isolates were classified as susceptible (S), susceptible dose-dependent (SDD) and resistant (R), according to Clinical and Laboratory Standards Institute (CLSI) (7); for ITC: ≥1 mg/ L was used for R, 0.25-0.5 mg/L for SDD, and ≤0.125 mg/L for S; for KTC: ≥1 mg/ L was used for R, 0.25-0.5 mg/L for SDD, and ≤0.125 mg/L for S.

As the interpretive criteria for AMB was not established by EUCAST or CLSI, isolates with MICs ≤1 mg/L were considered susceptible and those with MICs ≥2 mg/L, resistant (34, 43, 45). Positive control was performed using *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 for each batch of isolates tested.

Statistical analysis. Differences in antifungal MIC medians were evaluated using Kruskal – Wallis followed by Dunn's test for multiple comparisons. Comparisons of frequencies were carried out by Chi-square. All tests were performed using the statistical analysis system (SAS), version 6.12 (Institute Inc. USA) (52). Significance was set at $p < 0.05$.

RESULTS

A total of 300 oral *Candida* spp. isolates from HIV-positive individuals were included in this study.

Prevalence of *Candida* spp.. Prevalence of *C. albicans* (267 isolates) was 89.0%, *C. glabrata* (18 isolates), 6.0%; *C. tropicalis* (13 isolates), 4.3%; and *C. krusei* (2 isolates), 0.7%. The identification of all *C. albicans* was confirmed by PCR using amplification of the primers CAL5 and NL4CAL (Figure 1).

Antifungal susceptibility testing. All tested organisms grew after 24h incubation. Susceptibility results for *Candida* isolates are summarized in Tables 1 and 2. MICs for the quality control strains were within the accepted limits

(Eucast). MIC distribution of AMB, FCL, KTC and ITC for 300 isolates of *Candida* spp. is shown in Figure 2A and B, and Figure 3 A to D. MIC (mg/L) median for fluconazole was lower in *C. albicans* than in *C. glabrata* and *C. tropicalis* ($p<0.001$), which were not statistically different; as to ketoconazole, MIC was higher in *C. glabrata* than in *C. albicans* and *C. tropicalis* ($p<0.001$), which were not statistically different; as to itraconazole, MIC in *C. albicans* was lower than that in *C. glabrata* ($p<0.001$), while in *C. tropicalis* it was not statistically different from the other two species; and as to AMB, MIC in *C. albicans* was lower than that in *C. tropicalis* ($p<0.001$), while in *C. glabrata* it was not statistically different from the other species. These data are shown in Table 3.

Prevalence of resistance and susceptibility dose-dependence, taken together, concerning fluconazole was 0.75% for *C. albicans*, 50.0% for *C. glabrata*, 0.0% for *C. tropicalis*, and 100.0% for *C. krusei*, [*C.glabrata*>(*C. albicans*=*C. tropicalis*); $p=0.001$]; as to ketoconazole, it was 0.75% for *C. albicans*, 0.0% for *C. tropicalis*, 0.0% for *C. glabrata*, and 50% for *C. krusei* [(*C. albicans*=*C. glabrata*=*C. tropicalis*); $p>0.05$]; as to itraconazole, it was 1.9% for *C. albicans*, 72.2% for *C. glabrata*, 0.0% for *C. tropicalis*, and 50.0% for *C. krusei*. Comparing *C. albicans*, *C. glabrata* and *C. tropicalis*, higher prevalence of resistance was observed in *C. glabrata* [*C. glabrata* > (*C. albicans* = *C. tropicalis*); $p<0.05$]. All isolates were susceptible to amphotericin B [(*C. albicans* = *C. glabrata* = *C. tropicalis*); $p>0.05$]. All *C. tropicalis* isolates were susceptible to the antifungal agents tested.

Of the 267 *C. albicans* isolates, two were resistant to FLC (MIC ≥ 64.0 mg/L) and KTC (MICs equal to 1.0 and ≥ 8.0 mg/L) and presented susceptible dose-dependence (SDD) and resistance to ITC, with MIC equal to 0.25mg/L and ≥ 8.0 mg/L, respectively. Three *C. albicans* isolates susceptible to FLC and KTC were SDD to ITC. Eight *C. glabrata* isolates showed resistance to FLC, according to EUCAST; one of them was also resistant and the other seven were SDD to ITC. Susceptibility dose-dependence to FLC was observed for one *C. glabrata* isolate which was also resistant to ITC. The two *C. krusei* isolates were resistant to FLC; one of them was SDD to ITC and KTC, whereas the other one was resistant to KTC and susceptible to ITC. Cross-resistance to the three azole compounds was observed for two *C. albicans* isolates. Cross-resistance to two azole compounds was shown by nine *C. glabrata* isolates.

The correlations between the susceptibility profiles of the different antifungal compounds are presented in Figures 4 and 5.

DISCUSSION

Oropharyngeal candidiasis has been considered the most common opportunistic infection among patients with human immunodeficiency virus (HIV) (12). Due to recurrent episodes of candidiasis, the patients are frequently subjected to long-term prophylaxis or repeated courses of fluconazole therapy (3,11, 55). As a consequence, the susceptibility pattern to fluconazole by *C. albicans,* the major etiologic agent of fungal oropharyngeal infections, has been

changing (32, 48, 56). In some regions, resistance to antifungal agents is a problem concerning the most commonly isolated *Candida* species (41-44). Since susceptibility profile is usually predictable based on *Candida* species, culture results that identify the organism to the species level can be very useful to help guide therapy choices, including *C. krusei*, which is inherently resistant to azoles (33, 38, 46), and *C. glabrata*, which easily develops resistance to azole compounds (27, 41, 46, 56).

In our study, 300 *Candida* spp. isolates were recovered from the oral cavity of HIV-infected individuals. *C. albicans* was the predominant species, identified in 84.0% of the samples, confirming previous findings of a frequency between 78.0 and 94.8% (2, 11, 18, 49, 51, 53). It is noteworthy that our results disagreed with those of two recent Brazilian studies which reported *C. albicans* prevalence of 50.0 and 65.0% (8,30) in the oral cavity of HIV-positive individuals.

The prevalence of non-*Candida albicans* was 11.0% in our study, and was distributed among *C. glabrata* (6.0%), *C. tropicalis* (4.3%), and *C. krusei* (0.7%). Prevalence rates of *C. glabrata* ranged from 0.0 to 32.7%, *C. tropicalis* from 0.0 to 17.6%, and *C. krusei* between 0.0 and 8.0% at this site (2, 49,53,57) according to the world literature. These findings showing higher prevalence in the world literature suggest we take care in the transference of these data to our patients.

Previous Brazilian studies on the oral cavity of HIV-positive individuals identified *C. glabrata* in 0.0% to 9.2% of the isolates (10,29,31,47). Our values were lower than those of Milan and coworkers (31), who reported 9.2%. As to

C. tropicalis, the prevalence rates showed by Menezes and coworkers (30) and Costa and coworkers (8), 27.5 and 20.9%, respectively, were five-to-six-fold higher than ours. On the other hand, our findings are comparable to the 4.6% of Melo and coworkers (29), and the 2.5% of Delgado and coworkers (10). *C. krusei* isolation from the oral cavity of HIV-positive individuals varied from 0.0 to 7.5% (10,17,29,31,47). Interestingly, the two highest prevalence values were observed for patients with oral erythematous candidiasis in São Paulo City, Brazil – 6.5% (31), and in HIV individuals colonized or infected by this fungus in Campinas City, Brazil – 7.5% (29), which are geographically very close. On the other hand, another study evaluating only colonization in Campinas City, Brazil, showed a lower prevalence – 1.2% (10). These apparently contradictory results could be related to the unknown immunological status of the HIV-infected individuals and antifungal compounds and antiretroviral agents they have used.

Our findings revealed that *Candida* spp. are highly susceptible to amphotericin B, with MICs varying between 0.03 and 1.0 mg/L, which confirms previous reports in the world (5,49) and in the Brazilian literature (8,10). In addition, our findings showed higher MICs for *C. tropicalis* than for *C. albicans* and *C. glabrata*. Few *Candida* spp. isolates with *in vitro* resistance to amphotericin B were recovered from patients previously treated with this antifungal compound (23, 34).

Some recent studies reported fluconazole resistance for *Candida* spp. isolated from HIV-infected patients with oral candidiasis (4,5,9,26,50). The emergence of *Candida* spp. with reduced susceptibility to this agent was also

observed in cases of invasive candidiasis (20,27,33,36,37). These findings can be related to the frequent use of this antifungal compound.

In our study, 99.3% of the *C. albicans* isolates were found to be susceptible to FLC, confirming some Brazilian (10,17,31) and African (51) studies. On the other hand, our findings are in marked contrast to those from Mexico (49), Argentina (26) and also Brazil (8,29), with susceptibility dose-dependance and resistance, taken together, of 8.1%, 31.2%, 12.9% and 29.0%, respectively.

The prevalence of resistance and susceptibility dose-dependance to FLC, taken together, for *C. glabrata* isolates was 50.0% in our study, and ranged from 10.0% in São Paulo, Brazil, to 75.0% in Rosario – Santa Fe, Argentina (10,26,29,31,49,51). Studies carried out in cases of invasive candidiasis demonstrated a decreased susceptibility of *C. glabrata* to FLC (37,40-43, 59).

All *C. krusei* isolates evaluated in our study were resistant to FLC, which is consistent with the results obtained by other authors (10, 26, 31) and the finding of a natural resistance (20,46).

Our results with ketoconazole showed 90.0% susceptibility of *Candida* spp isolates. The prevalence of resistance and susceptibility dose-dependance, taken together, as to species revealed 0.75% for *C. albicans,* 0.0% for *C. glabrata* and *C. tropicalis,* and 50.0% for *C. krusei*, in agreement with other studies (4,23,51). Conversely, some Brazilian studies reported a prevalence of susceptibility dose-dependance to KTC of 85.7% for *C. glabrata*, 100.0% for *C. tropicalis,* and 100.0% for *C. krusei* (10), and a prevalence of resistance and

susceptibility dose-dependance, taken together, of 60.0% for *C. glabrata* and 71.4% for *C. krusei* (31).

Several studies have reported high prevalence of susceptibility to ITC for *C. albicans* isolates (8,10,31), in agreement with our findings (98.1%). However, two studies revealed increased frequency of SDD or ITC-resistant isolates (26,49). A prevalence of 62.5% was observed for a cohort of 16 patients, but six of the 10 non-susceptible isolates were from patients previously treated with FCL or ITC (26). A prevalence of 29.4% was observed for *C. albicans* isolated from patients with pseudomembranous or erythematous candidiasis, or denture stomatitis; however, no data about previous antifungal treatment were available. In our study, the prevalence of resistance and susceptibility dose-dependance to ITC, taken together, was 72.2% for *C. glabrata*, 0.0% for *C. tropicalis*, and 50.0% for *C. krusei*, as also revealed by other investigators (8,10,51), The exception is related to *C. tropicalis*, with five SDD and two resistant isolates out of 11 [63.7%] (49).

