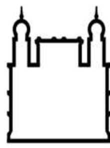

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CARLOS ROBERTO SOBRINHO DO NASCIMENTO

Estudo da estrutura populacional, virulência e métodos de preservação em cepas mistas de *Cryptococcus neoformans* e *Cryptococcus gattii* de origem ambiental e clínica

Rio de Janeiro
2018

CARLOS ROBERTO SOBRINHO DO NASCIMENTO

ESTUDO DA ESTRUTURA POPULACIONAL, VIRULÊNCIA E MÉTODOS DE PRESERVAÇÃO EM CEPAS MISTAS DE *Cryptococcus neoformans* E *Cryptococcus gattii* DE ORIGEM AMBIENTAL E CLÍNICA

Tese de doutorado apresentada ao programa de Pós-Graduação Strictu-Sensu do Instituto Nacional de Infectologia Evandro Chagas para obtenção do grau de Doutor em Ciências
Orientadores: Dra. Luciana Trilles e Dra. Marcia dos Santos Lazéra

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"Cada um de nós compõe a sua história, cada ser em si

Carrega o dom de ser capaz, e ser feliz"

Renato Teixeira e Almir Sater

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RESUMO

Cryptococcus neoformans e *Cryptococcus gattii* são os agentes etiológicos da criptococose, micose de natureza sistêmica que se destaca entre as infecções fúngicas humanas de maior letalidade, principalmente sob a forma de meningoencefalite, atingindo tanto indivíduos imunocomprometidos como imunocompetentes. Na maioria dos relatos sobre os agentes etiológicos da criptococose estes têm sido estudados a partir de uma única colônia isolada. Este trabalho avaliou a estrutura genética populacional e a virulência de cepas de origem ambiental e clínica, selecionadas a partir dos registros de cepas isoladas com fenótipos atípicos sugestivos de cepas mistas de *C. neoformans* e *C. gattii* depositadas e preservadas em coleções de culturas. Para as análises iniciais, 50 colônias puras de uma cepa ambiental e também de uma cepa clínica com fenótipos atípicos foram isoladas para realização dos testes fenotípicos, genotípicos e de virulência. Na população de origem ambiental observou-se a presença de ambas espécies, *C. neoformans* (VNI) e *C. gattii* (VGII) caracterizando cepa mista, todas as colônias foram haploides, *mating-type* α e colônia albina foi isolada e a mesma foi não virulenta para o modelo *in vivo* utilizado (*Galleria mellonella*). Inoculação simultânea de uma colônia de *C. neoformans* (VNI) e uma colônia de *C. gattii* (VGII) proveniente da cepa ambiental realizado em *G. mellonella* não revelou cruzamento interespécies. Já para as colônias isoladas da população clínica, apenas *C. neoformans* (VNI) foi identificado e todas as colônias foram *mating-type* α, no entanto, células haploides e diploides foram observadas, o que pode se relacionar com instabilidade fenotípica observada nesta população. Importante heterogeneidade fenotípica foi observada em relação aos fatores de virulência para ambas populações, como produção de melanina, espessura de cápsula e termotolerância. Para o sucesso de estudos futuros, importância deve ser dada aos métodos de preservação a fim de garantir a viabilidade de ambas as espécies em cepas mistas e/ou híbridas. Este estudo apontou a liofilização como melhor método para manutenção mútua de ambas as espécies conjuntamente.

Palavras chave: *Cryptococcus*, criptococose, virulência, coexistência, coinfecção, preservação

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ABSTRACT

Cryptococcus neoformans and *Cryptococcus gattii* are the etiological agents of cryptococcosis, systemic mycosis that stands out among the most lethal human fungal infections, mainly in the form of meningoencephalitis, reaching both immunocompromised and immunocompetent individuals. In the majority of reports of the etiological agents of cryptococcosis these have been studied from unique isolated colony. This work evaluated the genetical population structure and virulence of strains of environmental and clinical origin that were selected from the records of isolated strains with atypical phenotypes suggestive of mixed strains of *C. neoformans* and *C. gattii* deposited and preserved in culture collections. For the initial analyzes 50 pure colonies of an atypical phenotype environmental strain and also of a clinical strain were isolated for phenotypic, genotypic and virulence tests. In the population of environmental origin, the presence of both *C. neoformans* (VNI) and *C. gattii* (VGII) were characterized by mixed species, all colonies were haploid, mating-type α, and albino colony was isolated and the same was non-virulent for the *in vivo* model used (*Galleria mellonella*). Simultaneous inoculation of a colony of *C. neoformans* (VNI) and a colony of *C. gattii* (VGII) from the environmental strain carried out in *G. mellonella* did not reveal inter-species reproduction. For isolate colonies from clinical population only *C. neoformans* (VNI) was identified and all colonies were mating-type α, however haploid and diploid cells were observed, which may be related to phenotypic instability observed in this population. Significant phenotypic heterogeneity was observed in relation to virulence factors for both populations, such as melanin production, capsule thickness and thermotolerance. For the success of future studies, importance should be given to preservation methods to ensure the viability of both species in mixed and/or hybrid strains. This study pointed to lyophilization as best method for mutual maintenance of both species together.

Key words: *Cryptococcus*, cryptococcosis, virulence, coexisting, coinfection, preservation

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LISTA DE ABREVIATURAS

5FC – 5-fluorocitosina
aids – Síndrome da Imunodeficiência Adquirida (*Acquired immunodeficiency syndrome)*
AMB – anfotericina B
BLAST – Ferramenta básica de busca de alinhamentos locais (*Basic local Allignment Search Tool)*
bp – Pares de Base (*Base Pair)*
CAP59 – cápsula de polissacárido
CFP – Coleção de Fungos Patogênicos, INI, Fiocruz
CFRVS – Coleção de Fungos de Referência em Vigilância Sanitária, INCQS, Fiocruz
CGB – canavanina-glicina-azul de bromotimol
CIM – concentração mínima inibitória
CLSI – *Clinical Laboratory Standards Institute*
DNA – Ácido Desoxirribonucleico (*Deoxyribonucleic Acid)*
ECV - valor de corte epidemiológico (*Epidemiological* Cutoff *Values*)
FCZ – fluconazol
GPD1 – glicerol-3-fosfato desidrogenase
HIV – Vírus da Imunodeficiência Humana (*Human Imunodeficiency Virus)*
ICZ – itraconazol
IGS1 – gene espaçador da região intergênica
Kb – Kilobase
LAC1 – lacase
LCR – líquido cefalorraquidiano
L-DOPA – 3,4-dihidroxi-L-fenilalanina
MALDI – Ionização e dessorção a laser assistida por matriz *(Matrix-assisted laser desorption/ionization)*
MS – Ministério da Saúde
NSA – agar semente de niger (*niger seed agar*)
PBS – Tampão Fosfato Salina
PCR – Reação em Cadeia da Polimerase (*Polymerase Chain Reaction)*

PCZ – posaconazol

PLB1 – fosfolipase B1

SD2 - Sabouraud dextrose 2%

SNC – Sistema Nervoso Central

SOD1 – superóxido dismutase

TSA – testes de sensibilidade aos antifúngicos

UFC – Unidade Formadora de Colônias

URA5 – pirofosforilase monofosfato de orotidina

VCZ – voriconazol

YMA – agar extrato malte-levedura

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INTRODUÇÃO

1. INTRODUÇÃO

A criptococose representa importante problema de saúde pública no Brasil. É doença geradora de problema social grave devido a sequelas incapacitantes, como a perda ou redução da visão e hidrocefalia (OLIVEIRA-NETTO et al., 1993; ROZENBAUM et al., 1992). A falta de conhecimento do espectro clínico desta micose, tanto por médicos como por equipes de análises clínicas, gera dificuldades laboratoriais que levam a equívocos de diagnóstico ou diagnóstico tardio. Por outro lado, a criptococose não é doença de notificação compulsória, o que implica no desconhecimento da magnitude do problema.

A identificação de coinfecção ou híbridos de *C. neoformans* e *C. gattii* em um mesmo paciente tem sido observada e descrita no Brasil, desde que mais de uma colônia de uma mesma amostra sejam analisadas, o que não é rotina nos laboratórios de diagnóstico (Márcia Lazéra, comunicação pessoal), podendo representar uma frequência mais comum do que se supõe. Além das coinfecções de *C. neoformans* e *C. gattii*, outra importante observação é a coexistência das duas espécies em uma mesma amostra ambiental, principalmente em madeira em decomposição associada a cavidades em árvores (BARBOSA et al., 2013; LAZERA et al., 2000; TRILLES et al., 2003).

Estes achados ocasionais, detectados quando grande número de colônias de *Cryptococcus* sp. são analisados, sugerem que mecanismos de interação entre populações destes dois agentes podem ocorrer, desde processos de hibridização e heterocariose, até ciclo sexuado com produção de basidiósporos e geração de propágulos potencialmente infectantes e virulentos.

Neste contexto, o estudo destas cepas de origem clínica e ambiental isoladamente e conjuntamente permitirá avaliar se tal interação leva a uma maior virulência, o que apontará para um cuidado maior tanto no diagnóstico quanto no tratamento da criptococose em casos de coinfecção.

1.1. *CRYPTOCOCCUS NEOFORMANS* E *CRYPTOCOCCUS GATTII*

Cryptococcus neoformans é sapróbio de diversos substratos orgânicos, frequentemente associado ao habitat de aves, rico em fontes de nitrogênio como ureia e creatinina. Condições favoráveis ao crescimento abundante deste agente levam à formação de microfocos ambientais, notadamente em centros urbanos onde prevalecem aqueles relacionados a pombos. Porém, habitat de outras espécies de aves também são encontrados contaminados, sobretudo de aves gregárias e em cativeiro. O ambiente domiciliar, seja em área urbana ou rural, pode estar contaminado com este fungo, por diversas vezes encontrado na poeira doméstica, fator com implicação direta na patogênese da doença (ALVES et al., 2016; BRITO-SANTOS et al., 2015; PASSONI et al., 1998). Além disso, os estudos de Lazéra et al. (1998; 2000) verificaram a presença de *C. neoformans* no oco de madeira em decomposição de árvores vivas, sugerindo um novo habitat natural e um possível nicho ecológico primário para o fungo.

O habitat natural de *C. gattii* foi inicialmente associado a restos vegetais de *Eucalyptus camaldulensis* na Austrália (ELLIS; PFEIFFER, 1990), seguindo-se outros achados em eucaliptos: no Parque do Ibirapuera na cidade de São Paulo (SP) e em plantação experimental da Embrapa em Teresina (PI) (MONTENEGRO; PAULA, 2000; NISHIKAWA et al., 2003). No entanto, este conceito se ampliou e ficou evidente que esta espécie está relacionada também a árvores tropicais de diferentes gêneros no Brasil (cássia, oiti, ficus, mulungu, *Guettarda acreana*) (FORTES et al., 2001; LAZERA et al., 2000; LAZÉRA et al., 1998) e na Colômbia (algodoeiro-da-praia) (CALLEJAS et al., 1998). No Canadá, *C. gattii* foi encontrado em árvores nativas como elmo, cedro, espécies de pinheiros e carvalho, mas não em eucaliptos (KIDD et al., 2004). Portanto, não há habitat ou associação específica de *C. gattii* com eucaliptos, mas sim diferentes padrões geográficos de ocorrência do fungo em madeira em decomposição (LAZERA et al., 2000; NISHIKAWA et al., 2003; TRILLES et al., 2003).

A descoberta de ciclos sexuados distintos, com diferente forma de basidiósporos, levaram ao reconhecimento de duas espécies separadas, *C. neoformans* e *C. gattii*. A taxonomia de *C. neoformans* e *C. gattii* sofreu mudanças importantes devido ao rápido desenvolvimento de técnicas de biologia molecular, que mostraram que estas espécies são, na realidade, dois "complexos de espécies". O complexo de espécies *Cryptococcus neoformans* inclui duas variedades ou duas espécies de acordo com a nova taxonomia proposta: *C. neoformans* var. *grubii* identificado pelos tipos moleculares VNI, VNII e VNB, *C. neoformans* var.

neoformans identificado pelo tipo molecular VNIV, além de híbridos inter-varietais (diplóides ou aneuplóides) identificados pelo tipo molecular VNIII. O complexo de espécies *C. gattii* inclui quatro tipos moleculares VGI, VGII, VGIII e VGIV. Verifica-se também a complexidade dos agentes da criptococose pois as duas espécies têm diversidade intraespecifica, bem como híbridos intra e interespécies (MEYER et al., 2003, 2009, KWON-CHUNG et al., 2017).

Várias técnicas moleculares têm sido propostas para realizar a diferenciação genética intraespecífica das espécies *C. neoformans* e *C. gattii*. Destacando-se PCR *fingerprinting* do minissatélite do fago M13; polimorfismo no comprimento de fragmento de restrição (RFLP, do inglês, *Restriction Fragment Length Polymorphism*); polimorfismo de tamanho de fragmento amplificado (AFLP, do inglês *Amplified Fragment Length Polymorphism*) (HAGEN et al., 2010); e tipagem por sequenciamento por multilocus (MLST, do inglês *Multilocus Sequence Typing*) (MEYER et al., 2003, 2009).

A técnica de RFLP tem como princípio a amplificação do DNA por reação de PCR seguida de digestão dupla com as endonucleases para o corte das regiões alvo do DNA. Os cortes realizados durante a incubação com as enzimas de restrição dão origem a fragmentos de DNA de tamanhos diferentes para cada genótipo. O perfil obtido é comparado visualmente ao perfil de cepas padrão para determinação do genótipo de cada isolado estudado. O gene orotidina-monofosfato pirofosforilase (URA5), é o gene alvo da técnica de RFLP para genotipagem de *C.neoformans* e *C.gattii* (MEYER et al., 2003).

Com base em regiões variáveis de sete loci genéticos independentes: cápsula de polissacárido (*CAP59*), glicerol-3-fosfato desidrogenase (*GPD1*), lacase (*LAC1*), fosfolipase B1 (*PLB1*), superóxido dismutase (*SOD1*), orotidina-monofosfato pirofosforilase (*URA5*) e o gene espaçador da região intergênica (*IGS1*), aproveitando o alto poder discriminatório e boa reprodutibilidade entre diferentes laboratórios, em 2009, a comunidade científica adotou o MLST, como o método de tipagem preferido, com o objetivo de estabelecer uma tipagem padronizada globalmente. O alelo (AT) e tipos de sequência (ST) podem ser determinados através do banco de dados disponível em http://mlst.mycologylab.org/ (MEYER et al., 2009).

Uma outra classificação importante para o entendimento deste trabalho é a classificação baseada nos cinco sorotipos capsulares. A classificação do sorotipo é

baseada nas reações de aglutinação dos antígenos polissacarídeos capsulares, onde VNI, VNII e VNB são sorotipo A, VNIII sorotipo AD, VNIV sorotipo D e, para *C. gattii*, os tipos moleculares denominados VGI, VGII, VGIII e VGIV podem ser sorotipos B ou C, não sendo observada correlação entre os sorotipos e os tipos moleculares (LITVINTSEVA, 2005; MEYER et al., 2003).

O emprego de técnicas de biologia molecular tem auxiliado a entender melhor a patogenicidade, a epidemiologia, o modo de transmissão e o tratamento das infecções fúngicas e seus agentes, pois fornecem informações sobre a distribuição e o grau de relacionamento dos isolados dentro de uma população, permitindo diferenciar entre recidiva e reinfecção, bem como detectar surtos. A detecção de variação genética entre os isolados de um determinado patógeno é importante porque esta pode traduzir-se em variações fenotípicas, tais como diferenças na composição antigênica, na virulência, na sensibilidade aos agentes terapêuticos, entre outros (PFALLER, 1995; POLACHECK; LEBENS; HICKS, 1992). O conhecimento da extensão da variabilidade genotípica entre isolados de um micro-organismo é essencial para a definição de sua estrutura populacional.

1.2. *MATING-TYPE*

O ciclo reprodutivo de *C. neoformans* pode ocorrer de forma sexuada ou assexuada. *C. neoformans* e *C. gattii* tiveram seus ciclos sexuais observados apenas em laboratório, e descritos há mais de quatro décadas, sendo tipicamente haploides com sistema heterotálico bipolar, contendo "tipos sexuais" (do inglês *mating types)* a e α (*MATa* e *MATα)*. O locus *mating type* (*MAT*) é uma região do genoma fúngico que regula o ciclo sexual e codifica para a forma alélica a ou α (BOVERS et al., 2008a) . O *mating* ocorre quando células haploides a e α se unem, assim, inicia-se uma transição dimórfica com o desenvolvimento de uma forma filamentosa que cresce como uma hifa dicarótica a-α. A hifa aérea pode formar um basídio e depois sofrer meiose dando origem a células haploides, os basidiósporos. *Cryptococcus* spp. podem também sofrer reprodução monocariótica, quando células do mesmo *mating* sofrem diploidização, ocorrendo meiose e formação de basidiósporos (LIN, 2009).

Existem evidências de que *C. neoformans* pode sofrer cruzamento entre os tipos sexuais do mesmo sexo na natureza (α-α). No entanto, uma limitação é que os híbridos αADα naturais têm uma capacidade prejudicada de realizar meiose e não produzir progênie haplóide, impedindo uma avaliação adicional com esses isolados no impacto deste estilo de vida na estrutura populacional haplóide e evolução do complexo de espécies *C. neoformans* (LIN et al., 2007).

O ciclo assexuado dessa levedura ocorre tanto em células MATα como nas células MATa, no entanto, na natureza, a maior prevalência ocorre em células MATα através da formação de corpos haplóides de frutificação em resposta à ausência de nitrogênio e à ausência de células MATa (TSCHARKE et al., 2003).

A ocorrência de cada *mating type MATα* e *MATa* em isolados clínicos e ambientais de *C. neoformans* diferem significativamente pois, o tipo *MATα* é 30 a 40 vezes mais frequente que o tipo *MATa.* Kwon-Chung et al. (1992), demonstraram que a progênie do tipo *MATα* é mais virulenta que a do tipo *MATa*, sugerindo que o tipo *MATα* apresenta vantagem seletiva na sobrevivência ambiental. A identificação dessas diferenças provavelmente proverá conhecimento de como *Cryptococcus* responde a vários tipos de estresses ambientais e como este causa doença em humanos e animais (NIELSEN; KWON-CHUNG, 2011).

1.3. HÍBRIDOS

Diversos métodos utilizados para a genotipagem de *C. gattii* e *C. neoformans* sugerem estrutura populacional predominantemente clonal, mas existem também evidências de recombinação ou hibridação entre as variedades de *C. neoformans* e também entre as espécies *C. neoformans* e *C. gattii*. A ocorrência de hibridação tem consequência na reprodução biológica das espécies, como um genótipo "novo" com a virulência alterada, sobrevivência devido à mudança ambiental, ou a sensibilidade a drogas antifúngicas e a substâncias xenobióticas (AMINNEJAD et al., 2012; BOEKHOUT et al., 2001; BOVERS et al., 2008b).

Vários híbridos haplóides e aneuplóides ocorrem no complexo *C. neoformans*-*C. gattii*. Os principais estudos relacionados envolvem o híbrido sorotipo AD, formados após conjugação de células de leveduras de *C. neoformans* var. *neoformans* (sorotipo D) e *C. neoformans* var. *grubii* (sorotipo A) (KWON-CHUNG,

2011). Híbridos BD provenientes de cruzamento entre *C. neoformans* (sorotipo D) e *C. gattii* (sorotipo B) e híbridos AB provenientes de cruzamento entre *C. neoformans* var. *grubii* (sorotipo A) e *C. gattii* (sorotipo B) também foram reportados (BOVERS et al., 2006, 2008b). Pouco se sabe sobre a patogenicidade e virulência relacionada a casos envolvendo híbridos.

Lengeler et al. (2001) demonstraram que alguns isolados de *C. neoformans* AD são aneuploides e que cepas diploides estáveis podem ser isoladas a partir de cruzamentos genéticos definidos. Os isolados ambientais e clínicos do sorotipo AD são aneuploides em vez de diploides verdadeiros e isto ocorre, provavelmente, devido a instabilidades genômicas em *C. neoformans*.

1.4. FATORES DE VIRULÊNCIA

A infecção fúngica resulta geralmente de um encontro acidental do fungo com um hospedeiro durante o seu ciclo de vida dos fungos, pois a grande maioria apresenta um ciclo de vida sapróbio. Entretanto, alguns fungos são capazes de causar doença no homem e animais devido à particular capacidade de sobreviver e multiplicar-se em tais hospedeiros, pela produção dos chamados fatores de virulência (CASADEVALL; PERFECT, 1998).

Avanços na biologia molecular de *Cryptococcus* confirmaram vários fatores de virulência, sendo a termotolerância, formação de cápsula e produção de melanina os três fatores de virulência clássicos e proeminentes (HULL; HEITMAN, 2002; PERFECT, 2005) e também de outros fatores tais como fosfolipase e urease (LEV et al., 2013; SINGH et al., 2013).

Os agentes de criptococose representam um modelo para o estudo de virulência, pois são capazes de crescer à temperatura de 37 °C e essa capacidade está relacionada com múltiplas vias de sinalização e enzimas adquiridas ou adaptadas ao longo do tempo para reter ou melhorar a sua patogenicidade em mamíferos. Possuem cápsula de natureza polissacarídica, que atua na resistência à fagocitose mediada por macrófagos, neutrófilos e monócitos. Produzem melanina, cuja síntese é catalisada pela enzima difenoloxidase, localizada na parede celular do fungo, conferindo à célula um efeito protetor contra reações oxidativas, atuando na defesa contra a radiação e o ataque das células de defesa (BUCHANAN; MURPHY, 1998; HAMILTON; HOLDOM, 1999; MAZIARZ; PERFECT, 2016).

Tanto as espécies *C. neoformans* e *C. gattii* e seus respectivos tipos moleculares principais possuem diferenças fisiológicas, epidemiológicas, moleculares, de susceptibilidade antifúngica e de virulência (NGAMSKULRUNGROJ et al., 2011; PERFECT et al., 2010; TRILLES et al., 2012). Além do mais, infecções causadas por *C. gattii* normalmente apresentam pior prognóstico do que aquelas causadas por *C. neoformans* (SORRELL, 2001).

Estudos dos fatores de virulência são fundamentais para melhor compreensão da patogenicidade dos agentes da criptococose e podem indicar novas estratégias para o controle desta micose sistêmica.

1.5. HETEROGENEIDADE FENOTÍPICA

No contexto da microbiologia, a heterogeneidade fenotípica refere-se ao fenômeno pelo qual células individuais dentro de populações clonais, que têm um fundo genético uniforme, podem mesmo assim exibir diferenças no fenótipo. A prevalência emergente e aparente importância da heterogeneidade fenotípica fúngica fornece um alerta de que certos aspectos, potencialmente centrais da biologia fúngica, ainda permanecem amplamente sub-explorados (HEWITT et al., 2016). A heterogeneidade no nível unicelular é tipicamente mascarada em estudos convencionais de populações microbianas, que se baseiam na média dos dados em milhares ou milhões de células em uma amostra e essa heterogeneidade se manifesta em uma ampla gama de fenótipos, muitos dos quais são fundamentais para a aptidão e o desenvolvimento do organismo. Por exemplo, células individuais de patógenos microbianos exibem graus variáveis de virulência, possuem graus variados de resistência a tratamentos antimicrobianos e outras tensões (AVERY, 2006).

A heterogeneidade não genotípica é uma característica importante para organismos no ambiente natural, contribuindo para a sua competitividade e pode ser o principal determinante da sobrevivência microbiana em condições adversas (HOLLAND et al., 2014). Ainda existem poucos estudos detalhados sobre os fatores responsáveis pela heterogeneidade fenotípica, mas essa heterogeneidade determina o destino das células individuais nas populações isogênicas e provavelmente o destino final das próprias populações. Os mecanismos moleculares que sustentam essa heterogeneidade (por exemplo, que determinam fenótipos

dependentes do ciclo celular ou da taxa de crescimento) na maioria dos casos permanecem por elucidar (SUMNER; AVERY, 2002).

1.6. MODELOS *IN VIVO*

Uma grande quantidade de informação pode ser recolhida a partir de ensaios *in vivo*, tais como os processos biológicos ou genes específicos de *Cryptococcus* spp. com capacidade de causar doença e como o hospedeiro responde à doença (CLEMONS; STEVENS, 2011).

Modelos animais em infecções fúngicas são fundamentais para melhor entender a patogênese da doença, resposta do hospedeiro, prevenção da doença e tratamento. Numerosos modelos *in vivo* têm sido desenvolvidos para investigar estas características da criptococose, sejam estes mamíferos (*Mus musculus, Rattus rattus, Cavia porcellus, Oryctolagus cuniculus*) ou não mamíferos (*Galleria mellonella, Drosophila melanogaster, Caenorhabditis elegans, Danio rerio*). Cada um destes modelos tem um papel a desempenhar para contribuir na compreensão da criptococose e seus agentes (CLEMONS; STEVENS, 2011; DAVIS et al., 2015; SABIITI; MAY; PURSALL, 2012; THOMPSON et al., 2014).

Dentre os modelos de mamíferos, o murino tem sido o mais utilizado nos estudos com *Cryptococcus* spp., especialmete por ser um modelo bem estabelecido e caracterizado em pesquisas médicas, além de existir uma grande variedade genética disponível (BARCHIESI et al., 2005; IROKANULO; AKUESHI, 1995; MUHAMMED et al., 2012; SABIITI; MAY; PURSALL, 2012).

Por outro lado, modelos não mamíferos podem ser excelentes e tem vantagens sobre os seus homólogos de mamíferos, o que inclui redução de custos de manutenção, menos restrições éticas, tempo de reprodução relativamente curto, e grande tamanho de ninhada (SABIITI; MAY; PURSALL, 2012).

Galleria mellonella tem sido usada como um sistema modelo por diversos motivos, dentre eles o fato de poder ser mantida na faixa entre 25ºC a 37ºC, poder ser armazenada em placas de Petri, o que permite o uso de estufas após inoculação e também a mortalidade da larva pode ser monitorada para determinação da patogenicidade do fungo em curto espaço de tempo (BINDER; MAURER; LASS-FLÖRL, 2016; FIRACATIVE; DUAN; MEYER, 2014; FUCHS et al., 2010; GARCÍA-RODAS et al., 2011; MYLONAKIS et al., 2005).

1.7. CRIPTOCOCOSE

É a micose de natureza sistêmica causada por *C. neoformans* e/ou *C. gattii*, adquirida por inalação de leveduras desidratadas e/ou basidiósporos, provenientes de fontes ambientais. Propágulos dispersados pelo ar, ao atingir os bronquíolos terminais e alvéolos pulmonares, iniciam a infecção sob a forma de leveduras capsuladas no pulmão do hospedeiro (KWON-CHUNG; BENNETT, 1992).

A criptococose coloca-se entre as infecções fúngicas humanas de maior letalidade, principalmente sob a forma de meningoencefalite. Apresenta-se como duas entidades distintas do ponto de vista clínico e epidemiológico: criptococose oportunista, cosmopolita, associada a condições de imunocomprometimento celular, causada predominantemente por *C. neoformans*, e criptococose primária, atingindo indivíduos imunocompetentes, causada predominantemente por *C. gattii* (CASADEVALL; PERFECT, 1998). Embora a criptococose seja descrita em pacientes de todas as idades, ela é mais frequente em adultos jovens e pessoas de meia idade do sexo masculino (ESPINEL-INGROFF; KIDD, 2015; FANG; FA; LIAO, 2015; ROZENBAUM; GONÇALVES, 1994; SMITH et al., 2015).

A criptococose causada por *C. neoformans* é de ocorrência cosmopolita, marcadora de imunodepressão, sendo a infecção pelo vírus da imunodeficiência humana (HIV) a condição predisponente mais comum para o desenvolvimento da doença. Outros fatores ou condições predisponentes do hospedeiro, tais como: uso de corticóides, lupus eritematoso sistêmico e outras doenças autoimunes, diabetes, tuberculose, alcoolismo, gravidez, transplante de órgãos sólidos, linfomas, câncer, sarcoidose e uso de drogas imunossupressoras utilizadas nestas condições, entre outros, são também condições predisponentes para o desenvolvimento da criptococose por *C. neoformans* (FRIES; COX, 2011).

Acreditava-se que a criptococose causada por *C. gattii* fosse restrita a regiões tropicais e subtropicais, ocorrendo na América Latina (México, Colômbia, Venezuela, Brasil, Peru, Argentina), sul dos Estados Unidos, países da África Central, Austrália, Nova Guiné e sudeste da Ásia (Índia, Tailândia, Camboja) (KWON-CHUNG; BENNETT, 1992). Porém, a única epidemia humana e animal de criptococose descrita na literatura teve início em 1999, na Ilha de Vancouver, no Canadá. Até 2006, foram diagnosticados mais de 100 casos humanos, com 6 óbitos, e também um grande

número de cães, gatos, furões, lhamas e cetáceos infectados (LINDBERG et al., 2007; STEPHEN et al., 2002). Em sua grande maioria a infecção ocorreu em hospedeiros sem imunodepressão evidente, sendo *C. gattii* VGII o agente em todos os casos. A ocorrência de tal epidemia numa região temperada indicou a ocorrência de uma mudança na distribuição geográfica de *C. gattii* (KIDD et al., 2004).

De modo geral, aproximadamente 95% dos casos de criptococose são causados por cepas de *C. neoformans* (sorotipo A) e os demais 4% a 5% das infecções são causados por *C. neoformans* (sorotipo D) ou *C. gattii* (sorotipos B/C) (MAZIARZ; PERFECT, 2016).

Ambas as espécies causam meningoencefalite, de evolução grave e fatal, acompanhada ou não, de lesão pulmonar evidente, fungemia e focos secundários para pele, ossos, rins, suprarrenal, entre outros. A letalidade da criptococose é estimada em 10% nos países desenvolvidos, chegando a 43% nos países em desenvolvimento como a Tailândia e 70% na África (KON et al., 2008; MOLLOY et al., 2018).

Globalmente, a meningite criptocócica foi responsável por 15% das mortes relacionadas à síndrome da imunodeficiência adquirida (aids). Estudo realizado com pessoas com contagem de células CD4 inferior a 100 células por µL demonstrou que a prevalência média global de antígeno criptocócico nesta população é de 6% e, globalmente, havia 278.000 pessoas com antigenemia criptocócica e 223.100 casos incidentais de meningite criptocócica em 2014. A África Subsaariana foi responsável por 73% dos casos estimados de meningite criptocócica em 2014, sendo que as mortes globais anuais por meningite criptocócica foram estimadas em 181.100. Estima-se que na América Latina chegue a 7.000 o número de pessoas com antigemia criptocócica, a 5.300 os casos de meningite criptocócica e 2.400 o número de mortes por meningite causada por *Cryptococcus.* Neste estudo o número total de pessoas com antigenemia criptocócica foi estimado usando estudos de prevalência de antigenemia criptocócica no sangue de 1989 a 2016 (RAJASINGHAM et al., 2017). Porém todos esses números são provavelmente subestimados, já que a criptococose não é doença de notificação compulsória na América Latina.