Cross-resistance to the three azole compounds was observed for two *C. albicans* and one *C. krusei*. Cross-resistance to two azole compounds was shown by one *C. krusei* and nine *C. glabrata* isolates. Clinical isolates exhibiting cross-resistance to azoles have been reported by several authors (31,26, 49,58). The involved resistance mechanisms appear to be related to altered cellular efflux and reduced permeability of the membrane to azoles or enhanced activity of 14 DM cellular contents (13, 59). Studies that will contribute to a greater understanding of the relationships between organisms and antimicrobials should be carried out in order to elucidate the complex

mechanisms involving microbial resistance to drugs. Our results show predominance of *C. albicans* in the oral cavity of HIV-positive individuals, the presence of cross-resistance to azole compounds, and a prevalence of resistance that suggests the evaluation of antifungal susceptibility testing whenever antifungal treatment with azoles is planned.

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TABLES AND FIGURES

TABLE 1. *In vitro* antifungal susceptibilities of oral *Candida* isolates.

Species	Antifungal agent	MIC (mg/L)					I or SDD (%)	R (%)
		Median	Range	GM	MIC_{50}	MIC_{90}		
C. albicans (267 isolates)	AMB	0.25	0.03 - 1.0	0.23	0.25	0.5	-	-
	FLC	0.25	0.125 - 64.0	0.26	0.25	0.5	-	2 (0.75)
	KTC	0.02	0.015 - 8.0	0.0	0.015	0.015	-	2 (0.75)
	ITC	0.02	0.015 - 8.0	0.0	0.015	0.03	4 (1.50)	1 (0.37)
C. glabrata (18 isolates)	AMB	0.50	0.06 - 1.0	0.35	0.5	1.0	-	-
	FLC	3.00	0.5 - 8.0	3.30	2.0	8.0	1 (5.55)	8 (44.44)
	KTC	0.03	0.015 - 0.125	0.03	0.03	0.06	-	-
	ITC	0.25	0.015 - 4.0	0.17	0.25	1.0	11(61.11)	2 (11.11)
C. tropicalis (13 isolates)	AMB	0.50	0.05 - 1.0	0.59	0.5	1.0	-	-
	FLC	0.50	0.25 - 1.0	0.56	0.5	1.0	-	-
	KTC	0.02	0.015 - 0.06	0.03	0.03	0.06	-	-
	ITC	0.03	0.015 - 0.06	0.03	0.03	0.06	-	-
C. krusei (02 isolates)	AMB	1.00	1.0	1.0	1.0	1.0	-	-
	FLC	16.00	16.0	16.0	16.0	16.0	-	2(100.00)
	KTC	0.62	0.025 - 0.5	0.5	0.25	1.0	1(50.00)	1(50.00)
	ITC	0.16	0.06 - 0.12	0.12	0.06	0.25	1(50.00)	-

MIC - Minimal inhibitory concentration
GM – Geometric means
I – Intermediate; SDD – susceptible dose dependent; R – resistant.

TABLE 2 A. Distribution of amphotericin B (AMB), ketoconaloze (KTC) and itraconazole (ITC) minimum inhibitory concentration (MIC) for *Candida* spp isolates tested by EUCAST procedure.

Species no. (%)	Antifungal	MIC (mg/L)									
		≤0.015	**0.03**	**0.06**	**0.125**	**0.25**	**0.5**	**1.0**	**2.0**	**4.0**	**≥8.0**
C. albicans 267 (89.0)	AMB	0	1	4	50	175	35	2	0	0	0
	KTC	261	2	1	1	0	0	1	0	0	1
	ITC	157	100	4	1	4	0	0	0	0	1
C. glabrata 18 (6.0)	AMB	0	0	1	3	3	8	3	0	0	0
	KTC	5	6	6	1	0	0	0	0	0	0
	ITC	3	1	0	1	10	1	1	0	1	0
C. tropicalis 13 (4.3)	AMB	0	0	0	0	0	10	3	0	0	0
	KTC	13	0	0	0	0	0	0	0	0	0
	ITC	2	9	2	0	0	0	0	0	0	0
C. krusei 02 (0.7)	AMB	0	0	0	0	0	0	2	0	0	0
	KTC	0	0	0	0	1	0	1	0	0	0
	ITC	0	0	1	0	1	0	0	0	0	0

TABLE 2 B. Distribution of fluconazole (FCL) minimum inhibitory concentration (MIC) for *Candida* spp isolates tested by EUCAST procedure.

Species no. (%)	**MIC (mg/L)**									
	≤0.125	**0.25**	**0.5**	**1.0**	**2.0**	**4.0**	**8.0**	**16**	**32**	**≥64**
C. albicans 267 (89.0)	32	207	22	4	0	0	0	0	0	2
C. glabrata 18 (6.0)	0	0	1	2	6	1	8	0	0	0
C. tropicalis 13 (4.3)	0	1	9	3	0	0	0	0	0	0
C. krusei 02 (0.7)	0	0	0	0	0	0	0	2	0	0

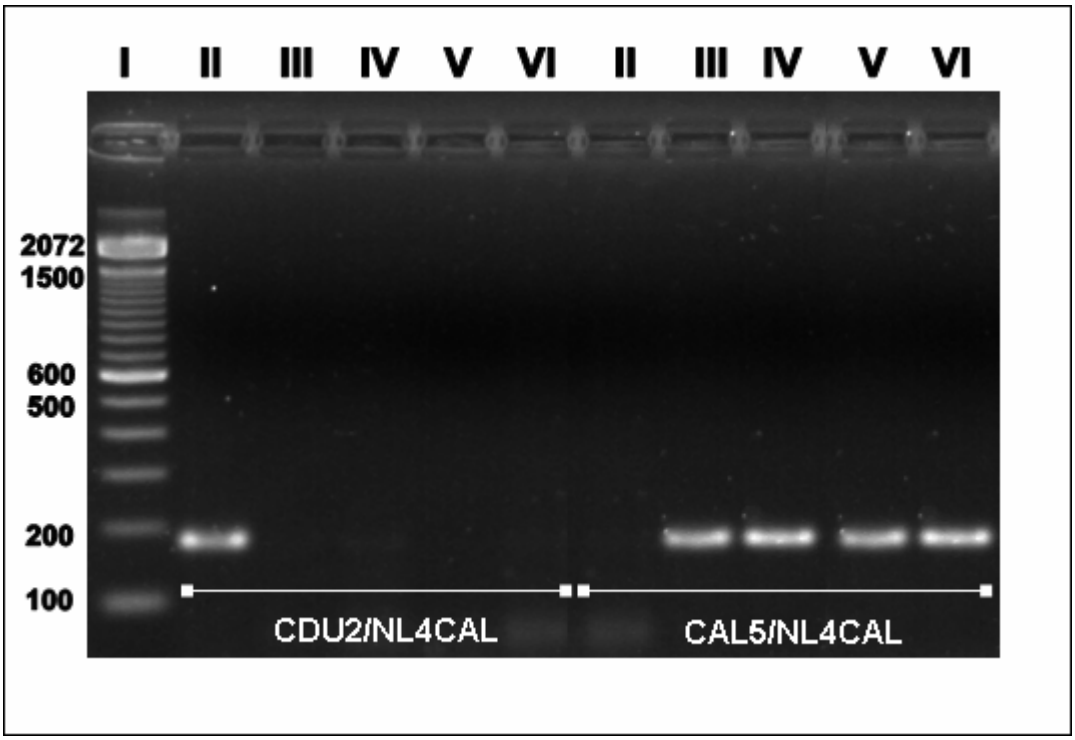

FIGURE 1 – Molecular differentiation between *C. albicans* and *C. dubliniensis* by amplification primer CAL5/NL4CAL and CDU2/NL4CAL by the polymerase chain reaction (PCR), product of 175bp. Line I, 100-bp lade; lines II, *C. dubliniensis* (CBS 9768); line III, *C.albicans* (ATCC 76615); lane IV and VI *C. albicans* isolates from the oral cavity of HIV patients.

TABLE 3 – Median and range of minimal inhibitory concentration (MIC) of amphotericin (AMB), fluconazole (FLC), ketoconazole (KTC), and itraconazole (ITC) evaluated in *Candida* spp. isolates from the oral cavity of HIV infected individuals. Comparison between species were carried out by Kruskal-Wallis and Dunn's test.

Species	**(no.)**	**MIC (mg/L)**							
		AMB		**FLC**		**KTC**		**ITC**	
		Median	Range	Median	Range	Median	Range	Median	Range
C. albicans	(267)	0.25B	0.03 -1.0	0.25B	0.125 - ≥64.0	0.02B	0.015 - 8.0	0.02B	0.015 - 8.0
C. glabrata	(18)	0.50AB	0.06 -1.0	3.00A	0.5 - 8.0	0.03A	0.015 - 0.12	0.25A	0.015 - 4.0
C. tropicalis	(13)	0.50A	0.5 - 1.0	0.50A	0.25 - 1.0	0.02B	0.015	0.03AB	0.015 - 0.06
***P* value**		<0.001		<0.001		<0.001		<0.001	

Capital letters compare medians in the same column.

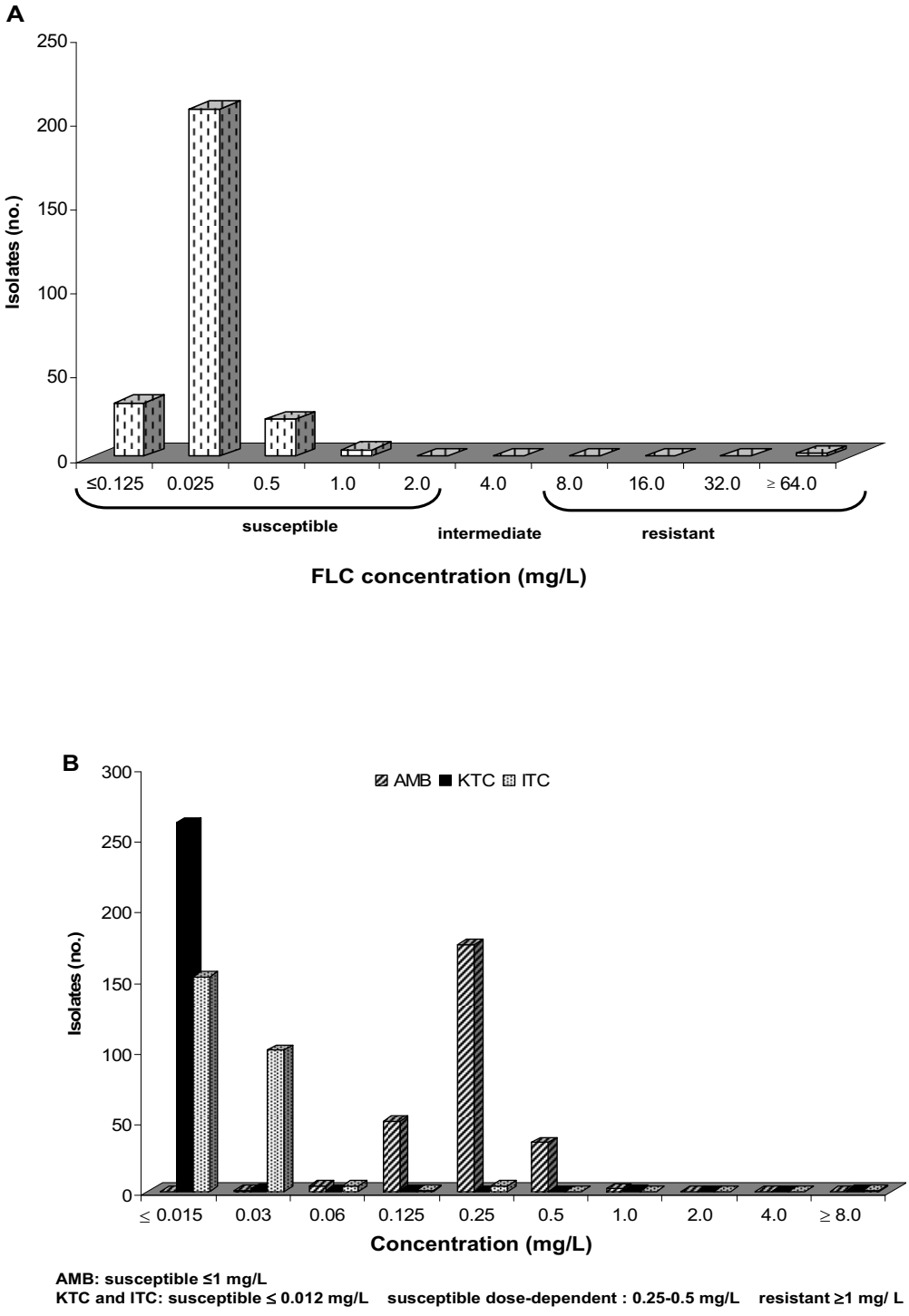

FIGURE 2 – Distribution of fluconazole (FCL), amphotericin B (AMB), ketoconaloze (KTC) and itraconazole (ITC) minimum inhibitory concentration (MIC) for 267 isolates of the *Candida albicans* tested by EUCAST procedure.