A presença conjunta de múltiplas cepas e espécies no ambiente torna provável que várias cepas possam ser inaladas por hospedeiros humanos. Estudos mostraram a presença simultânea de *C. neoformans* e *C. gattii* isolados no mesmo paciente (BOVERS et al., 2006; DESNOS-OLLIVIER et al., 2010; MANDAL et al.,

2005). A escassez de relatos de infecção mista também pode ser devida a um viés técnico. De fato, a prática clínica rotineira de analisar a população infectante dos agentes da criptococose a partir de uma única colônia obtida de um único sítio anatômico torna improvável a descoberta de infecções mistas (DESNOS-OLLIVIER et al., 2010).

1.7.1. Criptococose no Brasil

Duas grandes tendências epidemiológicas foram identificadas no Brasil: *C. gattii* ocorre predominantemente na macrorregião norte, especialmente nos estados do Amazonas, Pará, Roraima, Pernambuco, Piauí e Bahia. Já *C. neoformans* ocorreu predominantemente na macrorregião sul, especialmente nos estados de Mato Grosso do Sul, Minas Gerais, São Paulo, Rio de Janeiro, Paraná e Rio Grande do Sul (TRILLES et al., 2008). Outro dado importante é que dependendo da região, a proporção de infecção por *C. neoformans* e *C. gattii* chega a aproximados 50% (DOS SANTOS et al. 2008).

Dados do Ministério da Saúde (MS) mostram que dos 215.810 casos de aids notificados no Brasil de 1980 a 2002, 6% apresentaram criptococose como doença definidora para o diagnóstico da aids (PAPPALARDO; MELHEM, 2003).

Estudo realizado no Brasil com 3.583 óbitos por micoses sistêmicas (paracoccidioidomicose, criptococose, aspergilose, coccidioidomicose e zigomicoses), no período de 1996 a 2006, apontou que a criptococose é a segunda causa diretamente relacionada ao óbito, representando 24,5% no início do estudo e alcançando 26,8% no final do período, sendo a principal micose (50,9%) associada ao óbito no grupo aids (PRADO et al., 2009).

A criptococose *neoformans* associada à aids predomina nas Regiões Sul, Sudeste e Centro-Oeste do Brasil (IGREJA et al., 2004; ROZENBAUM; GONÇALVES, 1994) sendo relatada em diversos estados como por exemplo Mato Grosso do Sul, Minas Gerais, Pará, Amazonas, Piauí, Maranhão, São Paulo, Rio de Janeiro, Paraná e Rio Grande do Sul (ALMEIDA et al., 2007; ALVES et al., 2016; CASALI et al., 2003; DOS SANTOS et al., 2008; FIGUEIREDO et al., 2016; LEAL et al., 2008; LEIMANN; KOIFMAN, 2008; LINDENBERG et al., 2008; MARTINS et al., 2011; MORA et al., 2010; SEVERO; OLIVEIRA; LONDERO, 1999; SPINA-TENSINI et al., 2017). Estes estudos apontam para uma maior incidência em pacientes do

sexo masculino, portadores do vírus HIV, sendo que no Brasil, mais de 70% dos pacientes com aids vivem nas Regiões Sul e Sudeste do país (BRITO; CASTILHO; SZWARCWALD, 2001). Já *C. gattii* apresentou menor prevalência em pacientes sororeativos para o HIV, ou seja, afeta principalmente hospedeiros imunocompetentes (TRILLES et al., 2008). Diferentemente de outros países, são descritos números significativos de criptococose infantil na Região Norte e Nordeste do Brasil (DOS SANTOS et al. 2008, MARTINS et al., 2011).

1.8. TRATAMENTO E SUSCEPTIBILIDADE AOS ANTIFÚNGICOS

A escolha do esquema terapêutico deve considerar a disponibilidade dos antifúngicos, o sítio anatômico da infecção e o estado imunológico do hospedeiro. As drogas disponíveis para o tratamento da criptococose são: anfotericina B (AMB) (e seus derivados lipossomais), fluconazol (FCZ), itraconazol (ICZ) e 5-flucitosina (5FC), sendo este último de difícil importação no Brasil. A AMB reduziu a mortalidade da criptococose em 30%. Para meningite criptocócica, o consenso internacional preconiza o tratamento com uma divisão em três fases: uma terapia de indução inicial de 2 semanas com regime de AMB fungicida + 5FC, seguido por 8 semanas de terapia de consolidação e, posteriormente, terapia de manutenção com FCZ, continuado por 6-12 meses e/ou até o restabelecimento da imunidade do hospedeiro. O FCZ deve ser iniciado na fase de consolidação, quando o paciente apresentar melhora clínica definida por: melhora do status mental, febre, cefaleia, sinais meníngeos e/ou negativação da cultura do líquido cefalorraquidiano (LCR) na 2ª semana. Para indivíduos com intolerância ao FCZ, o itraconazol (ICZ) é uma alternativa aceitável (KON et al., 2008; PERFECT et al., 2010; SAAG et al., 2000).

Outras drogas como voriconazol (VCZ) e posaconazol (PCZ), azólicos de terceira geração, também têm tido sua eficácia avaliada e com resultados promissores para o tratamento da criptococose (BARCHIESI, 2001; G. FLORES et al., 2012; VAN DUIN et al., 2004; YAO et al., 2015).

Na escolha de um antimicrobiano, a susceptibilidade subjacente de um microrganismo é um dos primeiros fatores a serem considerados. Essa necessidade levou à padronização da metodologia de testes de suscetibilidade antimicrobiana e ao uso do termo “CIM” – concentração inibitória mínima. CIM é a menor concentração de um agente antimicrobiano que impedirá uma quantidade pré-

determinada de crescimento (geralmente uma diminuição de 50 a 100% no crescimento em relação a um controle não tratado) de um organismo testado *in vitro*. O próximo passo na evolução dos testes de determinação da CIM foi o desenvolvimento de pontos de corte (*breakpoints*) da CIM. Um *breakpoint* é usado como um valor preditivo de CIM para determinar se um microrganismo é ou não capaz de responder *in vivo* a um concentração alcançável de um antimicrobiano no local da infecção quando administrado utilizando um esquema de dosagem pré-definido. É difícil e caro gerar o tipo de dados necessários para a determinação de *breakpoints*. No entanto, existe um valor alternativo que, embora não seja preditivo da eficácia *in vivo*, pode ser utilizado para determinar se um isolado possui alta probabilidade de apresentar mutações que confiram resistência antifúngica em relação à sua resposta *in vitro* a um determinado agente antifúngico. Este valor é o valor de corte epidemiológico (ECV) (LOCKHART; GHANNOUM; ALEXANDER, 2017)

A maioria dos testes de sensibilidade aos antifúngicos (TSA) utilizam técnicas de diluição em caldo e dentre os métodos de referência, se destacam os recomendados pelo *Clinical Laboratory Standards Institute* – CLSI dos Estados Unidos da América. O CLSI vem desenvolvendo faixas de CIM de referência para testes de microdiluição de antifúngicos estabelecidos e recém-introduzidos, no entanto *breakpoints* clínicos não estão disponíveis para o complexo de espécies *Cryptococcus neoformans - Cryptococcus gattii*, essas distribuições CIM foram determinadas utilizando cepas de *Cryptococcus* com distribuição multicêntrica e global para estabelecer valores de corte epidemiológicos para *C. neoformans* e *C. gattii* versus AMB, 5FC, FCZ, ICZ, PCZ e VCZ (ESPINEL-INGROFF et al., 2012a, 2012b).

JUSTIFICATIVA

2. JUSTIFICATIVA

O presente trabalho resultou da necessidade de compreender melhor as características fenotípicas e genotípicas de cepas mistas de origem clínica e ambiental dos agentes da criptococose que eventualmente temos a oportunidade de estudar. Tais cepas são difíceis de identificar por seus resultados discordantes nas metodologias de rotina para a caracterização dos agentes da criptococose, portanto necessitam de testes adicionais.

Tal situação deve ser identificada e analisada, pois pode estar relacionada a dificuldades de identificação de espécie, erros de diagnóstico, quadros clínicos mais graves e com resposta terapêutica antifúngica inadequada. Podem ser também fator de confundimento, simulando erro de diagnóstico laboratorial, quando na verdade trata-se de fenótipos instáveis, aspectos fundamentais que precisam ser identificados em uma coleção de cultura. No entanto, pouco tem se avaliado sobre a importância e frequência de infecção mista como agentes da criptococose.

Alguns casos de criptococose por cepas híbridas *C. neoformans*/*C. gattii* foram confirmados (AMINNEJAD et al., 2012), mas acredita-se que tais casos sejam mais comuns do que se pensa, principalmente em regiões onde as duas espécies são endêmicas, como no Norte e Nordeste do Brasil. Dos Santos et al. (2008) verificou que cerca de 10% dos isolados clínicos no estado do Pará apresentaram fenótipos atípicos no meio canavanina-glicina-azul de bromotimol (CGB) e na sorotipagem, sugerindo colônias mistas ou híbridas. No Rio de Janeiro há também relato de infecção mista por *C. neoformans* /*C. gattii* isolados do mesmo paciente, porém de amostras clínicas distintas (IGREJA et al., 2004).

Neste contexto, duas cepas com histórico de coexistência/coinfecção foram selecionadas. A cepa CFRVS 71012 (78MC1) de origem ambiental proveniente de oco de árvore *Guettarda acreana* na Ilha de Maracá no Estado de Roraima (FORTES et al., 2001) foi sorotipada pela CFRVS apresentando-se compatível com os sorotipos A (*C. neoformans*) e B (*C. gattii*) e CGB positivo (*C. gattii*), não conseguindo o isolamento de colônias de *C. neoformans* na ocasião. Já a cepa de origem clínica foi isolada de amostra de urina homem com 47 anos, HIV positivo do Rio de Janeiro, em 1994. No momento da sorotipagem pelo CFRVS apresentou

resultados duvidosos, sorotipos A (*C. neoformans*) e B (*C. gattii*), e o meio canavanina-azul-glicina (CGB) foi positivo (*C. gattii*), mas o isolamento de ambas as espécies não foi possível.

Com isso, a solução encontrada foi isolar e analisar o fenótipo e o genótipo de diferentes colônias da mesma cepa para tentar esclarecer os resultados encontrados e verificar as consequências desses isolados para a virulência e suscetibilidade antifúngica.

Outro importante aspecto está relacionado à preservação destas cepas, visto que a metodologia usada deve ser capaz de manter viável ambas as espécies para estudos futuros, sendo assim quatro metodologias de preservação foram testadas para avaliar a mais adequada na preservação de *C. neoformans* e *C. gatti* concomitantemente.

OBJETIVOS

3. OBJETIVOS GERAIS

Avaliar a estrutura populacional e a virulência de isolados de *C. neoformans* e *C. gattii* de origem clínica e ambiental, onde foram observados a presença de ambas espécies concomitantemente e/ou resultados sorológicos controversos e determinar a estabilidade das características fenotípicas e genotípicas destas populações.

3.1. OBJETIVOS ESPECÍFICOS

1. Caracterizar diferentes colônias presentes nas cepas de *C. neoformans* e *C. gattii* de origem ambiental e clínica por métodos fenotípicos e genotípicos;
2. Identificar o tipo sexuado das colônias isoladas;
3. Avaliar a virulência das colônias isoladas;
4. Avaliar a capacidade de cruzamento *in vivo* de cepas da mesma população;
5. Determinar a estabilidade de colônias isoladas após cruzamento *in vivo*;
6. Avaliar metodologias de preservação capazes de manter as duas espécies conjuntamente preservadas.

CAPÍTULO 1

CAPÍTULO 1

Este capítulo avaliou a heterogeneidade genotípica e fenotípica, bem como os fatores de virulência, em uma população ambiental coexistente de *C. neoformans* e *C. gattii* depositada na Coleção de Fungos de Referência em Vigilância Sanitária (Fiocruz / CFRVS) que apresentou divergências em suas análises preliminares, na ocasião do isolamento.

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Phenotypic heterogeneity in a single coexistent *Cryptococcus neoformans* and *C. gattii* environmental strain.

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ABSTRACT

Cryptococcosis is an infectious disease with a worldwide distribution and a wide variety of clinical presentations caused by encapsulated yeasts *Cryptococcus neoformans* and *C. gattii* acquired by inhalation of dehydrated yeasts and/or basidiospores, from environmental saprobic sources. It has not been uncommon in environmental studies of *C. neoformans* and *C. gattii* reports of coexistence of both species in environmental isolates, mainly in decaying wood associated to cavities in trees. Isolated colonies are selected and in the common sense these isolates have the presence of only one species, which in fact occurs in most cases. However, it has been observed the presence of unstable phenotypes that generate a confounding factor, simulating error by the laboratory analysis. This study aims to evaluate the phenotypic heterogeneity of an environmental isolate where confounding factors

were observed during identification in order to better understand the possibilities of coexistence between *C. neoformans* and *C. gattii* in a unique colony. The study clearly demonstrates important phenotypic variations related to the virulence factors when more than one colony from the same isolate is selected. It contributes to a better understanding about the potential of phenotypic heterogeneity of *C. gattii* in the environment, where populations with high cell-cell variability may be better prepared to deal with environmental stresses, leading to a better adaptation to new environments and ability to cause outbreaks.

Introduction

Cryptococcosis is one of the most lethal human fungal infections, especially in the form of meningoencephalitis. It is presented as two distinct entities from the clinical and epidemiological point of view: opportunistic cryptococcosis, cosmopolitan and associated with immunocompromised conditions, predominantly caused by *C. neoformans* species complex, and primary cryptococcosis, affecting immunocompetent individuals, predominantly caused by *C. gattii* species complex[1].

These agents can be classified into five capsular serotypes and eight main molecular genotypes. Analysis of nucleic acid fragments generated by restriction enzymes produce specific molecular types for *C. neoformans* called VNI and VNII (serotype A), the hybrid VNIII (serotype AD), and VNIV (serotype D). The molecular types of *C. gattii* are denominated VGI, VGII, VGIII and VGIV, with no correlation with the serotypes (B and C)[2,3]. The species complexes differ in their epidemiological and ecological features, clinical presentations and therapeutic outcomes. Previous studies have documented genetic and phenotypic instability among *C. neoformans* and *C. gattii* strains [4–8], including phenotypic heterogeneity, that can be a phenomenon whereby individual cells within clonally derived populations can display differences in phenotype [9]. Nevertheless, most reports has been studied from isolated cultures and little attention is given about the variation that can exists between individual cells [5,10]. Besides, the routine clinical practice characterize one single colony per isolate, hindering the discovery of coexistence.

Thus, this work aims to evaluate the genotypic and phenotypic heterogeneity, including virulence factors, in a coexistent *C. neoformans* and *C. gattii* environmental population deposited in the Collection of Reference Fungi in Sanitary

Surveillance (Fiocruz/CFRVS) that showed disagreements in their preliminary phenotypic characterizations.

Results

The strain CFRVS 71012 (78MC1) was isolated from *Guettarda acreana* tree hollow in Maracá Island, in the State of Roraima, North Brazil [11]. It was serotyped as A and B and the CGB medium revealed *C. gattii* at the time of isolation.

The strain was tested for genotypic stability over 12 months, and the isolated colonies from the strain were tested for melanin production, thermotolerance, survival in *Galleria mellonella* model, capsule and cell size before and after animal inoculation, ploidy, major molecular type by *URA5*-RFLP, molecular subtype by PCR-fingerprinting and multi locus sequence typing (MLST).

The PCR-fingerprinting analysis of the strain over 12 months demonstrated relative molecular stability. In all months, the strain was CGB positive (*C. gattii*) molecular type VGII, but the band profile of the first month present one different band (figure 1).

The initial 50 colonies underwent phenotipic and molecular identification analysis using the CGB media and PCR-fingerprinting with M13 primer. From the 50 dark brown colonies on niger seed agar (NSA) selected for the study, one was identified as *C. neoformans* VNI (78MC1-42), while all the other 49 colonies were identified as *C. gattii* on CGB medium. A white-mucoid colony (78MC1-B) was identified on NSA and this phenoloxidase negative colony was identified as *C. neoformans* on Vitek2 and VGII by PCR-fingerprinting. The *C. gattii* colonies were all VGII. All 50 colonies were identified as mating alpha.

As most of the colonies did not show significant molecular difference using M13 PCR-fingerprinting (data not shown), 25 colonies were selected to undergo the other tests, including the colonies 78MC1-42 (*C. neoformans*) and 78MC1-B (albino). *URA5*-RFLP profiles of 25 colonies confirmed the results obtained by PCR-fingerprinting, 78MC1-42 was VNI and the others, including 78MC1-B, were VGII. Determination of ploidy using flow cytometry analysis showed that all 25 isolates were haploid.

There were no thermotolerance differences among the VGII colonies at 37 °C, as all of them were considered 3+ growth and presented growth at the three different

concentrations inoculated (10^6, 10^5 and 10^4). The isolate VNI grew only in two concentration points (2+). None of the colonies presented growth at 40 °C, but after re-incubation of the plates at 25 °C, a considerable polymorphism was observed. The profiles varied as follows: 5 isolates, including 78MC1-B, showed no growth at any of the inoculated concentration points and were considered negative (-); 9 colonies, including 78MC1-42, presented growth in only one inoculated concentration point (1+); 8 colonies showed growth at two concentration points (2+); and 3 colonies and the positive control CFP 411 presented growth in the three concentration points (3+) (figure 2).

The melanin production showed all colonies with optimal growth at 25 °C and 37 °C at the three inoculated sites on NSA, being considered positive (3+). As expected, on NSA 78MC1-B did not show melanin production (-) in the three points inoculated at 25 °C, and at 37 °C showed slightly beige coloration and was considered slightly positive (1+) (figure 3). The low melanin production of this colony was confirm on L-dopa and cafeic acid media (data not show). At 40 °C, none of the colonies showed growth and this result was maintained after re-incubation of the plates at 25 °C.

The cell size among the *C. gattii* colonies was significantly different ($p < 0.0001$). *C. gattii* mean cell diameter was 7.92 μm with mean values ranging from 5.19 μm (78MC1-B) to 10.51 μm (78MC1-77). *C. neoformans* isolate 78MC-42 had a cell diameter ranging from 4.77 μm to 5.77 and mean 5.29 μm (figure 4-A).

The differences among the capsule thickness of *C. gattii* colonies was also significant ($p < 0.0001$). The mean of measurements varied between 5.97 μm (78MC1-27) and 1.54 μm (78MC1-38) and the mean value was 3.49 μm. The capsule of the isolate 78MC1-B presented mean of 2.09 μm. *C. neoformans* 78MC1-42 presented mean of 2.57 μm in capsule thickness (figure 4-B).

The *in vivo* virulence test was carried out especially to evaluate the pathogenicity of the albino colony, and it was performed with three colonies using the *G. mellonella* model: 01 colony phenoloxidase positive of *C. gattii* (78MC1-21); 01 phenoloxidase negative *C. gattii* (78MC1-B); and *C. neoformans* (78MC1-42). The strain CFP 411 (VGIIa) was used as positive control. Furthermore, to verify if virulence would change after passage in *G. mellonella* model, cell size and capsule thickness were evaluated.

The dead units of the infected animals in the period of 10 days is represented in table 1. The test was interrupted after the emergence of the first adult. Mixtures of two distinct colonies were performed to evaluate possible increase in virulence. The mixed colonies were: 78MC1-21+78MC1-42, 78MC1-42+78MC1-B and 78MC1-21+78MC1-B.

Table 1. Values comparing survival curves and median survival times of *Galleria mellonella* larvae after being infected with CFP411 (VGIIa) and 78MC1 colonies. (-) strains that did not kill any larvae during the time of the experiment, (+) strains that killed at least one larva and were less virulent than control strain CFP411 (p <0.05), (++) strains that were of comparable virulence as CFP411 (p >0.05) and (+++) strains that were more virulent than control strain CFP411 (p <0.05), (ND) not determined, (NA) not applicable.

isolate	number of deaths	median survival (h)	***p*-value**	virulence
78MC1-21	4	ND	0.1475	++
78MC1-42	3	ND	0.0755	++
78MC1-B	0	ND	0.0012	-
78MC1-21+ 78MC1-42	3	ND	0.0963	++
78MC1-42+ 78MC1-B	4	ND	0.2139	++
78MC1-21+ 78MC1-B	5	240	0.2141	++
CFP411	7	216	NA	++

No colony tested was consider hipervirulent (+++). Survival curves and median time of survival of the larvae inoculated with different colonies demonstrated compatible virulence among the VGIIa strain CFP411 and two colonies tested, including the colonies mixtures (p>0.05) (figure 5). The isolate phenoloxidase negative *C. gattii* (78MC1-B) was significantly less virulent than the benchmark VGIIa strain CFP411 (p<0.05). The colony 78MC1 kept the albino phenotype after passage in *G. mellonella*. The mixtures demonstrated a virulence profile compatible with the most virulent colony in the mixture (Table 1).

The colonies 78MC1-42 e 78MC1-B increased the cell size after animal passage ($p<0.05$). 78MC1-B also had the capsule increased after *G. mellonella*, but the capsule thickness of 78MC1-21 decreased ($p<0.05$). There was no difference between cell and capsule size of 78MC1-21 isolate and capsule thickness of 78MC1-42 isolate before and after inoculation (figure 6).

No ploidy change was observed after passage in *G. mellonella*, with the maintenance of the colonies haploidy. For evaluation of a possible conjugation of cells during animal passage, flow cytometry of re-isolated colonies was performed and all the isolates were haploid.

Five colonies of the VGII genotype (78MC1-21, 27, 59, 82 and 78MC1-B) were submitted to MLST and all isolates showed ST 124.

Discussion

In the laboratory routines, one or two microbial colonies are usually isolated, thus strains in smaller quantities may not be detected. The isolation of both *C. gattii* and *C. neoformans* in this population points to the need for care especially in the preservation of primary cultures for a more accurate determination of the real prevalence of mixed cultures, which have been previously reported [5,10].

The stability analysis did not detect the presence of the two species concomitantly, although a distinct band was visualized in the first month. This limitation may have occurred due to the low incidence of the *C. neoformans* species in the preserved material, since of the 50 isolated colonies only one was identified as *C. neoformans*. Although PCR-fingerprinting is able to discriminate individual strains, it was not discriminatory enough for the studied population. Moreover, all *C. gattii* colonies showed the same band profile, indicating no genetic variation. The MLST is a more discriminating methodology than PCR-fingerprinting, but it was performed in only five *C. gattii* colonies. Nevertheless, MLST also demonstrate the same genetic profile (ST124).

The colony 78MC1-B did not present melanin production capacity on NSA, and low melanin production in L-dopa and cafeic acid media (data not show). Its identification as *C. gattii* was confirmed both phenotypically and molecularly. It should be noted that melanin-deficient mutants can occur with some frequency and attention should be given, especially to white strains with mucus production, and it was

essential to demonstrate that isolate is *C. gattii* instead of other yeasts [5,8]. In the present study, the colony called "albino" demonstrated very low capacity of melanin production at 37ºC, indicating the main laccase genes (*LAC1* and *LAC2*) are functional and probably the repression or induction of those genes are deficient. Besides, the gene *LAC1*, which expresses the dominant laccase activity, turned out to be an allele identical to the melanized colonies.

G. mellonella has been successfully used in many virulence studies of *C. neoformans*[12,13] and *C. gattii*[14–16]. In fact, melanization was an important virulence factor in the infection process in the current study, since only the non-melanin-producing 78MC1-B did not induced mortality in the model used. Previous study presented similar results, where the VGII strain that produced the minor amount of melanin did not kill any larvae[16]. Other studies have also shown that, even in other animal models, non-melanized-isolate survived significantly longer than those infected with melanin-producing-isolates[7,8,17]. The albino colony also demonstrated no viability at 40 ºC, smaller cell and one of the smallest capsule thickness before inoculation, melanin production was not observed after animal passage, despite the increase on capsule thickness, suggesting that low melanin production was essential for the avirulent profile.

Analysis of capsule thickness after animal passage had unexpected results. *C. neoformans* (78MC1-42) did not increase capsule thickness and the colony 78MC1-21 decreased capsule thickness after inoculation. Further studies with more strains are necessary to understand better the relation between capsule stimulation *in vitro* and *in vivo* using *G. mellonella* model.

Significant differences, both for cell size and capsule thickness, were detected among different colonies from the same strain, demonstrating a significant intra-population phenotipic diversity. In a recent study with strains of *C. gattii*, VGII mean cell size and capsule thickness were 8.4 µm and 2.6 µm, respectively, and included mostly clinical and veterinary strains from eight countries with only one environmental strain from Brazil[18]. Using the same parameter of the cited study, we observed similar results for our isolated colonies, confirming that the capacity to increase capsule thickness can be an important virulence factor.

C. neoformans and *C. gattii* are the only *Tremallelles* that can grow optimally above 30 °C. Strains of *C. neoformans* have been shown to have optimum and maximum growth temperatures of 32 and 40 °C, respectively. However, within the

various strains and varieties of *C. neoformans* the tolerance for high temperature can actually vary. Thermotolerance (growth at 35–40 °C) is a fundamental physical characteristic for invasive mycoses to produce disease within a mammalian host[19]. In the present study, the high temperature avoid the growth but kept viable cells in most colonies, which grew after reincubation at 25 °C, demonstrating variations on thermotolerance among the different VGII colonies obtained from one strain, and also the importance of reincubation at 25 ° C to detect the thermovariability and to confirm if the strain really lost the viability at 40 °C.

Fungal virulence evolved from selection imposed by environmental predators, and this study clearly demonstrates important phenotypic variations related to the virulence factors when more than one colony from the same isolate is selected. It contributes to a better understanding about the potential of phenotypic heterogeneity of *C. gattii* in the environment, where populations with high cell-cell variability may be better prepared to deal with environmental stresses[9], leading to a better adaptation to new environments and ability to cause outbreaks, as the unexpected emergence of *C. gattii* in Canada, a temperate region. In addition, the isolation of both species *C. gattii-C. neoformans* in the same population corroborates the possibility of a mixed initial infection since the agents can be acquired concomitantly or sequentially[5], and points to a greater care in the observations of identification processes as well as effective preservation methods to maintain the diversity of the initial culture.

Methods

Strain studied

The strain defined for this study was deposited in the Culture Collection of Reference Fungi in Sanitary Surveillance (Fiocruz/ CFRVS), strain CFRVS 71012 from the *Guettarda acreana* tree hollow in Maracá Island, in the State of Roraima, North Brazil [11]. It was serotyped by CFRVS and presented inconsistent results, serotypes A (*C. neoformans*) and B (*C. gattii*). The canavanine-blue-glycine (CGB) agar medium was positive (*C. gattii*), and only *C. gattii* was identified at that time. After 15 years preserved in ultra-freezer at -80 °C, the strain was recovered to evaluate the coexistence of different species and/or different genotypes or phenotypes among the different colonies. The colonies were tested for genotypic stability over 12 months, melanin production, thermotolerance, survival curve in

Galleria mellonella model, capsule and cell size before and after animal inoculation, ploidy, major molecular type by *URA5*-RFLP, molecular subtype by PCR-fingerprinting and multi locus sequence typing (MLST).

Stability

In order to evaluate the stability of this strain over time, one of the tubes of the reactivation protocol containing yeast malt agar (YMA) (Difco – USA) was maintained at 25 °C for 30 days. After that, a fraction of the cell mass was separated for DNA extraction and the other fraction was incubated on YMA at 25 °C for 30 days. This procedure was performed monthly during 12 months.

Colonies selection

From the second YMA tube at 25 °C after two days, a suspension in sterile purified water was prepared using 2 McFarland standard, and serial dilutions were performed. Volumes of 0.1 mL of the 10^{-2}, 10^{-4}, and 10^{-5} dilutions were inoculated onto niger seed (*Guizotia abyssinica*) agar (NSA) plates, which were incubated for five days at 25 °C. After the incubation period, 50 dark brown colonies were cultured on YMA and CGB media.

PCR-fingerprinting

The molecular typing of *Cryptococcus* isolates was performed according to the methodology described by Meyer [20], which is based on random amplification of DNA fragments generated by primer (M13) recognizing specific minisatellite sequences. This methodology was used to observe visually the band pattern and to analyze the molecular stability of the strain over 12 months and among the 50 dark brown colonies selected.

Identification of the molecular type

URA5-RFLP analysis using the enzymes *Hha*I and *Sau*96I was performed to verify the molecular type. URA5-RFLP patterns were assigned visually by comparing them with the patterns obtained from the standard strains WM 148 (VNI/AFLP1), WM 626 (VNII/ AFLP1A), WM 628 (VNIII/AFLP2), WM 629 (VNIV/AFLP3), WM 179 (VGI/AFLP4), WM 178 (VGII/AFLP6), WM 175 (VGIII/AFLP5), and WM 779 (VGIV/AFLP7) [2].

Determination of the mating type by PCR

Two pairs of mating-type specific primers were used, MFal- faU (5' TTCACTGCCATCTTCACCACC 3') and MFalfaL (5' TCTAGGCGATGACACAAAGGG 3') to identify the mating type alpha (MATalpha); and JOHE9787 (5' ACACCGCCTGTTACAATGGAC 3') and JOHE9788 (5' CAGCGTTTGAAGATGGACTTT 3') to identify the mating type a (MATa). The unique fragment corresponding to each mating type was visualized after electrophoresis in agarose gel [21].

Thermotolerance test

A cell suspension was adjusted to the standard 0.5 McFarland scale (1-5x10^6 CFU / mL) and serial dilutions were prepared to reach the concentrations of 10^5 and 10^4 CFU x mL^{-1}. Two microliters of these three concentrations were inoculated onto Sabouraud dextrose 2% (SD2) (Difco – USA) in different spots and incubated at 25 °C, 37 °C and 40 °C for 72h [22]. The plates at different temperatures were observed for the presence or absence of cell growth. All plates with no growth were reincubated at 25 ° C for 24 to 48 h to observe the presence of cell growth.

Melanin production

Similar methodology to that described for thermotolerance was used for the melanin production. However, the NSA medium was used and the plates were incubated for 5 days. The production of melanin was observed by the presence of dark brown colonies, phenoloxidase positive [23]. The strain *Cryptococcus gattii* CFP 411 was used as a positive control. The melanin production on NSA was analyzed visually and a scale from (-) negative to (++++) high melanin production were applied, where: (-) did not produce apparent melanin with white coloration; (+) produced a beige coloration; (++) light brown; (+++) brown; (++++) dark brown.

Cellular and capsular size

After growth of 48h at 25 °C on SD2, a cell suspension adjusted to the standard 0.5 McFarland scale (1-5x10^6 CFU x mL^{-1}) was prepared. Capsule production was induced by inoculating 0.1 ml of the suspension in SD2 broth, pH 7.0,

diluted 1:10 with 50 mM morpholino propanesulfonic acid buffer (MOPS) (Sigma Aldrich - USA), pH 7.3. The tubes were incubated at 36 °C for 48 h, without stirring [24].

After the incubation period, approximately 10 µL of the culture was placed on a microscopic slide with a drop of 1% nigrosine in glycerol covered with clear glass cover; cell and capsule sizes were measured directly under the optical microscope (Zeiss – Axio Scope.A1) and 40X magnification. The capsule was evidenced by the formation of a transparent halo around the cell in contrast to the dark background of the preparation. The thickness of the capsule was measured as the distance between the boundary of the cell (cell wall) and the transparent border produced by nigrosin. At least ten cells were evaluated.