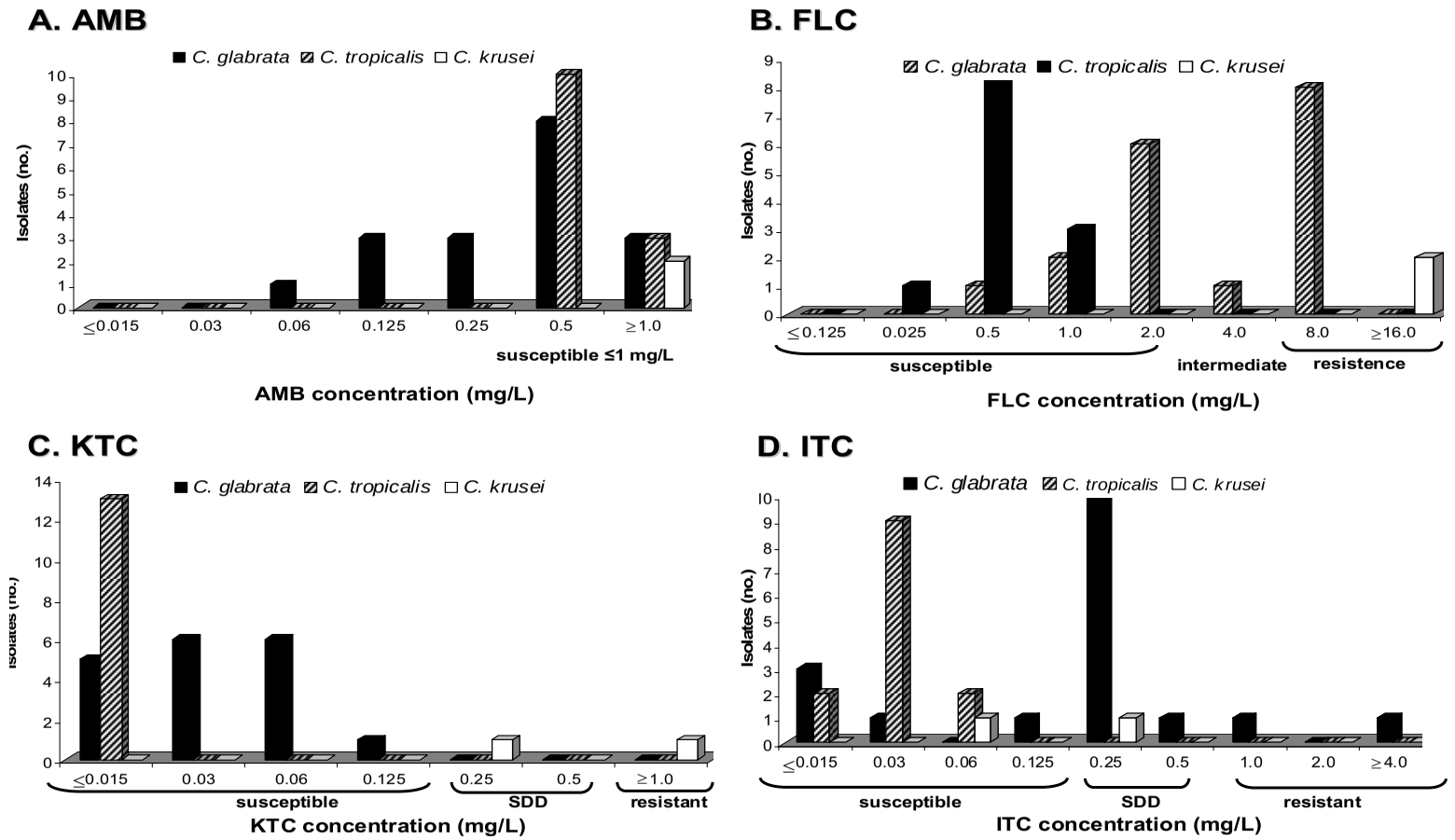

FIGURE 3 – Distribution of amphotericin B (AMB), fluconazole (FCL), ketoconaloze (KTC) and itraconazole (ITC) minimum inhibitory concentration (MIC) for 33 isolates of the non-*Candida albicans* isolates tested by the EUCAST procedure. SDD - susceptible dose dependent.

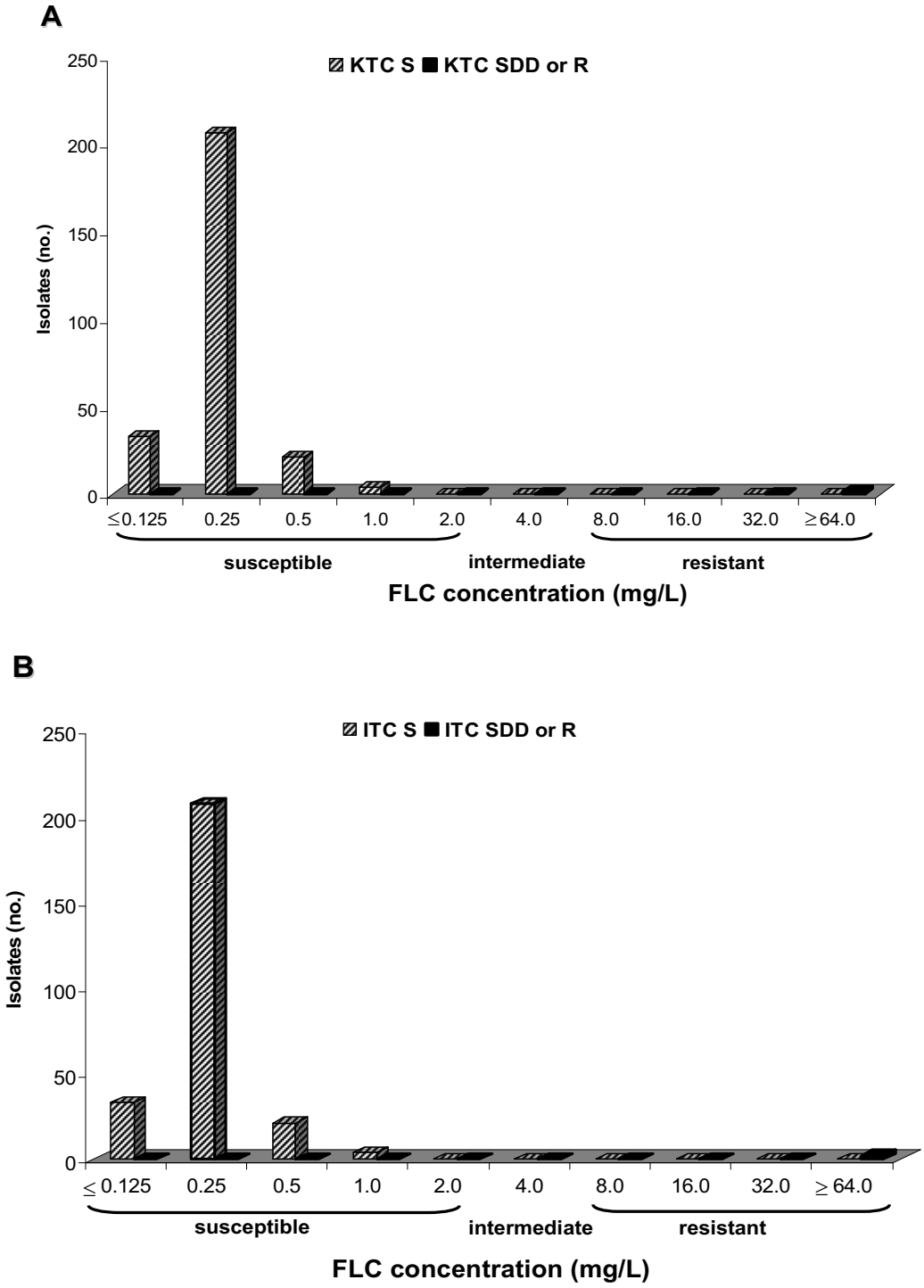

FIGURE 4 – Correlation between minimal inhibitory concentration (MIC) values of fluconazole (FCZ) and susceptibility profile to itraconazol (ITC) [A] and ketoconazole (KTC) [B]. Evaluation of 267 *Candida albicans* isolates from the oral cavity of HIV infected individuals. S – susceptible; SDD – susceptible dose dependent; R – resistant.

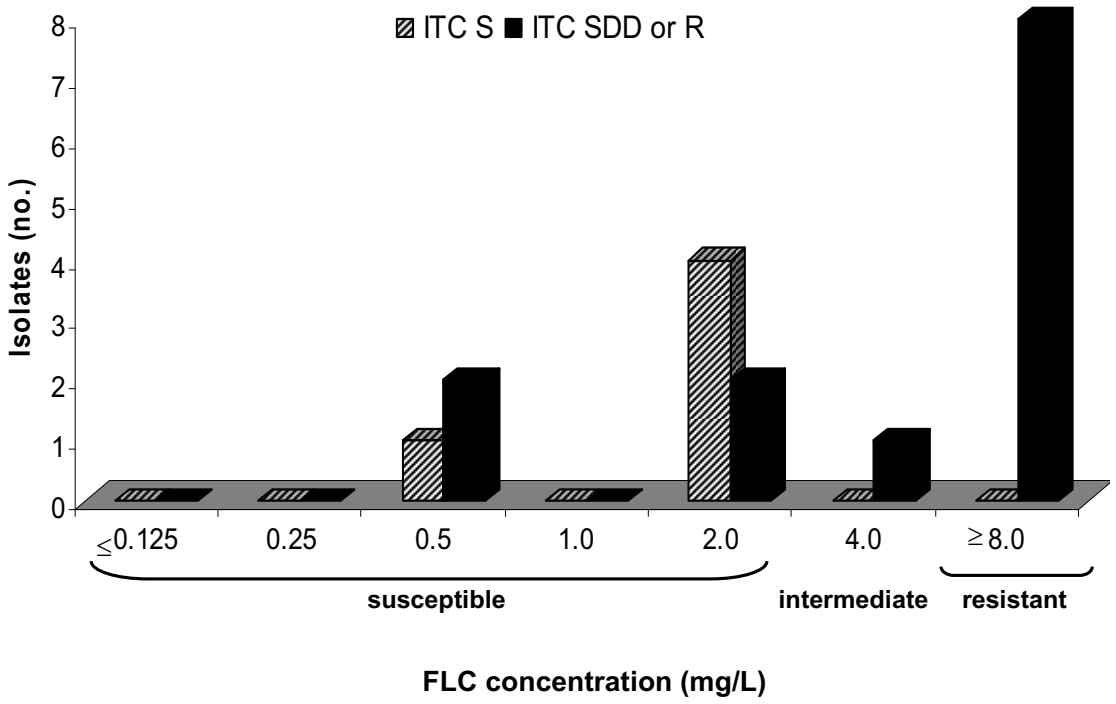

FIGURE 5 – Correlation between minimal inhibitory concentration (MIC) values of fluconazole (FCZ) susceptibility profile to itraconazol (ITC). Evaluation of *Candida glabrata* isolates from the oral cavity of HIV infected individuals. S – susceptible; SDD – susceptible dose dependent; R – resistant.