DNA Ploidy

The colonies were grown on YPD agar for 2 days at 25 °C. The obtained cells were inoculated into 5 mL of YPD broth and incubated for 16 hours at 25 °C. Two aliquots with 1 ml each were transferred to microtubes, the culture was subjected to centrifugation, the supernatant was discarded and the cells washed twice with 1 mL of distilled water. The cells were then pooled and fixed with 1 mL of 70% ethanol and kept at 4 °C overnight. After the ethanol fixation period, the cells were centrifuged and the supernatant discarded, then washed twice with 1 mL of distilled water and once with 1 mL of NS buffer (10 mM Tris (Sigma Aldrich - USA) (pH7.4), 0.25 M sucrose, 7 mM β-mercaptoethanol (Sigma Aldrich - USA), 0.4 mM phenylmethylsulfonyl fluoride (Sigma Aldrich - USA), 1 mM EDTA disodium (Sigma Aldrich - USA), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM $ZnCl_2$). Cells were suspended in 0.5 mL of staining solution (1 mg mL-1 of RNase A (Sigma Aldrich - USA) and 0.01 mg mL -1 of propidium iodide (Sigma Aldrich - USA) in NS buffer and incubated for 150 min at 37 °C. Then 0.05 ml of the stained cells were diluted in 2 ml of 50 mM Tris-HCl (pH 8.0) and sonicated for 10 seconds. DNA ploidy was access using the flow cytometer BD FACSCalibur™.

The wavelength of the laser beam was 488 nm and 10,000 cells were counted and the fluorescence intensities measured. The data were shown as histograms where the abscissa represents the channel numbers in the proportion of the fluorescence intensities and the ordinate shows the number of cells [25–27].

CFRVS 70297 (WM 628) and CFRVS 70302 (WM 178) were used as a diploid and haploid standard strains respectively.

In vivo virulence test *(Galleria mellonella* model)

Ten similar sized larvae (about 3g each) were selected to be inoculated by each colony. Each colony was previously grown on SD2 agar for 48 h at 25°C. Using a turbidimeter, an inoculum of 10^8 yeast cells/mL was prepared in Phosphate Buffered Saline (PBS) and 10 µL were inoculated into the hemocoel of each larva by injection into the last left pro-leg, using a 50 U Insulin Syringe with a 29-gauge needle. A group of 10 larvae was also inoculated with PBS to monitor potential effects on survival due to physical injury, while another 10 were not inoculated at all as an untreated control. After injection, the larvae were incubated in Petri dishes at 37 °C for 10 days and checked daily for any mortality [16].

Some mixtures of isolates were performed to evaluate the virulence of potential mixed lineages. The suspensions were prepared separately at the concentration of 10^8 yeast cells/mL and were mixtures in the proportion of 1:1.

Cellular and capsular size after inoculation

To observe the fungal structures, the killed larvae were crushed in 1mL PBS in a 50mL Falcon tube with a glass rod. The skin has been removed as much as possible. Cell and capsule sizes of the strains were measured as previously described.

Multilocus Sequence Typing (MLST)

Sub-typing and molecular polymorphism analysis were performed according to the ISHAM consensus multi-locus sequence typing scheme for *C. neoformans* and *C. gattii* including seven unlinked genetic loci, the genes: *CAP59*, *GPD1*, *LAC1*, *PLB1*, *SOD1*, *URA5* and the IGS1 region. All 7 loci were amplified as previously described[3]. The sequences were manually edited using the software Sequencher 4.10.1 (Gene Codes Corporation, MI, USA), and the allele types (AT) and the combined sequence types (ST) were identified via the MLST webpage http://mlst.mycologylab.org/. The corrected sequences were aligned using the program MEGA version 5.

Statistical analysis

Statistical significance was calculated using Kruskal-Wallis test followed by Dunn's multiple comparison test for statistical differences between cellular and capsule measurements.

Wilcoxon test was applied for comparison of cell and capsules size before and after passage in *G. mellonella*.

Survival curves per strain were graphed, median survival times were calculated and estimation of differences in survival was analysed by the Log-rank (Mantel-Cox) test.

When more than five larvae (50%) were alive at the end of the experiment, median survival times were not determined.

The survival curve of the larvae inoculated with the control strain CFP411 was used as a benchmark to determine the level of virulence of the remaining strains. Four groups of strains were thus defined and were represented with different numbers of crosses and dashes as follow: (-) strains that did not kill any larvae during the time of the experiment, (+) strains that killed at least one larva and were less virulent than control strain CFP411 ($p < 0.05$), (++) strains that were of comparable virulence as CFP411 ($p > 0.05$) and (+++) strains that were more virulent than control strain CFP411 ($p < 0.05$).

In all cases *P* values of 0.05 were considered significant. All data were analyzed and plots were carried out using Excel (Microsoft Corporation) or Prism 7 (GraphPad Inc.) software.

Acknowledgements

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References

1. Casadevall, A. & Perfect, J. R. *Cryptococcus neoformans*. (ASM Press, 1998).

2. Meyer, W. *et al.* Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. *Emerging infectious diseases* 189–195 (2003).

3. Meyer, W. *et al.* Consensus multi-locus sequence typing scheme for *Cryptococcus neoformans* and *Cryptococcus gattii*. *Medical Mycology* **47,** 561–570 (2009).

4. Franzot, S. P. *et al.* Microevolution of a Standard Strain of *Cryptococcus neoformans* Resulting in Differences in Virulence and Other Phenotypes. *Infection and immunity* **66,** 89–97 (1998).

5. Mandal, P., Banerjee, U., Casadevall, A. & Nosanchuk, J. D. Dual Infections with Pigmented and Albino Strains of *Cryptococcus neoformans* in Patients with or without Human Immunodeficiency Virus Infection in India. *Journal of Clinical Microbiology* **43,** 4766–4772 (2005).

6. Jain, N. & Fries, B. C. Phenotypic Switching of *Cryptococcus neoformans* and *Cryptococcus gattii*. *Mycopathologia* **166,** 181–188 (2008).

7. Kwon-Chung, K. J., Polacheck, I. & Popkin, T. J. Melanin-lacking mutants of *Cryptococcus neoformans* and their virulence for mice. *Journal of bacteriology* **150,** 1414–1421 (1982).

8. Rhodes, J. C., Polacheck, I. & Kwon-Chung, K. J. Phenoloxidase activity and virulence in isogenic strains of *Cryptococcus neoformans*. *Infection and Immunity* **36,** 1175–1184 (1982).

9. Hewitt, S. K., Foster, D. S., Dyer, P. S. & Avery, S. V. Phenotypic heterogeneity in fungi: Importance and methodology. *Fungal Biology Reviews* **30,** 176–184 (2016).

10. Desnos-Ollivier, M. *et al.* Mixed Infections and In Vivo Evolution in the Human Fungal Pathogen *Cryptococcus neoformans*. *mBio* **1,** e00091-10-e00091-10 (2010).

11. Fortes, S. T., Lazéra, M. S., Nishikawa, M. M., Macedo, R. C. L. & Wanke, B. First isolation of *Cryptococcus neoformans* var. *gattii* from a native jungle tree in the Brazilian Amazon rainforest. *Mycoses* **44,** 137–140 (2001).

12. García-Rodas, R., Casadevall, A., Rodríguez-Tudela, J. L., Cuenca-Estrella, M. & Zaragoza, O. *Cryptococcus neoformans* Capsular Enlargement and Cellular Gigantism during *Galleria mellonella* Infection. *PLoS ONE* **6,** e24485 (2011).

13. Mylonakis, E. *et al. Galleria mellonella* as a Model System To Study *Cryptococcus neoformans* Pathogenesis. *Infection and Immunity* **73,** 3842–3850 (2005).

14. Byrnes, E. J. *et al.* First Reported Case of *Cryptococcus gattii* in the Southeastern USA: Implications for Travel-Associated Acquisition of an Emerging Pathogen. *PLoS ONE* **4,** e5851 (2009).

15. Chen, Y.-L., Lehman, V. N., Lewit, Y., Averette, A. F. & Heitman, J. Calcineurin Governs Thermotolerance and Virulence of *Cryptococcus gattii*. *G3: Genes|Genomes|Genetics* **3,** 527–539 (2013).

16. Firacative, C., Duan, S. & Meyer, W. *Galleria mellonella* Model Identifies Highly Virulent Strains among All Major Molecular Types of *Cryptococcus gattii*. *PLoS ONE* **9,** e105076 (2014).

17. Kwon-Chung, K. J. & Rhodes, J. C. Encapsulation and melanin formation as indicators of virulence in *Cryptococcus neoformans*. *Infection and Immunity* **51,** 218–223 (1986).

18. Fernandes, K. E., Dwyer, C., Campbell, L. T. & Carter, D. A. Species in the *Cryptococcus gattii* Complex Differ in Capsule and Cell Size following Growth under Capsule-Inducing Conditions. *mSphere* **1,** e00350-16 (2016).

19. Perfect, J. R. *Cryptococcus neoformans* : the yeast that likes it hot. *FEMS Yeast Research* **6,** 463–468 (2006).

20. Meyer, W., Mitchell, T. G., Freedman, E. Z. & Vilgalys, R. Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. *J Clin Microbiol* **31,** 2274–2280 (1993).

21. Chaturvedi, S. *et al.* Direct PCR of *Cryptococcus neoformans* MATα and MATa Pheromones To Determine Mating Type, Ploidy, and Variety: a Tool for Epidemiological and Molecular Pathogenesis Studies. *J Clin Microbiol* **38,** 2007–2009 (2000).

22. Rosario, I. *et al.* Isolation of *Cryptococcus* species including *C. neoformans* from cloaca of pigeons. *Mycoses* **48,** 421–424 (2005).

23. Kwon-Chung, K. J. Chapter 114 - *Filobasidiella* Kwon-Chung (1975) A2 - Boekhout, Cletus P. KurtzmanJack W. FellTeun. in *The Yeasts (Fifth Edition)* 1443–1455 (Elsevier, 2011).

24. Zaragoza, O. & Casadevall, A. Experimental modulation of capsule size in *Cryptococcus neoformans*. *Biol Proced Online* **6,** 10–15 (2004).

25. Tanaka, R., Taguchi, H., Takeo, K., Miyaji, M. & Nishimura, K. Determination of ploidy in *Cryptococcus neoformans* by flow cytometry. *Journal of medical and veterinary mycology* **34,** 299–301 (1996).

26. Sia, R. A., Lengeler, K. B. & Heitman, J. Diploid Strains of the Pathogenic Basidiomycete *Cryptococcus neoformans* Are Thermally Dimorphic. *Fungal Genetics and Biology* **29,** 153–163 (2000).

27. Lin, X. *et al.* Diploids in the *Cryptococcus neoformans* Serotype A Population Homozygous for the α Mating Type Originate via Unisexual Mating. *PLoS Pathogens* **5,** e1000283 (2009).

Figures:

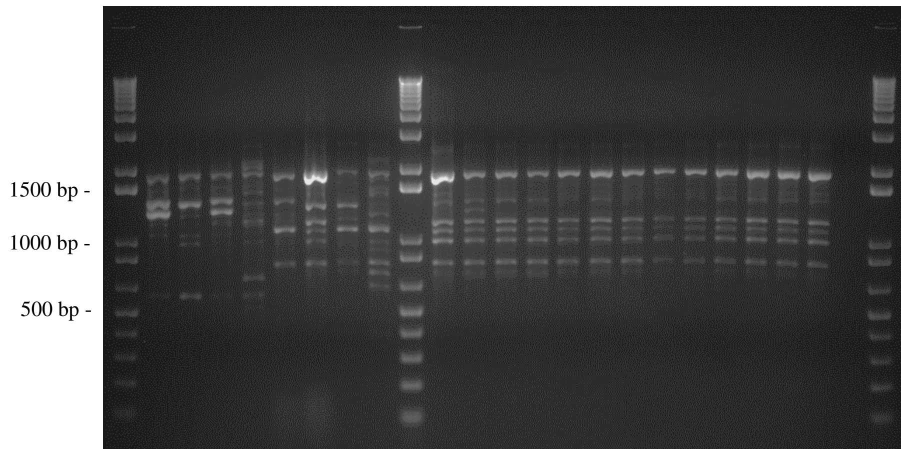

Figure 1. PCR-fingerprinting of the strain 78MC over one year. P. molecular weigth; a-h. Standard strains for molecular typing a - WM 148 (serotype A, VNI), b - WM 626 (serotype A, VNII), c - WM 628 (serotype AD, VNIII), d - WM 629 (serotype D, VNIV), e - WM 179 (serotype B, VGI), f - WM 178 (serotype B, VGII), g - WM 161 (serotype B, VGIII) and h - WM 779 (serotype C, VGIV); 0-12. Month 0 to 12; N. negative control. The arrow points the different band observed in the first month.

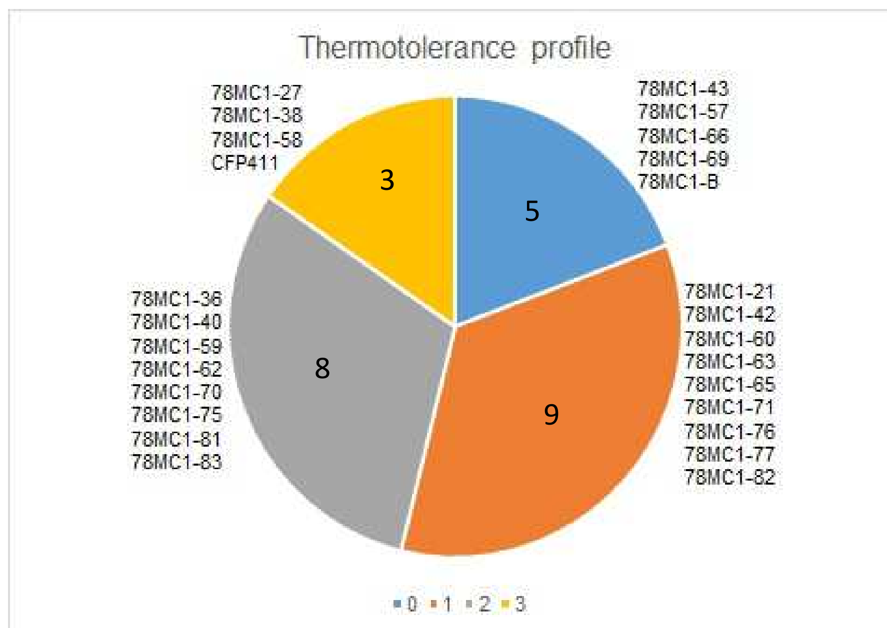

Figure 2. Thermotolerance profile after incubation at 40°C and re-incubation at 25°C, where: 0 - no growth at any of the inoculated concentration points (5 isolates); 1 - presented growth in only one inoculated concentration point (9 isolates); 2 - showed growth at two

concentration points (8 isolates); and 3 - presented growth in the three concentration points (3 isolates).

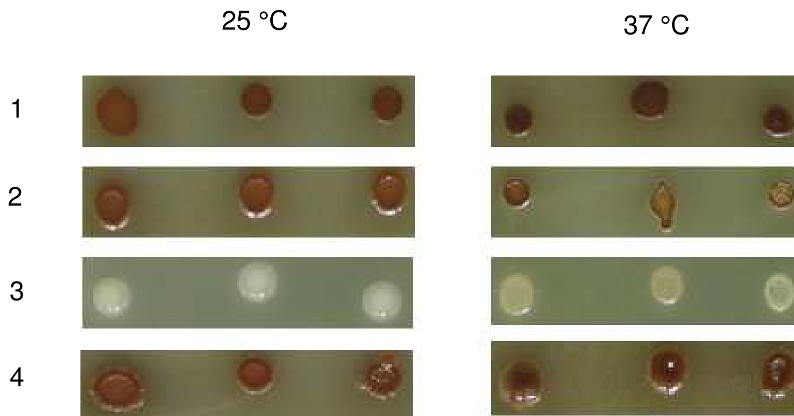

Figure 3. Melanin production at 25 °C and 37 °C of representative samples: 1 – 78MC1-21; 2 – 78MC1-42; 3 – 78MC1-B; 4 – CFP 411 (positive control).

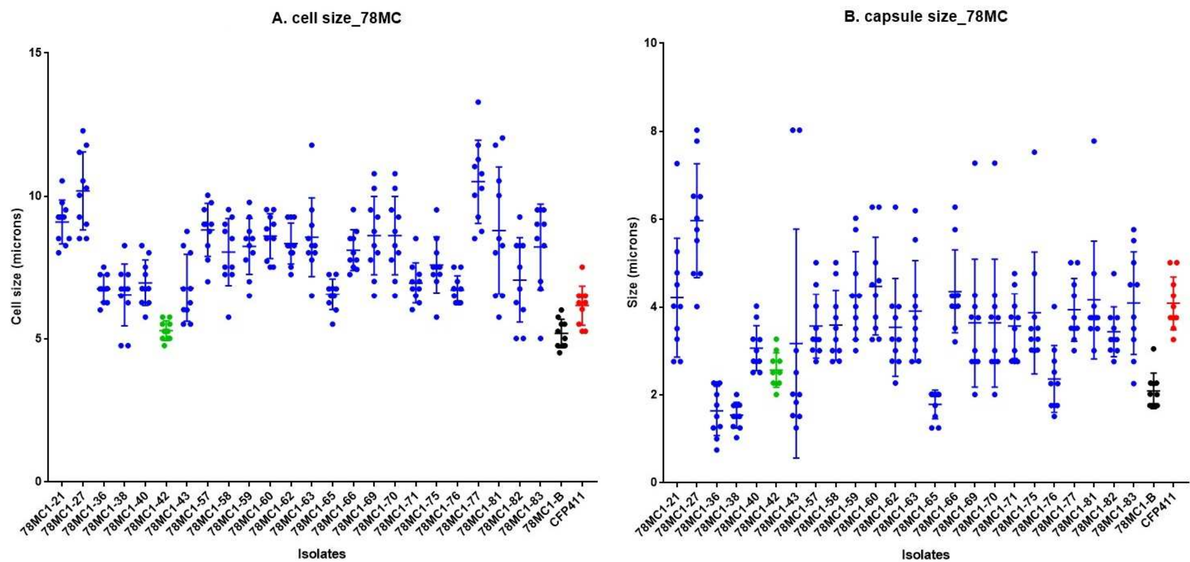

Figure 4. Cell size (A) and capsule thickness (B) distribution of twenty five isolates from *Cryptococcus gattii* (in blue), albino strain (in black), *C. neoformans* (in green) from 78MC1 population and positive control CFP411 (in red).

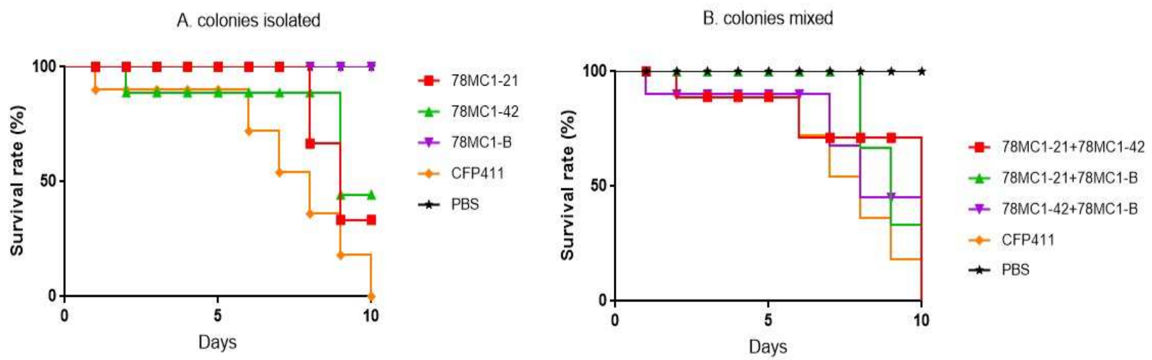

Figure 5. Survival curve of *Galleria mellonella* larvae inoculated with: A. colonies 78MC1-21 (*C. gattii*), 78MC1-42 (*C. neoformans*), 78MC1-B (*C. gattii* - phenoloxidase negative), strain CFP411 (VGIIa) and PBS (negative control) and B. Mixed colonies 78MC1-21+78MC1-42, 78MC1-21+78MC1-B, 78MC1-42+78MC1-B, strain CFP411 (VGIIa) and PBS (negative control).

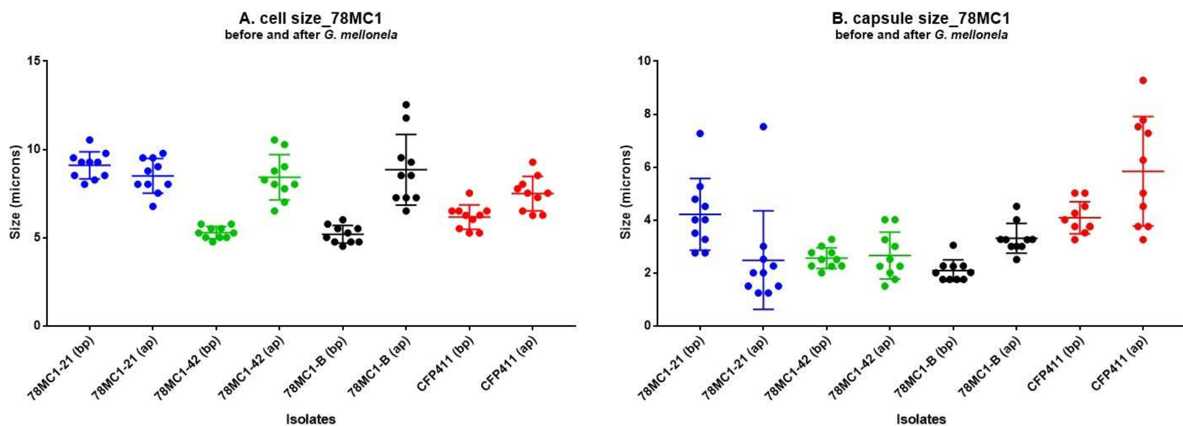

Figure 6. Cell size (A) and capsule thickness (B) distribution before (bp) and after (ap) passage in *G. mellonella*, where: in blue *C. gattii* 78MC1-21; in green *C. neoformans* 78MC1-42; in black the albino strain 78MC1-B; and in red the positive control CFP411.

CAPÍTULO 2

CAPÍTULO 2

O foco principal deste capítulo foi analisar a estrutura populacional e virulência de um isolado clínico para determinar a presença simultânea de diferentes complexos de espécies *C. neoformans* e *C. gattii* e também a possibilidade de heterogeneidade fenotípica e genotípica destes microrganismos.

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Clinical Microbiology and Infection **(CMI)**

Original article

Phenotypic heterogeneity in a clinical *Cryptococcus neoformans* strain

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Abstract

Cryptococcus neoformans and *C. gattii* are the etiological agents of cryptococcosis, a systemic mycosis, that stands out among the most lethal human fungal infections, mainly in the form of meningoencephalitis, reaching both immunocompromised and immunocompetent individuals. The identification of coinfection by *C. neoformans* and *C. gattii* has been observed since more than one colony of the same sample or serial isolates are analyzed, which is not a routine in the diagnostic laboratories. Therefore, coinfections may be more common than is assumed. The aim was to analyze the population structure of one clinical isolate to determine simultaneous presence of different species complexes *C. neoformans* and *C. gattii*, and/or phenotypic and genotypic heterogeneity. The strain was tested for genotypic stability over 12 months and the different colonies were analyzed as for melanin production, thermotolerance, capsule and cell size, mating type, ploidy, major molecular type by *URA5*-RFLP and

molecular subtype by PCR-fingerprinting. Phenotypic heterogeneity was observed in virulence factors especially in the production of melanin at 37 °C and capsule thickness. Thermotolerance showed difference between the isolates at reincubation at 25 °C after testing at 40 °C. Variety in ploidy with presence of haploid, diploid and suspected aneuploid cells were also observed. Of the antifungal agents evaluated only AMB presented resistance mutations/mechanisms as non-WT. This study confirms the importance in the evaluation of more than one colony in the same population.

Introduction

Cryptococcus neoformans and *C. gattii* are the etiological agents of cryptococcosis, a systemic mycosis that stands out among the most lethal human fungal infections, mainly in the form of meningoencephalitis, reaching both immunocompromised and immunocompetent individuals (1,2).

Phylogenetic studies suggest that *C. gattii* and *C. neoformans* strains are a complex of multiple phenotypically cryptic species, which present differences in their ecology, epidemiology, virulence, genetics and clinical outcome. The major groups for the two species complexes have most commonly been designated molecular types VNI (AFLP1, serotype A), VNII (AFLP1A/IB, serotype A), VNIII (AFLP3, serotype AD), and VNIV (AFLP2, serotype D) for *C. neoformans* and molecular types VGI (AFLP4), VGII (AFLP6), VGIII (AFLP5), and VGIV (AFLP6) for *C. gattii* (serotypes B and C) (3).

Phenotypic heterogeneity can be a phenomenon whereby individual cells within clonally derived populations, can nevertheless display differences in phenotype (4). Furthermore, phenotypic heterogeneity within clonal microbial cultures is potentially of great significance in fungal biology and ecology, and fundamental to organismal fitness and development (5). Mixed infections in humans are more common than previously thought, occurring in almost 20% of patients diagnosed with cryptococcosis. These mixed infections are composed of different mating types, serotypes, and/or genotypes (6). Multiple ploidy states can leads to phenotypic changes, but the advantages or disadvantages of these different ploidy states are not well stablished for human pathogens (7).

The identification of coinfection by *C. neoformans* and *C. gattii* has been observed (8), since more than one colony of the same sample or serial isolates are

analyzed, which is not a routine in the diagnostic laboratories. Therefore, coinfections may be more common than is assumed. Thus, our main goal was to analyze the population structure of one clinical isolate to determine simultaneous presence of different species complexes *C. neoformans* and *C. gattii*, and/or phenotypic and genotypic cell heterogeneity.

Methods

A clinical isolate, strain CFRVS 71013 (LMM21), deposited at the Culture Collection of Reference Fungi in Sanitary Surveillance (Fiocruz/ CFRVS) was selected for the study. The strain was isolated from urine sample of a 47 years old, male patient, HIV positive from Rio de Janeiro, in 1994. At the time of diagnosis, it was serotyped by CFRVS and presented dubious results, serotypes A (*C. neoformans*) and B (*C. gattii*), and the canavanine-blue-glycine medium (CGB) was positive (*C. gattii*), but the isolation of both species was not possible. The original strain was reactivated from ultra-freezer at -80 °C to evaluate the coexistence of both species in different isolated colonies. The strain was tested for genotypic stability over 12 months and the different colonies were analyzed as for melanin production, thermotolerance, capsule and cell size, mating type, ploidy, major molecular type by *URA5*-RFLP, molecular subtype by PCR-fingerprinting and multi locus sequence typing (MLST).

The strain stored at -80°C was cultured on Yeast Malt Agar (YMA) (Difco – USA) at 25 °C for 30 days. Part of the cell mass was collected for subsequent DNA extraction and PCR-fingerprinting, and new culture on YMA medium was incubated at 25 °C for 30 days. The procedure was performed monthly until the end of 12 months in order to evaluate the genotypic stability of this population over time.

The cell density of the strain CFRVS 71012 was adjusted visually by adding sterile purified water to that produced by 2 McFarland standard. Volumes of 0.1 ml from 10^{-2}, 10^{-4}, and 10^{-5} dilutions were inoculated onto niger seed (*Guizotia abyssinica*) agar (NSA) plates and incubated for five days at 25 °C. After the incubation period, 50 isolated colonies that showed melanin production were picked and subcultured on YMA and CGB media.

The molecular stability of the strain over 12 months and the analysis of the 50 *Cryptococcus* colonies was performed according to the methodology described by Meyer (9), which is based on random amplification of DNA fragments generated by

using the minisatellite-specific core sequence of the wild-type phage M13. Molecular grouping was defined as VNI, VNII, VNIII and VNIV to *C. neoformans*, and VGI, VGII, VGIII and VGIV to *C. gattii* by the comparison with the standards strains WM 148 (serotype A, VNI/AFLP1), WM 626 (serotype A, VNII/AFLP1A), WM 628 (serotype AD, VNIII/AFLP2), WM 629 (serotype D, VNIV/AFLP3), WM 179 (serotype B, VGI/AFLP4), WM 178 (serotype B, VGII/AFLP6), WM 175 (serotype B, VGIII/AFLP5), and WM 779 (serotype C, VGIV/AFLP7) (10).

The major molecular types were confirmed by *URA5*-RFLP analysis using the enzymes *Hha*I and *Sau*96I. The band patterns were assigned visually by comparing them with the patterns obtained from the standard strains cited above (10).

The mating types were determined by the polymerase chain reaction of specific pheromone loci using the primers MFal- faU (5' TTCACTGCCATCTTCACCACC 3'); MFalfaL (5' TCTAGGCGATGACACAAAGGG 3') for mating type alpha (MATalpha) and JOHE9787 (5' ACACCGCCTGTTACAATGGAC 3'); JOHE9788 (5' CAGCGTTTGAAGATGGACTTT 3') for mating type a (MATa). The unique fragment corresponding to each mating type was visualized after electrophoresis of agarose gel (11).

For the thermotolerance test, the cell suspension was adjusted to the standard 0.5 McFarland scale (1-5x10^6 CFU x mL-1), followed by serial dilutions of 10^5 and 10^4 CFU x mL-1. Two microliters of each dilution were spot inoculated on Sabouraud dextrose 2% (SD2) (Difco™ – USA), incubated at 25 °C, 37 °C and 40 °C for 72h. All plates with no growth were reincubated at 25 °C for 24 to 48 h.

Melanin production methodology was similar to that described for thermotolerance test, and it was carried out twice. However, the NSA medium was used and the plates were incubated for 5 days. The production of melanin was observed by the presence of phenoloxidase positive (dark brown) colonies (12). The strain *Cryptococcus gattii* CFP 411 was used as a positive control. The melanin production on NSA was analyzed visually and a scale from (-) negative to (++++) high melanin production were applied, where: (-) did not produce apparent melanin with white coloration; (+) produced a beige coloration; (++) light brown; (+++) brown; (++++) dark brown.

Capsule production was induced by inoculating 0.1 ml of a suspension adjusted to the standard 0.5 McFarland on SD2 broth, pH 7.0, diluted 1:10 with 50

mM morpholino propanesulfonic acid buffer (MOPS) (Sigma-Aldrich - USA), pH 7.3. The cultures were incubated at 36 °C for 48 h (13). The capsule was evidenced by the formation of a transparent halo around the cell in contrast to the dark background of the preparation of 10 µl of culture and a drop of 1% nigrosine in glycerol. The cellular size and thickness of the capsule was measured. Thickness of the capsule was the distance between the boundary of the cell (cell wall) and the transparent border produced by nigrosine; at least ten cells were evaluated under optical microscope at 400X magnification. Statistical significance was calculated using Kruskal-Wallis test followed by Dunn's multiple comparison test for statistical differences between cellular and capsule measurements. P values ≤ 0.05 were considered significant. All data were analyzed and plots were carried out using Excel (Microsoft Corporation) or Prism 7 (GraphPad Inc.) software.