4. Genotyping and antifungal susceptibility profile of sequential *Candida albicans* isolated from the oral cavity of HIV-infected individuals.

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Running title: Characteristcs of *C. psilosis* isolated from HIV - infected individuals

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ABSTRACT

Although the most HIV-infected individuals are colonized or infected by multiples strains of oral *Candida albicans,* little is known of their micro-evolution over time. This study aimed to evaluate the genotypic profiles of oral *C. albicans* sequentially isolated during the HIV infection course, and to determine its minimal inhibitory concentration (MIC) to four antifungal compounds. A total of 142 oral-swab samples were isolated from the 59 HIV-infected individuals with or without symptoms of oropharyngeal candidiasis, and later evaluated as to susceptibility to four antifungal compounds using the microdilution method as to AFST/EUCAST. Oral samples were obtained from all individuals during 48 month period up to five visits. *C. albicans* isolates were phenotypically and genotypically identified, and the genetic similarities of yeast isolates within and between sequential clones of *C. albicans* were assessed by DNA fingerprinting through random amplification of polymorphic DNA (RAPD). Twenty clusters were identified during the study period, with multiple genotypes isolated simultaneously from the HIV infected individuals. Analysis of the profiles revealed that yeasts isolated over sequential visits from symptomatic individuals showed a 78.0% of relatedness and from asymptomatic individuals showed a 87.0% of relatedness. The degree of similarity among *C. albicans* was higher for isolates from colonization than for those from infection. Genetically identical *C. albicans* samples also formed connected subclusters in sequential visits. Susceptibility studies showed that all isolates were susceptible to amphotericin B, fluconazole, ketoconazole, and itraconazole. These data point to varying evolutionary genetic trends in *C. albicans* sequentially isolated from de oral cavity from HIV infected individuals. In addition, all the isolates were susceptible to the antifungal compound tested.

INTRODUCTION

Host defense mechanisms against *Candida albicans* are complex. Many studies have shown that deficiencies in both phagocytic cell functions and classical cell-mediated or humoral immune responses have been linked to the increased susceptibility of the host to *C. albicans* infection (20). The impairment of cell-mediated immunity caused by the human immunodeficiency virus (HIV) predisposes these individuals to oral candidiasis and other opportunistic infections (12).

The prevalence of *C. albicans* colonization and the recurrence of oral candidiasis increase with the advance of HIV infection (23,48). For HIV-infected individuals, oral candidiasis has been recognized as an early expression of their immunodeficiency (19) while esophagic candidiasis is considered a clinical predictor of AIDS (22). The repeated use of short- or long-term antifungal azoles, particularly fluconazole (FLC), to treat oropharingeal candidiasis can lead to the development and/or selection of resistance among clinical isolates (41).

This, in turn, implies that subclones of *C. albicans* with variable genotypes may simultaneously colonize the oral cavity. In this case, it is difficult to determine with certainty the contribution, if any, of individual clones to infection (21,44), and the presence of more than one clonal type may have important therapeutic implications (25,31,36,37). However, few clonal variations are known for *C. albicans* during either recurrent episodes of oral candidiasis or asymptomatic carriage over a prolonged course of HIV infection. Recent advances in molecular techniques have generated several typing methods for

genetic assessment of *C. albicans* strain relatedness, which in turn have facilitated detailed studies on the molecular epidemiology of this yeast (45). In addition, a variety of molecular methods have indicated that *C. albicans* strains tend to be genetically similar when isolated from the same specific groups, such as HIV-positive and negative patients from geographically related locations (49).

These molecular techniques include Cp3-13-specific probe, hybridization, pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) (2,5,31, 40), amplified fragment length polymorphism (AFLP) (1), and multilocus sequence typing (MLST) (6,46). These techniques have helped delineate subtypes of colonizing and/or infecting *C. albicans* strains over sequential evaluations of HIV-infected individuals. This study aimed to evaluate the genotypic profiles of oral *C. albicans* isolated over sequential clinical visits during the HIV infection course, as well as to determine its minimal inhibitory concentration (MIC) to four antifungal compounds.

MATERIALS AND METHODS

Samples were collected from the oral cavity of HIV-positive individuals from June 2002 to July 2006. The patients were assisted at the Special Outpatient Clinic for Infectious and Parasitic Diseases or at the Ward of Tropical Diseases of Botucatu Medical School, São Paulo State University (UNESP). On each visit, samples were collected for culture independently of the clinical signs and symptoms of oral candidiasis. Each of the 59 patients attended the clinic

between two and five times during this period. The characteristics of the 58 HIV-infected individuals from which *C. albicans* was isolated are shown in Tables 1A and 1B. In total, 142 oral-swab samples were obtained during the observation period, as described below. Approval was obtained from the Research Ethics Committee, Botucatu Medical School, UNESP, and from the Ethics Committee on Human Research, Adolfo Lutz Institute (IAL). Written informed consent was obtained from every patient for this study.

Fungal isolates.

A total of 169 yeast isolates was obtained from a cohort of 59 HIV-infected individuals. *Candida* spp. were obtained by swabbing the oral cavity of these individuals. The swabs were then placed on plates containing Sabouraud agar (Difco, Detroit, Michigan) supplemented with 0.05% chloramphenicol. The plates were incubated at 30°C for 24h. The morphology of colonies of each sample was analyzed, and morphologically distinct colonies from each sample were subcultured in Sabouraud agar for species differentiation and DNA fingerprinting. The isolates were plated on CHROMagar *Candida* (CHROMagar, Paris France) at 35ºC for 48h. The color and morphology of colonies of each isolate was then analyzed to establish the presence of colored colonies, whereas green colonies grown on the agar plate were likely *C. albicans* or *C. dubliniensis*. To differentiate and identify *Candida* spp. at the microscopic level, isolates were incubated in cormeal agar (Oxoid, Ltda, Brasil) supplemented with 1% Tween 80 (Synth, Labsynth, Brasil) to verify their morphology (25) and in API 20C AUX (BioMérieux Marcy l'Etoile, France), according to the manufacturer's instructions, to determine their biochemical characteristics. Other *Candida* species, rather than *C. albicans*, were not analyzed in this study.

Molecular differentiation between *C. albicans* and *C. dubliniensis*

C. albicans and *C. dubliniensis* were differentiated by polymerase chain reaction (PCR), as previously described by Mannarelli and Kurtzman (26).

DNA extraction. Each isolate was plated on Sabouraud Dextrose Agar (Difco, Detroit, Michigan) and incubated at 30ºC for 24h. The yeast cells were then transferred to a microcentrifuge tube containing 1ml of 50mM EDTA pH8, vortexed and centrifuged at 13,000 x g for 15 min. The supernatant was discarded and the pellet was resuspended in 200µl of 50mM EDTA (Sigma Chemical, USA) pH8 containing 40µl lysis enzyme obtained from *Trichoderma harzianum* (10mg/ml) (Sigma Chemical, USA), followed by incubation at 37ºC for 2-3h. Subsequently, this mixture was centrifuged at 13,000 x g for 10 min and the pellet was dissolved in lysis buffer containing 10mM Tris-HCl (Sigma Chemical, USA) pH 8, 10mM EDTA (Sigma Chemical, USA), 0.5% sodium dodecyl sulfate (SDS) (Synth, Labsynth, Brasil), 0.01% N-laurylsacozyl (Sigma Chemical, USA) and 10µg/ml proteinase K (Sigma Chemical, USA). The mixture was then vortexed and incubated at 56ºC for 2-3h. DNA was extracted by phenol/chloroform/isoamyl alcohol and precipitated with isopropanol according to Sambrook and coworkers (38). After washing the pellet with 70% ethanol for 10min at 10,000 x g, DNA was dissolved in 50 µl ultra pure water. DNA concentrations were determined by using a NanoDrop1000 (Thermo Fisher Scientific, USA) apparatus, as described by Sambrook et al (38). For PCR and RAPD amplification, 1µl of each DNA sample (approximately 100ng) was used. DNA was frozen at -20ºC until use.

PCR. The protocol used for PCR was previously described by Mannarelli and Kurtzman (26). Two pairs of primers (Invitrogen Brasil Ltda) were chosen, for *C. albicans*: sense (CAL5- 5´TGTTGCTCTCTCGGGGGCGGCCG-3´) and anti-sense (NL4CAL– 5´AAGATCATTATGCCAACATCCTAGGTAAA3´), and for *C. dubliniensis:* sense (- 5´AGTTACTCTTTCGGGGGTGGCCT-3´) and anti-sense (NL4CAL– 5´AAGATCATTATGCCAACATCCTAGGTAAA3´). The following amplification conditions were adopted: a first denaturation cycle at 94ºC for 5min, followed by 35 denaturation cycles at 94ºC for 30s, annealing at 65ºC for 45s and elongation at 72ºC for 30s, with a final extension step at 72ºC for 10min. Amplified DNA products were separated by electrophoresis on 2% agarose gel in TBE (89 mM Tris/HCl, 89mM boric acid, 2 mM EDTA, pH 8) buffer at 100V for 35 min and a 100-bp DNA ladder was used as molecular size marker (Invitrogen, Brasil, Ltda). The gel was stained with ethidium bromide (0.5mg/ml) and DNA bands were visualized by UV transillumination Mini Bis Pro (Bio-Imaging). The band profiles in PCR for *C. albicans* and *C. dubliniensis* clinical isolates were compared to those for the type strains *C. albicans* of the American Type Culture Collection (ATCC) 76615 and *C. dubliniensis* of the Centraalbureau voor Schimmelcultures (CBS) 9768.