Ploidy was estimated by the measure of the relative DNA contents in the flow cytometry FacsCalibur, BD. The colonies were grown on YPD agar for 2 days at 25 °C. The obtained cells were inoculated into 5 ml of YPD broth and incubated for 16 hours at 25 °C. The cells were washed twice with distilled water and fixed with 1 mL of 70% ethanol at 4 °C overnight. The cells were washed twice with 1 mL of distilled water and once with 1 mL of NS buffer (10 mM Tris (pH7.4), 0.25 M sucrose, 7 mM β-mercaptoethanol, 0.4 mM phenylmethylsulfonyl fluoride, 1 mM EDTA disodium, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM $ZnCl_2$). After centrifugation, the pellet was suspended in 0.5 mL of staining solution (1 mg mL-1 of RNase A and 0.01 mg mL -1 of propidium iodide in NS buffer) and incubated for 150 min at 37 °C. Then, 0.05 mL of the stained cells were diluted in 2 mL of 50 mM Tris-HCl (pH 8.0) and sonicated for 10 seconds. The wavelength of the laser beam was 488 nm, 10,000 cells were counted and the fluorescence intensities measured. The data were shown as histograms where the abscissa represents the channel numbers in the proportion of the fluorescence intensities and the ordinate shows the number of cells(14–16). The DNA content of diploid cells should display values of 2*n* and *4n* with respect to the *1n* and 2*n* DNA content of haploid strains. In contrast, aneuploid strains have DNA contents between the expected values for haploid and diploid strains (17). The strains CFRVS 70297 (WM 628) was used as a diploid standard and CFRVS 70302 (WM 178) as a haploid standard.

The broth microdilution method was used to determine the minimal inhibitory concentration (MIC) values according to CLSI (Clinical and Laboratory Standards

Institute) M27-A3 guidelines (18), with a final concentration of 0.5×10^3 to 2.5×10^3 cells/ml and RPMI 1640 medium buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic. Growth and sterility control wells were included for each isolate tested. The following antifungal drugs were obtained as assay powders; itraconazole (ICZ), fluconazole (FCZ) and voriconazole (VCZ) (Sigma-Aldrich - USA); one polyene derivative, amphotericin B (AMB) (Sigma-Aldrich - USA); and a fluorinated pyrimidine, flucytosine (5FC) (Sigma-Aldrich - USA). The MIC of amphotericin B was defined as the lowest concentration that caused 100% inhibition of growth, and the MICs of all other antifungal drugs were described as the lowest concentrations that produced 50% reduction in growth as compared to controls. The *Candida parapsilosis* strain INCQS 40038 (ATCC 22019) and the *Candida krusei* strain INCQS 40147 (ATCC 6258) were included as controls each time that a set of isolates was tested.

In the absence of clinical breakpoints, Epidemiological Cutoff Values (ECVs) for the molecular type VNI were applied for the detection of colonies with resistance mechanism (19,20). The ECVs used for fluconazole was 8 µg/ml; itraconazole 0.25 µg/ml; voriconazole 0.25 µg/ml; amphotericin B 0.5 µg/ml; and flucytosine, 8 µg/ml. The geometric mean (GM) of MICs, defined as the MIC that encompassed at least 50 and 90% of isolates in the distribution, were calculated.

Results

The molecular stability of the stored strain at -80°C was observed by PCR-fingerprinting monthly and showed the same band profile over the 12 months, compatible to the major molecular type VNI. All 50 selected colonies from the original strain were identified as *C. neoformans* by the CGB medium, MATα, and as molecular type VNI by PCR-fingerprinting and *URA5*-RFLP. No different band pattern was observed in the PCR-fingerprinting analysis, thus 25 colonies were randomly selected for the ploidy, thermotolerance, melanin production, capsule and cell size measure and antifungal susceptibility.

The ploidy of the 25 colonies varied considerably, as 19 colonies were identified as haploid, 1 diploid (LMM21-42), and 5 demonstrated important cellular distribution between channels 360-560, indicating the presence of possible aneuploid and diploid cells (LMM21-12, LMM21-22, LMM21-27, LMM21-34, LMM21-44) (figure 1).

All twenty five VNI colonies were considered 3+ growth at 37 °C, since they presented growth at the three different concentrations. There was no growth at 40 °C, but after reincubation of the plates at 25 °C, the growth profiles were varied as follows: 12 isolates presented growth at only one inoculated point (1+); 8 isolates showed growth at two inoculated point (2+); and 5 isolates and the positive control CFP 411 presented growth in the three inoculated points (3+).

All isolates showed high melanin production at 25 °C, being considered positive (+++). However, at 37°C the profiles varied, 2 isolates did not produce melanin (-); 8 isolates demonstrated low (beige coloration) melanin production (+); 10 isolates were light brown (++); 4 isolates were brown (+++); 2 isolates, including positive control, showed high (dark brown) melanin production (++++) (figure 2).

The cell size and capsule thickness variation was significant among the 25 colonies ($p < 0.0001$). The cell size of the colonies ranged from 3.01-7.53 µm, with mean of 5.07 µm (figure 3A). Capsule thickness ranged from 0.5-2.26 µm and the mean of total measurements was 1.14 µm (figure 3B).

According to the epidemiological cutoff values (ECV), all colonies were considered as wild-type (WT) for 5-FC, FCZ, VCZ and ICZ, however 100% of the colonies demonstrated resistance mutations/mechanisms (non-WT) to AMB. The MIC ranges, geometric mean, MIC90 and MIC50 are shown on table 1.

Table 1. Variation of MIC, MIC 50, MIC 90 and GM values of the antifungal tested for twenty five isolates from *Cryptococcus neoformans* LMM21 population according to CLSI protocol M27-A3 (2008).

Isolate (n° of isolates)	MIC parameter	MIC (µg/ml) of:				
		AMB	5-FC	FCZ	VCZ	ICZ
C. neoformans LMM21 (26)	Range	0.5-2	1-4	<0.125-0.5	<0.015-0.015	<0.015-0.125
	GM	0.65	2.47	0.12	0.015	0.02
	50%	0.5	2.0	0.25	0.015	0.015
	90%	1.0	4.0	0.42	0.015	0.03

Discussion

Cryptococcosis by *C. neoformans* is most often associated with immunocompromised hosts and is considered one of the most important fungal diseases in this population. Due to the inefficient immunological status, it is not rare

or even expected to find coinfections in this population, especially with tuberculosis or other fungal infection as histoplasmosis (21). The present work analyzed a clinical *C. neoformans* strain isolated from urine specimen of an immunocompromised patient in 1994. A single colony was isolated at the time of diagnosis and the species and serotype identification were inconclusive. To evaluate the coexistence of different species, presence of molecular and phenotypic heterogeneity, or even the presence of hybrids, several colonies were isolated from the original strain for phenotypic and molecular study.

PCR-fingerprinting, mating type and *URA5*-RFLP did not highlight any variability among the colonies tested. Thermotolerance at 37 °C is critical for the yeasts to grow at mammalian physiological temperature, and some isolates can be thermotolerant at higher temperatures (22). At first there was no variation in thermotolerance, and any isolates presented direct growth at 40 °C, but after reincubation at 25 °C, the variation could be observed, showing the colonies with better resistance to higher temperatures. The higher thermal tolerance of cells could confer a survival advantage in warmer climates and predominance of these cells among clinical isolates could also reflect some selection by the temperatures associated with high fever (23).

The etiological agents of cryptococcosis are presumptively identified by their ability to produce dark, melanin-like pigments by enzyme laccase with various phenolic compounds as substrates (24), and this enzyme is more active at 30 °C than at 37 °C (25). The present results demonstrates equal production of melanin at 25 °C among the colonies, but at 37 °C the melanin production varied greatly, pointing to a strategy of maintaining viability under stressed conditions.

Extracellular polysaccharide capsules play a significant role in the pathogenicity of *Cryptococcus neoformans* (26). The capsule of *C. neoformans* contributes to virulence through several mechanisms that confer protection to yeast cells against host phagocytic cells and interfere with host immune mechanisms (27). Capsule thickness presented significant difference among *C. neoformans* colonies from CFRVS 71013. The colonies also respond differently against the antifungal drugs, although the difference of 2 folds observed for AMB, 5-FC and VCZ is not considered significant. The difference of 3 fold was observed for ICZ and FCZ, even though, the difference among the colonies was not significant. All tested colonies were considered resistant against AMB according to the ECV breakpoint in both

performed assays. Bennet et al. (2014) stated *C. neoformans* appear to be well adapted for propagation in multiple ploidy states with novel pathways driving ploidy variation leading to additional phenotypic changes (7). It seems the emergence of antifungal heteroresistant colonies of *C. neoformans* is caused by the formation of disomy of chromosomes that contain key genes associated with azole resistance (28). The present results evidenced the existence of ploidy plasticity in a clinical isolate, with haploid, diploid and aneuploid colonies coexisting in the same population, and may be the origin of the phenotypical heterogeneity found. The ploidy shift can arise as consequence of parasexual or sexual cicles, or even as result of defect in mitosis. However, mixed ploidy in a fungal population is considered unusual in the fungal kingdom, limited studies evaluating ploidy variations in natural fungal populations suggest that polyploidy may be more common among fungi (16). Considering that in the present study, the majority were haploid, one diploid and few possible aneuploid, the selection of only one isolated colony for genetic or phenotypic studies may favor the selection of the dominant profile in the case of haploid cells.

In this study, individual cells within supposedly clonal microbial culture of *C. neoformans* exhibited marked phenotypic and molecular heterogeneity. Such variability in expression confers a benefit in constant stressful conditions for yeast populations because it generates, in the absence of stress, a phenotypic diversity that makes the presence of preadapted cells more probable (29). Furthermore, the selection of one colony will impact greatly in the key highlights in fundamental research, including genomics-based studies of cryptococcal evolution, resistance and metabolic adaptation of *C. neoformans* in the host.

Reference

1. Kwon-Chung KJ, Bennett JE. Cryptococcosis. In: Medical Mycology. Lea & Febiger; 1992. p. 392–446.

2. Casadevall A, Perfect JR. *Cryptococcus neoformans*. ASM Press; 1998. Washington, DC.

3. Kwon-Chung KJ, Bennett JE, Wickes BL, Meyer W, Cuomo CA, Wollenburg KR, et al. The Case for Adopting the “Species Complex” Nomenclature for the Etiologic Agents of Cryptococcosis. Lorenz M, editor. mSphere. 2017 Feb 22;2(1):e00357-16.

4. Hewitt SK, Foster DS, Dyer PS, Avery SV. Phenotypic heterogeneity in fungi: Importance and methodology. Fungal Biology Reviews. 2016 Oct;30(4):176–84.

5. Avery SV. Microbial cell individuality and the underlying sources of heterogeneity. Nature Reviews Microbiology. 2006 Aug;4(8):577–87.

6. Desnos-Ollivier M, Patel S, Spaulding AR, Charlier C, Garcia-Hermoso D, Nielsen K, et al. Mixed Infections and In Vivo Evolution in the Human Fungal Pathogen *Cryptococcus neoformans*. mBio. 2010 May 18;1(1):e00091-10-e00091-10.

7. Bennett RJ, Forche A, Berman J. Rapid Mechanisms for Generating Genome Diversity: Whole Ploidy Shifts, Aneuploidy, and Loss of Heterozygosity. Cold Spring Harbor Perspectives in Medicine. 2014 Oct 1;4(10):a019604–a019604.

8. Kassi FK, Mallié M, Kouakou GA, Reynes J, Bertout S, Doumbia A, et al. First case of mixed infection with *Cryptococcus deuterogattii* and *Cryptococcus neoformans* VNI in an Ivorian HIV-positive patient. JMM Case Reports [Internet]. 2016 Jul 11 [cited 2018 Apr 3];3(4). Available from: http://www.microbiologyresearch.org/content/journal/jmmcr/10.1099/jmmcr.0.005037

9. Meyer W, Mitchell TG, Freedman EZ, Vilgalys R. Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. J Clin Microbiol. 1993 Sep;31(9):2274–80.

10. Meyer W, Castañeda A, Jackson S, Huynh M, Castañeda E, Arechavala A, et al. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. Emerging infectious diseases. 2003;9(2):189–195.

11. Chaturvedi S, Rodeghier B, Fan J, McClelland CM, Wickes BL, Chaturvedi V. Direct PCR of *Cryptococcus neoformans* MATα and MATa Pheromones To Determine Mating Type, Ploidy, and Variety: a Tool for Epidemiological and Molecular Pathogenesis Studies. J Clin Microbiol. 2000 May;38(5):2007–9.

12. Kwon-Chung KJ. Chapter 114 - *Filobasidiella* Kwon-Chung (1975) A2 - Boekhout, Cletus P. KurtzmanJack W. FellTeun. In: The Yeasts (Fifth Edition)

[Internet]. London: Elsevier; 2011. p. 1443–55. Available from: http://www.sciencedirect.com/science/article/pii/B9780444521491001142

13. Zaragoza O, Casadevall A. Experimental modulation of capsule size in *Cryptococcus neoformans*. Biol Proced Online. 2004 Mar 3;6:10–5.

14. Tanaka R, Taguchi H, Takeo K, Miyaji M, Nishimura K. Determination of ploidy in *Cryptococcus neoformans* by flow cytometry. Journal of medical and veterinary mycology. 1996;34(5):299–301.

15. Sia RA, Lengeler KB, Heitman J. Diploid Strains of the Pathogenic Basidiomycete *Cryptococcus neoformans* Are Thermally Dimorphic. Fungal Genetics and Biology. 2000 Apr;29(3):153–63.

16. Lin X, Patel S, Litvintseva AP, Floyd A, Mitchell TG, Heitman J. Diploids in the *Cryptococcus neoformans* Serotype A Population Homozygous for the α Mating Type Originate via Unisexual Mating. Andrianopoulos A, editor. PLoS Pathogens. 2009 Jan 30;5(1):e1000283.

17. Lengeler KB, Cox GM, Heitman J. Serotype AD Strains of *Cryptococcus neoformans* Are Diploid or Aneuploid and Are Heterozygous at the Mating-Type Locus. Infection and Immunity. 2001 Jan 1;69(1):115–22.

18. Clinical and Laboratory Standards Institute, editor. Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard. 3rd ed. Wayne, PA: National Committee for Clinical Laboratory Standards; 2008. 25 p. (Documents / Clinical and Laboratory Standards Institute).

19. Espinel-Ingroff A, Aller AI, Canton E, Castañón-Olivares LR, Chowdhary A, Cordoba S, et al. *Cryptococcus neoformans-Cryptococcus gattii* Species Complex: an International Study of Wild-Type Susceptibility Endpoint Distributions and Epidemiological Cutoff Values for Fluconazole, Itraconazole, Posaconazole, and Voriconazole. Antimicrobial Agents and Chemotherapy. 2012 Nov;56(11):5898–906.

20. Espinel-Ingroff A, Chowdhary A, Cuenca-Estrella M, Fothergill A, Fuller J, Hagen F, et al. *Cryptococcus neoformans-Cryptococcus gattii* Species Complex: an International Study of Wild-Type Susceptibility Endpoint Distributions and

Epidemiological Cutoff Values for Amphotericin B and Flucytosine. Antimicrobial Agents and Chemotherapy. 2012 Jun;56(6):3107–13.

21. Nunes J de O, Pillon KRAP, Bizerra PL, Paniago AMM, Mendes RP, Chang MR. The Simultaneous Occurrence of Histoplasmosis and Cryptococcal Fungemia: A Case Report and Review of the Literature. Mycopathologia. 2016 Dec 1;181(11):891–7.

22. Martinez LR, Garcia-Rivera J, Casadevall A. *Cryptococcus neoformans var. neoformans* (Serotype D) Strains Are More Susceptible to Heat than *C. neoformans var. grubii* (Serotype A) Strains. Journal of Clinical Microbiology. 2001 Sep 1;39(9):3365–7.

23. Nurudeen TA, Ahearn DG. Regulation of melanin production by *Cryptococcus neoformans*. Journal of clinical Microbiology. 1979;10(5):724–729.

24. Jacobson ES, Emery HS. Temperature regulation of the cryptococcal phenoloxidase. Journal of Medical and Veterinary Mycology. 1991 Mar 1;29(2):121–4.

25. Kwon-Chung KJ, Rhodes JC. Encapsulation and melanin formation as indicators of virulence in *Cryptococcus neoformans*. Infection and Immunity. 1986;51(1):218–223.