Genotyping of *C. albicans* isolates

RAPD analysis. Amplification was carried out with the kit Ready-to-Go-RAPD Analysis Beads (GE Healthcare, United Kingdom). RAPD beads were composed of 1.5 units of Taq DNA polymerase, 10mM Tris-HCl pH 8.3, 30mM KCl, 3 mM $MgCl_2$, 400mM of each dNTP, and stabilizers such as bovine serum albumin. Each reaction was performed by adding each DNA template and

25pmol of the primer to a final volume of 25 μl. The selection of primers was determined after testing six primer sequences for exclusive use in RAPD (GE Healthcare, United Kingdom). The primer p4 (5'-AAGAGCCCGT-3') was selected for *C. albicans* isolates. The amplification was performed in an automated thermal cycler (Progene, USA) and consisted of one initial denaturation cycle at 95ºC for 5 min, 45 denaturation cycles at 95ºC for 1 min, annealing at 32ºC for 1 min, and extension at 72ºC for 2 min. The procedure was completed with a final extension cycle for 10min. DNA samples were tested in duplicate. Each amplification run contained a negative control (ultra pure water). After thermal cycles, PCR products were eletrophoresed on polyacrylamide gels in TBE buffer pH 8 and stained with ethidium bromide. The sizes of fragments were based on the comparison with a 100-bp DNA ladder (Invitrogen, Ltda, Brasil) and visualized by UV transillumination Mini Bis Pro (Bio-Imaging Systems, Ltda, Israel). The band profiles in RAPD for *C. albicans* clinical isolates were compared to those for the type strains *C. albicans* (ATCC) 76615. All experiments were carried out in duplicate to assess reproducibility.

Analysis of data and dendrogram generation.

RAPD profiles were analyzed by Bionumerics version 5.10 (Applied Maths, Kortrijk, Bélgica). The similarity coefficient (S_{AB}) between patterns for every pair of isolates A and B was computed with the formula $S_{AB} = 2_E/(2_E + a + b)$, where *E* is the number of common bands in the patterns A and B; *a* is the number of bands in pattern A with no correlates in pattern B; and *b* is the number of bands in pattern B with no correlates in pattern A. Dendrograms

based on S_{AB} values were generated through dendrograms by unweighted pair-group method using arithmetic average (UPGMA) methods (1.5% optimization and 2% tolerance) implemented in the Bionumerics software. An S_{AB} value of 1.00 (100.0%) indicates that the banding patterns for strain A are identical to those for strain B; S_{AB} values of 0.80–0.99 (80.0–99.0%) represent highly similar but nonidentical strains and S_{AB} values below 0.80 represent unrelated strains (29).

Antifungal agent susceptibility testing. Standard antifungal susceptibility tests were carried out according to the reference microdilution method established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (14). Amphotericin B (AMB), ketoconazole (KTC), fluconazole (FLC) and itraconazole (ITC) were the antifungal compounds employed. These drugs were obtained as reagent-grade powders from Sigma-Aldrich Brasil Ltda. As regards susceptibility to FLC, the isolates were classified as susceptible (S) for MIC $\leq$ 8.0mg/L; intermediate (I) for MIC equal to 4.0mg/L; and resistant (R) for MIC $\geq$ 8.0mg/L, according to EUCAST (14). Considering the degree of susceptibility to KTC and ITC, the isolates were classified as susceptible (S), susceptible dose-dependent (SDD) and resistant (R), according to Clinical and Laboratory Standards Institute (CLSI) (8); for ITC: $\geq$1 mg/ L was used for R, 0.25-0.5 mg/L for SDD, and $\leq$0.125 mg/L for S; for KTC: $\geq$1 mg/ L was used for R, 0.25-0.5 mg/L for SDD, and $\leq$0.125 mg/L for S. As the interpretive criteria for AMB was not established by EUCAST or CLSI, isolates were considered susceptible with MICs $\leq$1 mg/L and resistant with MICs $\geq$2

mg/L (30,35). Positive control was performed using *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 for each batch of isolates tested.

Statistical analyses. Differences in antifungal MIC distributions were examined using the Wilcoxon test, Friedmann test and Kruskall-Wallis test followed by Dunn's test for multiple comparisons. Comparison of frequencies was carried out by Fisher's exact test. All tests were performed using the statistical analysis system (SAS), version 6.12 (Institute Inc. USA) (42). Significance was set at $p < 0.05$.

RESULTS

Six of the 59 HIV-infected individuals presented symptomatology of oral candidiasis in two or more visits during the study period, while the remaining patients were asymptomatic as to this condition. *Candida* spp. isolation was detected in two visits for 41 patients (69.50%), in three visits for 13 patients (22.0%), in four visits for four patients (6.8%), and finally in all five visits for only one patient (1.7%). Thus, 142 oral-swab samples were obtained during the observation period. However, 14 of them showed colonies with different morphology or green coloration and 13 with other colors; these 27 isolates were subcultured for species and genetic differentiation. Thus, each of the 27 swabs allowed the evaluation of two samples: one representative of most colonies and another one with different characteristics. Therefore, we studied 169 species isolated from sequential *Candida* spp. isolates. The sequence of isolated species is shown in Table 2, as follows: *C. albicans* alone occurred in 83.0%, *C. albicans* followed by *C. albicans* plus another species in 15.3%, and a change

in species was observed in only 1.7% of the cases. When different species were isolated from sequential samples, *C. glabrata* was followed by *C. tropicalis*; *C. albicans* by *C. krusei; C. albicans* by *C. parapsilosis; C. albicans* by *C. tropicalis* in three patients; *C. albicans* and *C. glabrata* also in three individuals; and finally *C. albicans* and *C. parapsilosis* followed by *C. albicans* and *C. metapsilosis*.

The identification of 156 *C. albicans* was confirmed by PCR using amplification of the primers CAL5/NL4CAL and CDU2/NL4CAL (Figure 1).

RAPD genotyping of sequential clinical *C. albicans* isolates

RAPD analysis was performed to determine the genetic variability of 156 oropharyngeal *C. albicans* samples. The visual and gross examination of the fingerprint profiles of sequential *C. albicans* isolates indicated 61 different molecular profiles, as shown in Figure 2.

Dendrogram analysis

A total of 156 *C. albicans* isolates recovered from the oral cavity of HIV-infected patients were grouped into twenty main clusters, showing 77.9.0% similarity (Figure 3). SAB ranged from 0.46 to 1.0 with mean plus standard deviation of 0.779 ± 0.178. Clusters (I to XX) were derived at a threshold S_{AB} of 80.0%.

Seventeen samples isolated from six individuals with symptomatic oral candidiasis were grouped into seven clusters with 78.0% similarity among them; S_{AB} ranged from 0.54 to 1.0, with mean plus standard deviation of 0.78 ± 0.174, as shown in Figure 4. Four of the seven clusters showed between two and six

subclusters with S_{AB} varying from 0.80 to 0.90 (Table 3). Clusters I and II included two strains with 70.0% similarity between them, suggesting unrelated isolates. Cluster V included one strain with 75.9% similarity to clusters III and IV, suggesting unrelated isolates. Finally, cluster VII grouped two isolates with 90.9% similarity, suggesting highly related strains (Table 3).

A similar dendrogram, based on the S_{AB} values of genetically related isolates, were generated for the other 44 samples from individuals without clinical manifestations of candidiasis. Due to the large number of studied strains, the RAPD profiles of these isolates were carried out only for samples that produced different profiles in the visual analysis (Figure 2). The Dendron database formed nine clusters with 87.0% similarity among them and S_{AB} ranging from 0.46 to 1.0, with mean plus standard deviation of 0.87 ± 0.123. Eight of the nine clusters showed two to 11 subclusters with S_{AB} varying from 0.81 to 1.0 (Table 4). Cluster II contained two identical strains (S_{AB} equal 1.0), and three other genetically related strains, with 93.2% similarity. Two identical strains were found for cluster III, together with four other strains connecting at an S_{AB} of 0·81. Cluster IV was composed of only one isolate and showed 70.7% similarity to the isolates of cluster III. Cluster VIII showed nine isolates with 87.0% similarity, and isolates connected to each other with high similarity (>93.0%); finally, cluster VIX with 100.0% similarity showed, however, 46.2% similarity to the other clusters, suggesting unrelated isolates.

Comparison of the similarity in sequential evaluation of *C. albicans* isolates as to host-parasite relationship. The degree of similarity among *C.*

albicans was higher for isolates from colonization than for those from infection (p=0.05), as shown in Table 5 and Figure 6.

Antifungal susceptibility testing. All tested organisms grew after 24h incubation. The results of susceptibility tests to AMB, FLC, KTC, and ITC for *C. albicans* isolates are presented in Tables 6 to 8. All isolates were susceptible to the different antifungal agents tested. In addition, differences in the MICs of all antifungal compounds tested were not observed for sequential *C. albicans* isolates (Table 6 and 7). The evaluation of MICs of 16 sequential *C. albicans* isolates from four patients indicated higher AMB values in all visits. In addition, in two visits the MICs of KTC were lower than those of ITC and, in the other two visits, the values did not differ (Table 8). Finally, differences in the MICs were not observed when the visits were compared to each other (Table 8).

DISCUSSION

The pathogenicity of *Candida* species is a result of the characteristics of the strains, the immunological status of the host, and the conditions of the infection sites (27). Oropharyngeal candidiasis has been considered the most common opportunistic infection among patients infected with the human immunodeficiency virus - HIV (11). Due to recurrent episodes of candidiasis, the patients are frequently subjected to repeated courses of FCL therapy (3,13,40). After the introduction of highly active antiretroviral therapy (HAART), the occurrence of oropharyngeal candidiasis has reduced (7,17). However, oropharyngeal candidiasis is frequent among patients with low compliance or poor response to the antiretroviral therapy (4,15,39).

Longitudinal studies on the oral colonization and infection patterns of the human pathogen *C. albicans* in HIV-infected individuals are scarce. Barchiesi and coworkers (2) used restriction fragment length polymorphism (RFLP) and Samaranayake and coworkers (37), the random amplification of polymorphic DNA (RAPD).

Barchiese and coworkers (2) carried out a sequential evaluation of five AIDS-patients with recurrent candidiasis during a 5-year period and observed that four of them maintained the similarity of isolates along time. Samaranayake and coworkers (37) studied sequential *C. albicans* isolates from 16 symptomatic HIV-infected individuals, with a total of 443 samples, during a 1-year period. They compared *C. albicans* prevalence as to colonization or infection, finding identical or high degree of similarity (80.0%) and higher prevalence of identity among patients with symptomatic candidiasis.

Our study showed higher prevalence of similarity for colonizing (75.0%) than infecting (33.3%) *C. albicans* strains from HIV-positive individuals. The degrees of similarity were 87.0% and 78.0%, respectively. To switch from saprophytic (colonization) to pathogenic (infection), *Candida* species has to increase its adhesive properties to attach to host components and must increase its production of lytic enzymes to penetrate the tissues (9). The main hydrolytic enzyme involved in its virulence is secretory aspartyl proteinase (SAP), which is a target for the protease inhibitors used in AIDS therapy (7, 24). Thus, in colonizing behavior, *Candida* species may be balanced and more stable, while in pathogenic behavior they may be unbalanced under the pressure of the cell-mediated immune response of the host and the antifungal

and antiretroviral compounds in use. This could be an explanation to our finding of higher similarity among colonizing than infecting *C. albicans* isolates.

Our findings cannot be compared to those of Barchiese and coworkers. (2), who evaluated only patients with candidiasis (infection). In addition, they cannot be compared to those of Saramanayake and coworkers (37) because the latter only evaluated degrees of similarity over 80.0%, whereas we compare isolates above and under 80.0% similarity.

It is difficult to explain the periodic emergence of genetic isotypes in one same individual. A possible failure in the isolation of these isotypes since the first visit cannot be discarded. Perhaps the evaluation of a larger number of isolates per visit can answer this question. Another reason for the presence of different isotypes could be the endogenous reinfection from a distant body site. On the other hand, identical *C. albicans* strains have been isolated from AIDS-patients with vaginitis (43).

Samaranayake and coworkers (37) observed strains with a similarity degree between 70 and 80%, similarly to our study, and suggested that genetic shuffling has occurred during HIV disease progression. Similar genetic shuffling in *C. albicans*, leading to evolution of subtypes during HIV disease progression, has been reported by other authors (5,28,43,31).

Finally, the sequential evaluation of *Candida* species has few times revealed non-*Candida albicans* species.

The development of methods to treat patients with candidiasis has routinely used susceptible yeasts for testing to select appropriate antifungal

compounds. The finding that resistant strains of *C. albicans* isolates from AIDS-patients with oral and esophageal candidiasis developed resistance to azole compounds suggested that fungal strains may become unresponsive to certain drug treatments (16). Thus, it was important to determine the sensitivity of sequential *C. albicans* strains isolates from HIV-positive individuals to establish an effective drug therapy. Our findings demonstrated the maintenance of the susceptibility profile of sequential isolates. It must be emphasized that only 12.7% patients had received previous antifungal treatment, which could explain these results. These findings are very important from a clinical point of view, constituting a safe guide for therapy.

The evidence that *C. albicans* reproduces in a clonal way (32, 47) suggests that it can adapt to its environments if such niches are stable. Alternatively, when exposed to frequent environmental changes, these microorganisms respond through microevolutionary selection based on their capacity to adapt to varied niches (47). Oral mucous membranes of HIV-infected individuals with reduced cell-mediated immune response, associated with the pressure of antifungal and antiretroviral compounds, can behave as a dynamic environment subjected to frequent changes.

We conclude that *C. albicans* remains the major species colonizing or infecting the oral cavity of HIV-positive individuals in our region; all isolates were susceptible to amphotericin B, ketoconazole, itraconazole and fluconazole; and the degree of similarity among the isolates was high, especially in case of colonization.

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TABLES AND FIGURES

TABLE 1A – Characteristics of the 59 human immunodeficiency virus infected individuals from whom *Candida* spp. were isolated in up to five different moments.

CHARACTERISTICS	INDIVIDUALS no. (%)	SWABS no. (%)
GENDER (n1=59)		
Male	38 (64.4)	-
Female	21 (35.6)	-
STAGE (n1=59)		
AIDS	46 (78.0)	-
non-AIDS	13 (22.0)	-
HOST- *Candida* RELATIONSHIP (n2=142)		
Colonization	-	125 (88.0)
Infection	-	17 (12.0)
ANTIFUNGAL THERAPY (n2=142)		
Yes	-	18 (12.7)
No	-	124(87.3)
INITIAL ANTI-RETROVIRAL THERAPY (n1=59)		
Yes	46 (78.0)	-
No	13 (22.0)	-

n1= number of individuals n2= number of oral swabs

TABLE 1B – Characteristics of the 59 human immunodeficiency virus individuals from whom *Candida* spp. was isolated at the moment of the first visit.

CHARACTERISTICS	no.	MEDIAN	RANGE
AGE	**59**		
		37.0	19.0 – 64.0
INITIAL $CD4^+$ T LYMPHOCYTES (no./mm^3)	**53**		
< 200	23	93.0	4.0 – 184.0
≥ 200	30	336.0	200.0 – 821.0
INITIAL HIV VIRAL LOAD (copies/mL)	**50**		
Undetectable	15	-	-
< 10,000	21	1,119	81.0 – 5,736
≥ 10,000	14	53,757	11,429 – 2 ,700,000
INTERVALS AMONG VISISTS (months)*	**59**	3.0	1-15

* Two or more visits

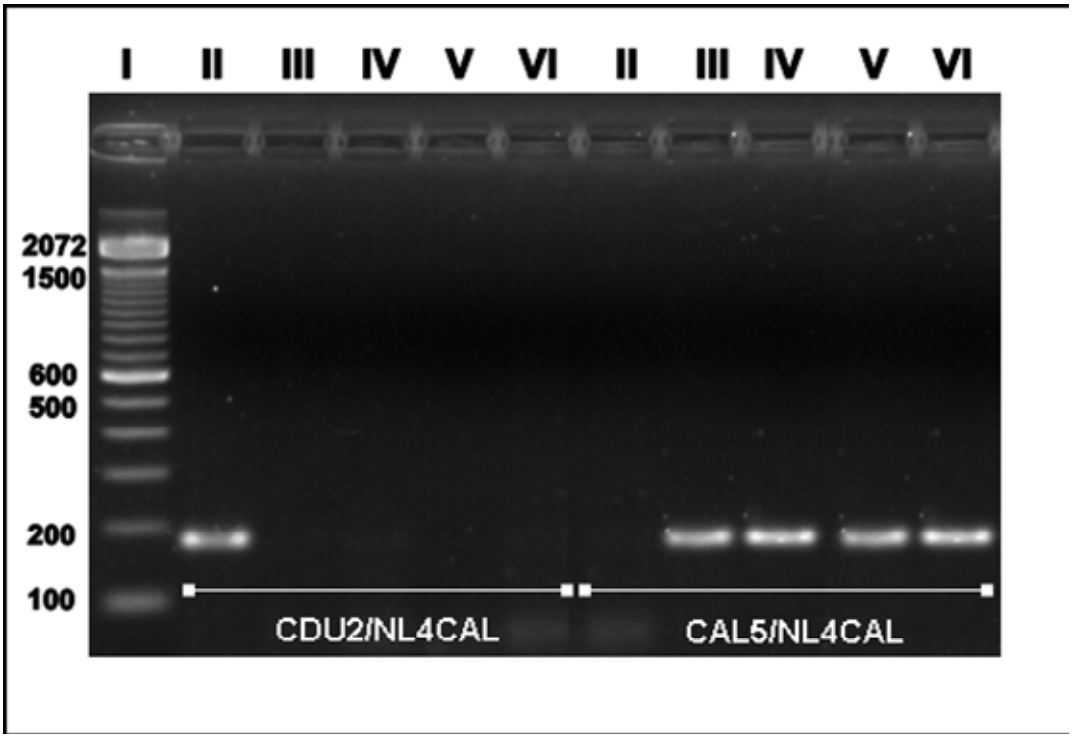

FIGURE 1 – Molecular differentiation between *Candida albicans* and *Candida dubliniensis* by amplification primer CAL5/NL4CAL and CDU2/NL4CAL by the polymerase chain reaction (PCR), product of 175bp. Line I, 100-bp ladde; lines II, *C. dubliniensis* (CBS 9768); line III, *C.albicans* (ATCC 76615); lane IV and VI *Candida albicans* isolates from the oral cavity of human immunodeficiency virus infected individuals.

TABLE 2 – Association of *Candida* species in sequential samples isolated from 59 human immunodeficiency virus infected individuals.

Identification	no.	(%)
Always the same species		
Only *C. albicans*	49	(83.0)
Non-*Candida albicans*	0	(0.0)
C. albicans followed by *C. albicans* plus another	9	(15.3)
Change of species.	1	(1.7)
Total	59	(100.0)

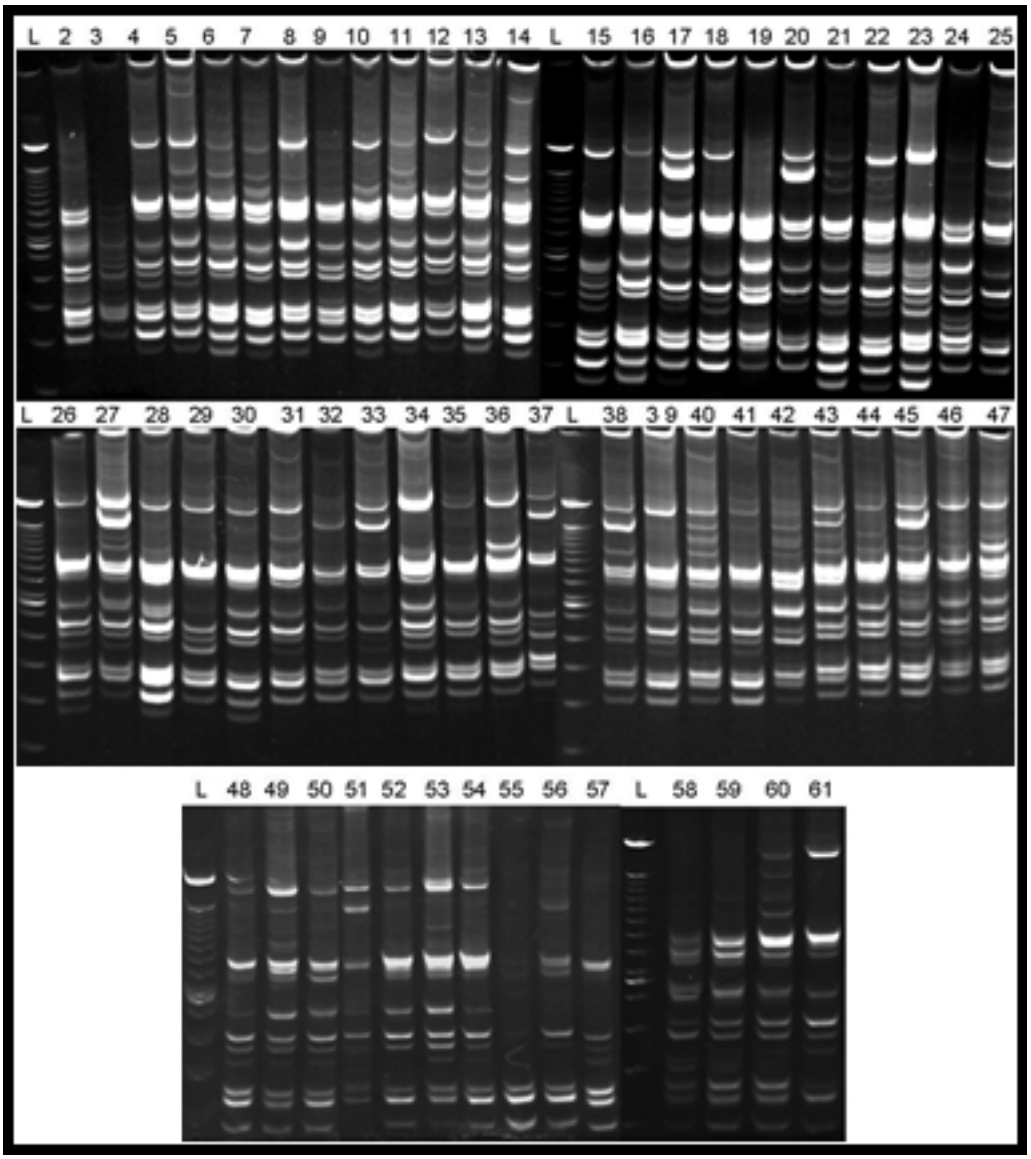

Figure 2 – Groups of 61 profiles of *Candida albicans* obtained from 156 isolates of human immunodeficiency virus individuals, by Randomly amplifield polymorphic DNA (RAPD) in polyacrylamide gel at 5%.

Dice (Opt: 1.50%) (Tol 2.0% - 2.0%) (H>0.0% S>0.0%) [0.0%-100.0%] - UPGMA
RAPD *Candida albicans*

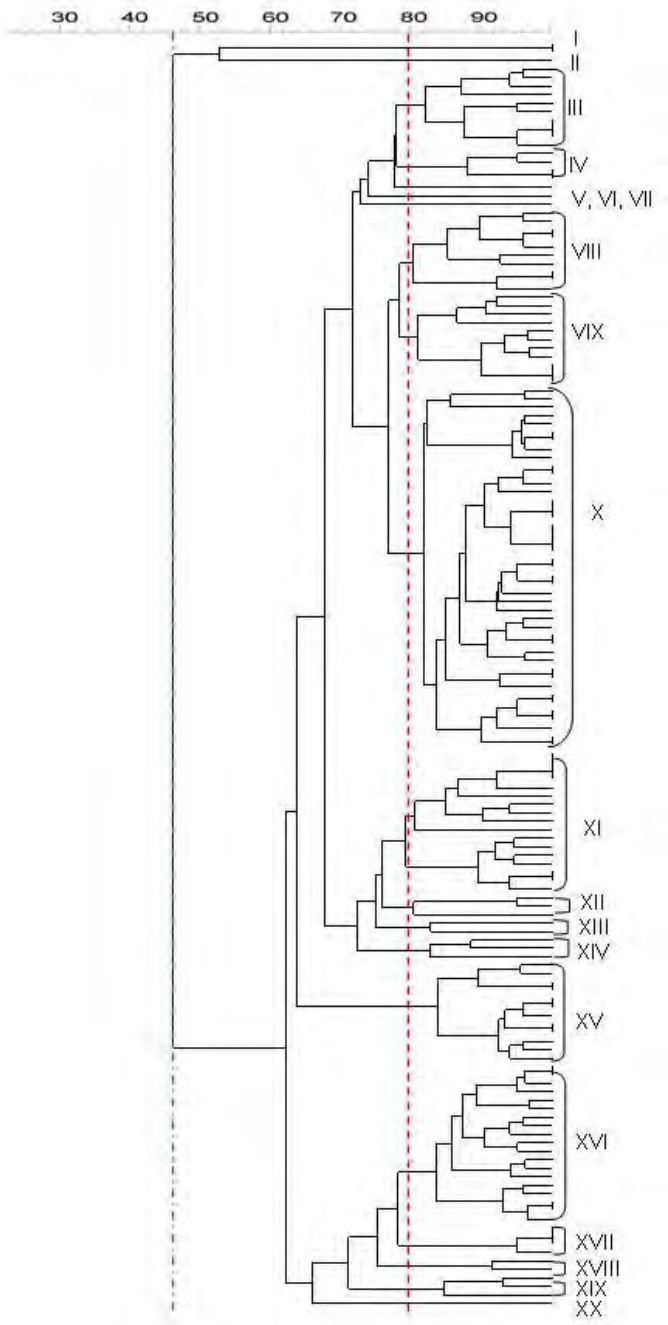

FIGURE 3 – Dendrograms generated for sequential *Candida albicans* from human immunodeficiency virus infected individuals. Vertical dashed line mark the positions of S_{AB} values of 0·46 and 0·80, clustering. Heavy vertical lines to the right of each dendrogram mark the position of clusters.

Dice (Opt: 1.50%) (Tol 2.0% - 2.0%) (H>0.0% S>0.0%) [0.0%-100.0%] - UPGMA

RAPD *Candida albicans*

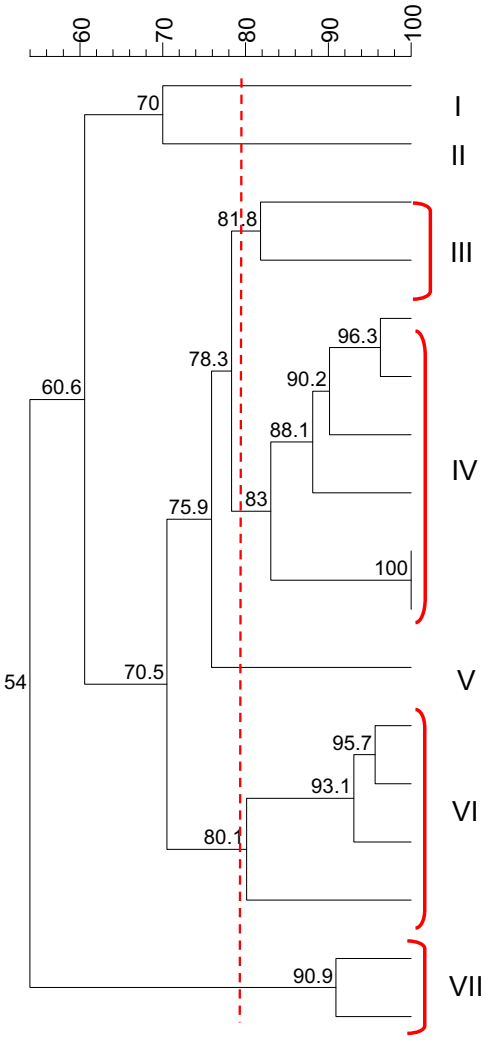

FIGURE 4 – Dendrograms generated for seventeen oral *Candida albicans* isolates from six human immunodeficiency virus patients with symptomatic oral candidiasis. Vertical dashed line mark the position of S_{AB} 0·80, clustering. Heavy vertical lines to the right of each dendrogram mark the position of clusters.

Dice (Opt: 1.50%) (Tol 2.0% - 2.0%) (H>0.0% S>0.0%) [0.0%-100.0%] - UPGMA

RAPD *Candida albicans*

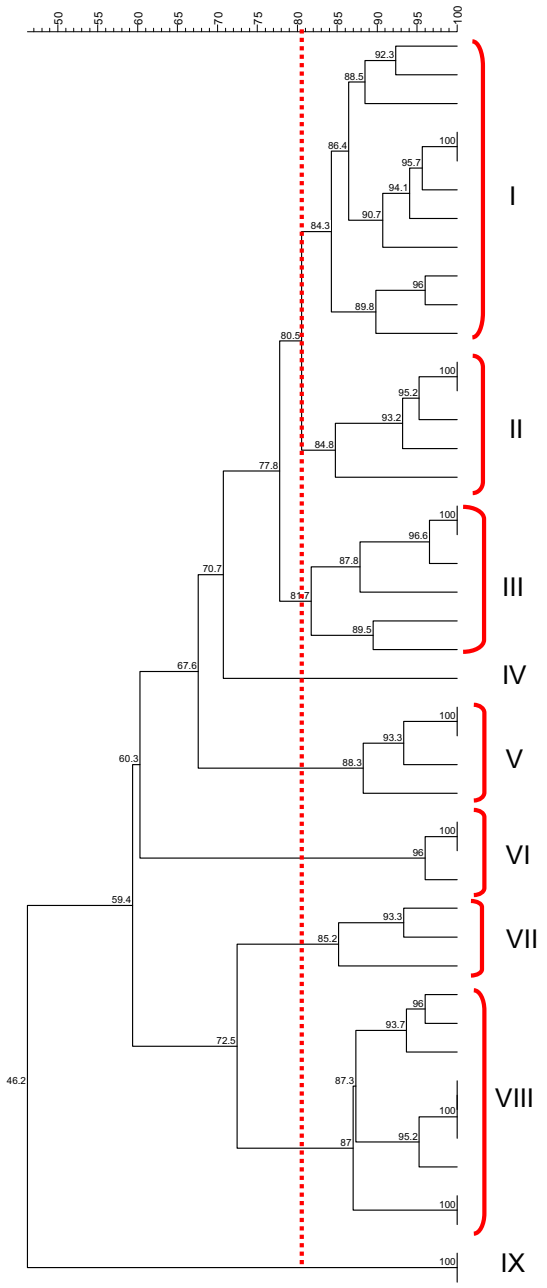

FIGURE 5 – Dendrogram generated for forty-four oral *Candida albicans* isolates from 40 individuals without symptomatic oral candidiasis. Vertical dashed line mark the positions of S_{AB} 0·80, clustering. Heavy vertical lines to the right of each dendrogram mark the position of clusters.

TABLE 3 – Distribution of seventeen oral *Candida albicans* isolates obtained by sequential evaluations of six human immunodeficiency virus individuals with symptomatic oral candidiasis, according to clusters and coefficient of similarity (S_{AB}) of the subclusters.

Cluster no.	Strains (no.)	S_{AB} / subclusters
I	1	-
II	1	-
III	2	0.81
IV	6	0.83
V	1	-
VI	4	0.80
VII	2	0.90
Total	17	-

TABLE 4 – Distribution of 44 oral *Candida albicans* isolates obtained by sequential evaluations of human immunodeficiency virus individuals with colonization by *Candida albicans*, according to clusters and coefficient of similarity (S_{AB}) of the subclusters.

Cluster no.	Strains (no.)	S_{AB} / subclusters
I	11	0.84
II	5	0.84
III	6	0.81
IV	1	-
V	4	0.88
VI	3	0.96
VII	3	0.85
VIII	9	0.87
VIX	2	1.0*
Total	44	-

* Identical strains

TABLE 5 – Evaluation of the degree of similarity of *Candida albicans* isolates obtained from 58 human immunodeficiency virus infected individuals as to of host-parasite relationship. Profiles obtained by randomly amplifield polymorphic DNA (RAPD) in polyacrylamide gel at 5%.

	S_{AB}		Total no. (%)
	< 0.80	≥ 0.80	
Colonization	**13 (25.0)**	**39 (75.0)**	**52 (100.0)**
Infection	**4 (66.7)**	**2 (33.3)**	**6 (100.0)**

Fisher's exact test ***p*** = 0,048

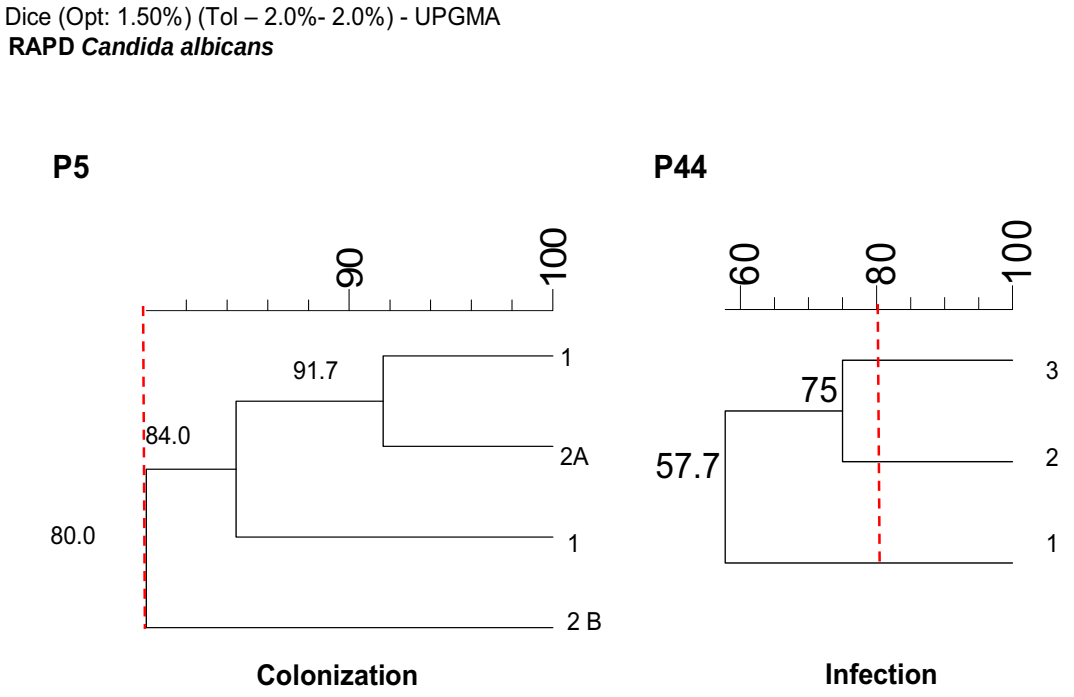

FIGURE 6 – Dendrogram generated for sequential oral *Candida albicans* from one human immunodeficiency virus infected individual with asymptomatic oral *Candida* carriage and, other with symptomatic oral candidiasis. Vertical dashed line mark the positions of S_{AB} 0·80.

TABLE 6 - Median of minimum inhibitory concentration (MIC), and MIC ranges of amphotericin B (AMB), fluconazole (FCL), ketoconaloze (KTC) and itraconazole (ITC) 80 sequential *Candida albicans* isolates, tested by EUCAST procedure.

ANTIFUNGAL	**VISITS**		***p*** value
	1	2	
	Median (range)	Median (range)	
FLC	0.25 [0.125 – 1.0]	0.25 [0.125 – 0.5]	$p > 0.05$
IITC	0.02 [0.015 – 0.06]	0.02 [0.015 – 0.06]	$p > 0.05$
KTC	0.02 [0.015 – 0.06]	0.02 [0.015 – 0.03]	$p > 0.05$
AMB	0.25 [0.03 – 0.5]	0.25 [0.125 – 0.5]	$p > 0.05$

Wilcoxon test

TABLE 7 - Median of minimum inhibitory concentration (MIC), and MIC ranges of amphotericin B (AMB), fluconazole (FCL), ketoconaloze (KTC) and itraconazole (ITC) 51 sequential *Candida albicans* isolates, tested by EUCAST procedure.

ANTIFUNGAL	**VISITS**			***p*** value
	1	2	3	
	Median [range]	Median [range]	Median [range]	
FLC	0.25 [0.25 – 0.5]	0.25 [0.125 – 0.5]	0.25 [0.125 – 0.5]	$p > 0.05$
IITC	0.03 [0.015 – 0.06]	0.0 [0.015 – 0.03]	0.02 [0.015 – 0.06]	$p > 0.05$
KTC	0.02 [0.015]	0.02 [0.015]	0.02 [0.015]	$p > 0.05$
AMB	0.25 [0.06 – 0.5]	0.25 [0.12 – 0.5]	0.25 [0.12 – 0.25]	$p > 0.05$

Friedmann test, followed by Dunn test

TABLE 8 - Median of minimum inhibitory concentration (MIC), and MIC ranges of amphotericin B (AMB), fluconazole (FCL), ketoconaloze (KTC) and itraconazole (ITC) 16 sequential *Candida albicans* isolates, tested by EUCAST procedure.

Visits	**Antifungals (Median [range])**			***P*** value
	ITC	KTC	AMB	
1	0.02 [00.15 – 0.06]b	0.02 [0.015]c	0.25 [0.25] a	<0.001
2	0.02 [00.15 – 0.06]b	0.02 [0.015]c	0.25 [0.12 – 0.5]a	<0.001
3	0.02 [00.15 – 0.06]b	0.02 [0.015]b	0.25 [0.25]a	<0.001
4	0.03 [00.15 – 0.06]b	0.02 [0.015]b	0.25 [0.25 –1.0]a	<0.001

Kruskall-Wallis test, followed by Dunn test.
Small letters compare medians in the same line.

Capítulo III - Conclusões

O presente projeto de pesquisa, apresentado sob a forma de quatro trabalhos, que avaliou 318 amostras de *Candida* spp. isoladas da cavidade oral de 214 indivíduos infectados pelo vírus da imunodeficiência humana (HIV), aids e não-aids, da Região de Botucatu, permitiu concluir o que se segue:

- *C. albicans, C. parapsilosis, C. tropicalis, C. glabrata* e *C. krusei* foram as espécies identificadas por métodos fenotípicos.
- A prevalência de espécies do complexo *C. psilosis* foi de 4.7% em 318 amostras de *Candida* spp., assim distribuídos : 2,2% *C. parapsilosis* e 2,5% *C. metapsilosis.* Todas as amostras do complexo *C. psilosis* foram sensíveis aos antifúngicos fluconazol, itraconazol, cetoconazol, voriconazol, caspofungina e anfotericina B. As amostras de *C. parapsilosis* apresentaram maiores médias de concentração inibitória mínima do que as de *C. metapsilosis.*
- A prevalência de *C. dubliniensis* foi de 0,9% em relação às 318 amostras e de 1,1% em relação às amostras de *C. albicans.* As amostras de *C. dubliniensis* foram sensíveis aos antifúngicos fluconazol, itraconazol, cetoconazol e anfotericina B.
- As prevalências das espécies de *Candida* spp. em 300 amostras foram: *C. albicans* 89,0%, *C. glabrata* 6,0%, *C. tropicalis* 4,3% e *C. krusei* 0,7%. As prevalências das espécies resistentes e sensíveis dose-dependente ao fluconazol foram: *C. albicans* 0,75%, *C. glabrata* 50,0%, *C. tropicalis* 0,0% e *C. krusei* 100,0% [*C.glabrata*>(*C. albicans*=*C. tropicalis*)]; para o cetoconazol: *C. albicans* 0,75%, *C.*

tropicalis 0,0%, *C. glabrata* 0,0% e *C. kruse* 100,0% [*C. albicans*=*C. glabrata*= *C. tropicalis*]; para o itraconazol: *C. albicans* 1,9%, *C. glabrata* 72,2%, *C. tropicalis* 0,0% e *C. krusei* 50,0% [maior incidência de resistência em *C. glabrata*]; finalmente, todas as amostras foram sensíveis à anfotericina B. A resistência cruzada para três azólicos foi observada em duas amostras de *C. albicans* e para dois azólicos, em nove amostras de *C. glabrata.*

- A análise das amostras de *Candida* spp. coletadas em episódios seqüenciais de colonização e infecção da cavidade oral revelou que *C. albicans* continua sendo a espécie prevalente. A análise dos genótipos de amostras de *C. albicans* coletadas em episódios seqüenciais revelou alta similaridade entre eles. No entanto, maior grau de similaridade foi observado em amostras isoladas de colonização do que de infecção da cavidade oral. Todas as amostras de *C. albicans* foram sensíveis aos antifúngicos fluconazol, itracanozol, cetoconazol e anfotericina B.

Resumo

A candidíase oral continua sendo a principal doença oportunística associada à infecção pelo vírus da imunodeficiência humana (HIV) e importante marcador da progressão da doença, com o aumento da imunodepressão. Os objetivos deste trabalho foram: Caracterizar por métodos fenotípicos as amostras de *Candida* spp. isoladas da cavidade bucal de indivíduos infectados pelo HIV; identificar a prevalência das espécies isoladas; identificar as espécies do complexo *C. psilosis* pelo método da reação em cadeia da polimerase (PCR) e análise do perfil genético gerado pela enzima de restrição "*Ban*I" pela técnica de restrição de fragmentos de DNA polimórfico (RFLP); identificar as amostras de *C. dubliniensis* pelo método de PCR e determinar sua prevalência; analisar a similaridade genética das amostras de *C. albicans* coletadas em episódios sequenciais de colonização e infecção, pelo método de amplificação aleatória de DNA polimórfico (RAPD); determinar o perfil de sensibilidade das amostras isoladas aos antifúngicos fluconazol (FLC), cetoconazol (CTC), itraconazol (ITC) e anfotericina B (AMB), utilizando-se o método EUCAST. Foram avaliadas 318 amostras isoladas de indivíduos infectados pelo HIV, atendidos no Ambulatório Especial de Moléstias Infecciosas e Parasitárias ou na Enfermaria de Doenças Tropicais da Faculdade de Medicina de Botucatu – UNESP. O material da cavidade bucal foi colhido por meio de *swabs* estéreis, e semeado em Sabouraud Dextrose. A identificação foi realizada pelo CHROMagar ®TM *Candida* e pelo sistema Api 20C AUX bioMeriaux (St. Louis, Mo). *C. albicans, C. parapsilosis, C.metapsilosis, C. tropicalis, C. glabrata*, *C. dubliniensis* e *C. krusei* foram as espécies identificadas. A prevalência do complexo *C. psilosis* foi de 4.7%, distribuídos em 2,2% de *C. parapsilosis* e 2,5% de *C. metapsilosis;* todas as amostras do complexo *C. psilosis* foram sensíveis aos antifúngicos FLC, CTC, ITC e AMB. As amostras de *C.*

parapsilosis apresentaram maiores medianas de concentração inibitória mínima (CIM) do que as de *C. metapsilosis. A* prevalência de *C. dubliniensis* foi de 0,9% em relação às 318 amostras e de 1,1% em relação às amostras de *C. albicans;* todas sensíveis ao FLC, CTC, ITC e AMB. As prevalências das demais espécies foram: *C. albicans* 89,0%, *C. glabrata* 6,0%, *C. tropicalis* 4,3% e *C. krusei* 0,7%. A prevalência de espécies resistentes e sensíveis dose-dependente, distribuídas segundo antifúngico, foram as seguintes: a) ao FCL: *C. albicans* 0,75%, *C. glabrata* 50,0%, *C. tropicalis* 0,0% e *C. krusei* 100,0% [*C.glabrata*>(*C. albicans*=*C. tropicalis*)]; b) ao CTC: *C. albicans* 0,75%, *C. tropicalis* 0,0%, *C. glabrata* 0,0% e *C. kruse* 100,0% [*C. albicans*=*C. glabrata*= *C. tropicalis*]; c) ao ITC: *C. albicans* 1,9%, *C. glabrata* 72,2%, *C. tropicalis* 0,0% e *C. krusei* 50,0% [maior incidência de resistência em *C. glabrata*]; d) à AMB: todas as amostras foram sensíveis. A resistência cruzada para três azólicos foi observada em duas amostras de *C. albicans*, e para dois azólicos, em nove amostras de *C. glabrata.* A análise dos genótipos de amostras de *C. albicans* coletadas em episódios seqüenciais revelou elevado grau de similaridade entre eles, que foi maior entre as isoladas de colonização do que em infecção da cavidade oral. Todas as amostras foram sensíveis aos antifúngicos FLC, CTC, ITC e AMB. Os achados do presente estudo permitem concluir que *C. albicans* é a espécie mais prevalente na cavidade bucal de indivíduos infectados pelo HIV da Região de Botucatu; que as amostras coletadas em episódios seqüenciais apresentaram grande similaridade genética, principalmente entre as isoladas de colonização; que a grande maioria das amostras revelou-se sensível aos antifúngicos estudados; e que pequena incidência de resistência, sensibilidade dose-dependente e resistência cruzada aos antifúngicos FLC, CTC e ITC foi detectada, em especial em relação à *C. glabrata.*