26. Casadevall A, Coelho C, Cordero RJB, Dragotakes Q, Jung E, Vij R, et al. The Capsule of *Cryptococcus neoformans*. Virulence. 2018 Feb 13;00–00.

27. Sionov E, Chang YC, Garraffo HM, Kwon-Chung KJ. Heteroresistance to Fluconazole in *Cryptococcus neoformans* Is Intrinsic and Associated with Virulence. Antimicrobial Agents and Chemotherapy. 2009 Jul 1;53(7):2804–15.

28. Blake WJ, KÆrn M, Cantor CR, Collins JJ. Noise in eukaryotic gene expression. Nature. 2003 Apr;422(6932):633–7.

Figures:

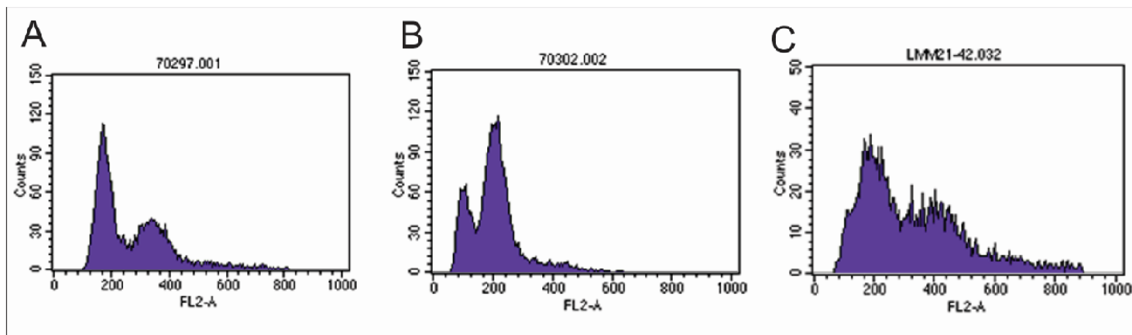

Figure 1. Representative histograms of DNA contents where the abscissa shows the channel numbers in the proportion of the fluorescence intensities and the ordinate shows the number of cells. The DNA content of diploid cells display values of 2*n* and *4n* (channel 200 and 400) (A and C); while haploid cells display *1n* and 2*n* (channel 100 and 200) (B).

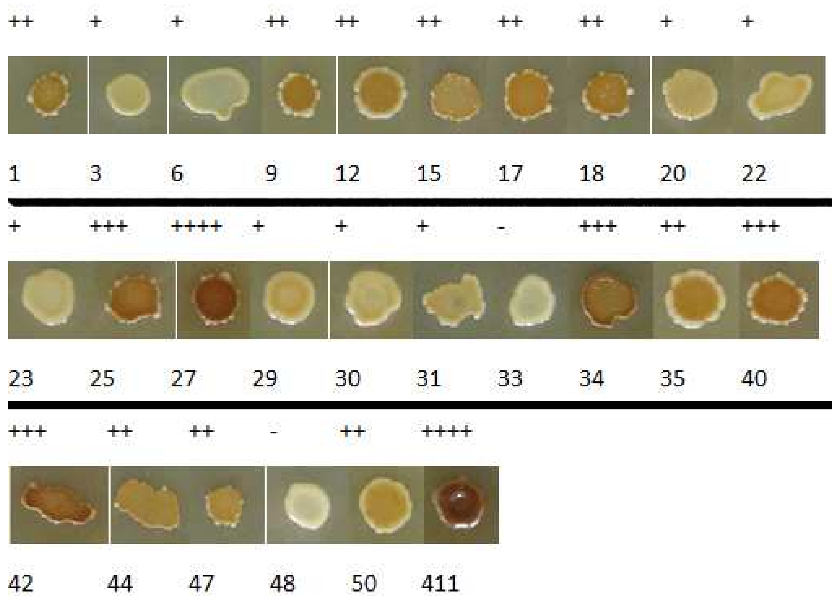

Figure 2. Variation of melanin production of twenty five isolates from *Cryptococcus neoformans* LMM21 population after 5 days incubation at 37 °C in NSA.

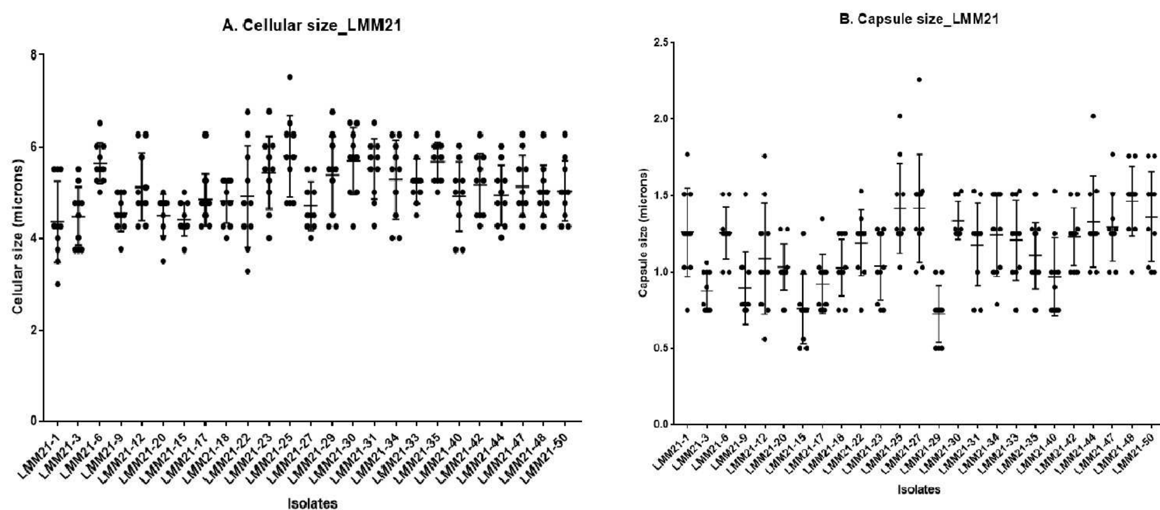

Figure 3. Cell size (A) and capsule thickness (B) distribution of twenty five isolates from *Cryptococcus neoformans* LMM21 population.

CAPÍTULO 3

CAPÍTULO 3

Neste capítulo, dentre quatro métodos de preservação testados, congelamento a -20 °C, liofilização e ultracongelamento a -80 °C e -196 °C foi avaliado quais apresentaram melhor desempenho na preservação de *C. neoformans* e *C. gatti* quando misturadas e submentidas em conjunto à preservação.

Artigo número 3, **submetido** para a revista:
Memórias do Instituto Oswaldo Cruz

Genotypic and phenotypic stability of mixed primary isolates of *Cryptococcus gattii* and *C. neoformans* – a comparative analysis of four preservation methods

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BACKGROUND The choice of a suitable preservation method is critical for long-term microorganisms' viability. The strains should be preserved for long periods using reliable and reproducible methods that minimize genotypic and phenotypic variations and viability losses. The methodologies are usually designed for a better performance in isolated microorganisms. However, atypical primary isolates of *C. neoformans* or *C. gattii*, such as mixed species or even different species of a species complex are a challenge for long-term preservation and taxonomic review studies.

OBJECTIVES The aim of this study was to evaluate which of the four preservation methods tested presented better performance in the preservation of simulated coexistence strains of *C. neoformans* and *C. gatti*.

METHODS Two environmental strains, one *C. gattii* and one *C. neoformans* were mixed *in vitro* to test four different preservation methods (freezing at -20 °C, -80 °C, -196 °C and freeze-drying). The CFU from each preservation method were evaluated and colonies were randomly selected and cultivated in CGB to evaluate the amounts of CGB positive (*C. gattii*) and CGB negative (*C. neoformans*) colonies resulting from each preservation method after 1 week, 15 days, 1 month, 6 months and 1 year preserved.

FINDINGS According to our results, cryopreservation at -20 °C demonstrated to favor *C. neoformans* species and further studies after long-term storage are

necessary. Recovery of yeast cells after freeze-dried preservation in skim milk is better for both species. Ultra freezing methods evaluated (-80 °C, -196 °C) also showed good results in the maintenance of both species, but adjustments are necessary.

MAIN CONCLUSIONS Freeze-drying should be preferred for the maintenance of multi-lineage isolates from the *C. neoformans* and *C. gattii* species complexes.

Keywords: *Cryptococcus* spp. – preservation – stability - mixed cultures

Cryptococcosis is a life-threatening mycosis, frequently causing meningoencephalitis. Only two of the species in the basidiomycetous yeast genus *Cryptococcus* cause mainly human diseases, these are the members of the *C. neoformans* and *C. gattii* species complexes, which are responsible globally for almost one million cryptococcosis cases yearly [(1)]. Mixed infections in humans are more common than previously thought, occurring in almost 20% of patients diagnosed with cryptococcosis [(2)]. These mixed infections are composed of different mating types, serotypes, and/or genotypes [(2)]. The epidemiology of *C. neoformans* is well characterized and this organism causes diseases mainly in immunocompromised hosts. *C. gattii*, on the contrary, has historically been considered as a pathogenic agent of apparently immunocompetent patients [(2)].

The maintenance and preservation of fungal cultures are essential for the development of systematic and biodiversity studies [(3)]. As fungi are a very diverse group, several cultivation and preservation methods are required to ensure the viability, morphological, physiological and genetic integrity of the cultures over time [(4)]. Therefore, many studies were carried out with the objective of evaluating efficient methods for the preservation of fungi [(5-10)]. Freezing methods are versatile and widely applicable. With freeze-drying (lyophilization), the fungal cultures are frozen and subsequently dried under vacuum. Freeze-drying and freezing below -139 °C are good methods for permanent preservation [(10)]. However, cryopreservation and lyophilization may result in deleterious impacts on viability, stability, and functionality of the microorganisms. Thus achieving a balance between stabilization and cell damage is critical in pursuing an efficient cell preservation strategy [(11)].

It is extremely important to protect the cells against ice crystal formation within the cell interior in cryogenic temperatures [(12)]. Several studies evaluating

cryoprotectants have been published [12-15]. Both cryopreservation and lyophilization have advantages and disadvantages, and the response to preservation varies according to the species [16]. As such a number of studies have been conducted to compare the different methodologies applied to certain species [17-19].

A pure culture theoretically should contain a single microorganism species, and the major objective of the preservation methods are designed to meet this demand. However, one isolated colony not always mean a single species. In the case of co-infections and environmental isolations, coexisting sibling species can be present in a single colony and the presented characters are determined by the predominant species.

The Collection of Reference Fungi in Sanitary Surveillance (Fiocruz/ CFRVS) and the Collection of Pathogenic Fungi (Fiocruz/CFP) have maintained cryopreserved and freeze-dried original clinical isolates from patients infected by different lineages of cryptococcosis agents, as well as environmental isolates cohabiting both species: *C. neoformans* and *C. gattii*. Divergent results in different periods have been observed in the analysis of those isolates. The present study was carried out to determine the best preservation method amongst the four most in fungal culture collections used ones (freezing at -20 °C, -80 °C, -196 °C and freeze-drying) to ensure phenotypic and genetic stability of the isolates to enable studies over time that allow a better understanding of the population structure of the agents of cryptococcosis.

MATERIALS AND METHODS

To determine the most effective preservation method to maintain the maximal genetic diversity of the original mixed culture, two environmental strains, one *C. gattii* (78MC2) and one *C. neoformans* (78MC3) isolated, both from a *Guettarda acreana* tree hollow in the Amazon region, Brazil [20], were mixed *in vitro* to test the different preservation methods.

An aliquot of 100 µL of the stock culture maintained in glycerol at -196 °C was inoculated separately into 9.9 mL of yeast extract-malt extract (YMB) broth for both strains. The tubes were homogenized and incubated at 25 °C for 48 h. The cells were washed twice with 0.5 mL of sterile purified water in order to remove residues from the culture medium [21]. Final cell concentrations were adjusted to 0.3 McFarland, this suspension was diluted in water (1:10) and another dilution was

performed directly on the cryoprotectant (1:100). Skim milk 20% was used as cryoprotectant for freeze-drying and frozen at -20 °C, and 15% glycerol for freezing -80 °C and -196 °C (liquid nitrogen).

The preservation methods tested were the most used ones in fungal culture collections (freezing at -20 °C, -80 °C, -196 °C and freeze-drying), which are also part of the routine in the CFRVS and CFP. Forty cryotubes or ampoules (freeze-drying) were prepared for each methodology according to the general protocols for freezing and lyophilization, with minimal modifications, established by Nakasone *et al.* [(4)].

To verify the viability before preservation of both suspensions of *C. gattii* and *C. neoformans* separately, as well as mixed, 0.1 mL of the final suspensions were inoculated into three plates containing YMA by the spread plate method. The plates were incubated at 25 °C for 48 h for subsequent counting of colony forming units per milliliter (CFU / mL). The CFU of two cryotubes/ampoules from each preservation method were evaluated and colonies were randomly selected and cultivated in CGB to evaluate the amounts of CGB positive (*C. gattii*) and CGB negative (*C. neoformans*) colonies resulting from each preservation method after 1 week, 15 days, 1 month, 6 months and 1 year preserved. The amount of colonies cultivated on CGB medium was calculated using Kruskal-Wallis test, followed by Dunn's multiple comparison test for statistical differences between different methods. Wilcoxon test was applied to compare the proportion CGB+ and CGB- over the times with the different methods. In all cases P values ≤ 0.05 were considered significant. All data were analyzed, and plots were carried out using Prism 7 (GraphPad Inc.) software.

RESULTS

The viability count before preservation in skim milk of *C. gattii* and *C. neoformans* separately were 47 CFU/100µL and 50.33 CFU/100µL, respectively. In glycerol they were 27.66 CFU/100µL and 32.66 CFU/100µL, respectively. After the mixing the species, the viable count was 40 CFU/100µL in skim milk and 32 CFU/100µL in glycerol. The recovery percentage of *C. neoformans* after mixture using CGB was 45.8% in skim milk and 54.7% in glycerol; *C. gatti* presented 54.1% and 45.3%, respectively. The viability counts of *C. neoformans*, *C. gattii* and mixed cells before preservation in both skim milk 20% and 15% glycerol suspensions did not show significant variations (Fig 1).

The viability of cells after preservation demonstrated that freeze-drying obtained the highest mean viability of mixed cultures, with a 33% loss in cumulative counts over 1 year. The strains preserved at -20 °C presented a loss of 52.25%. A higher loss (about 72-80%) was observed in the methodologies with glycerol at -80 °C and -196 °C, already at the first counting point (1 week), but maintained the viability counts stable in the other periods ($p=0.0530$ and $p=0.2261$, respectively).

The recovery of *C. gattii* and *C. neoformans* colonies varied according to the methodology employed and the period studied. The recovery of *C. neoformans* colonies was significantly higher than that of *C. gattii* colonies after preservation over 4 weeks at -20 °C, and only in 4 weeks for freeze-drying. On the other hand, *C. gattii* overcame *C. neoformans* viable count after 1 week at -80 °C and 48 weeks at -196 °C ($p<0.05$) (figure 2). Considering that the differences observed at only one point can be a consequence of a bias in randomly picked colonies, only cryopreservation at -20 °C demonstrated to favor *C. neoformans* species in a long-term storage. Freeze-drying showed a good performance maintaining the proportion of both species, as well as ultra-cryopreservation at -80 °C and -196 °C, although the last one showed the lowest mean viability counts.

DISCUSSION

The agents of cryptococcosis, members of the *C. neoformans* and *C. gattii* species complexes, are traditionally divided in 8 major molecular types. These molecular types differ in their epidemiological, ecological and molecular characteristics [(22)], antifungal susceptibility [(23)], clinical presentations and therapeutic outcomes [(24)]. Infections caused by *C. gattii* often have a worse prognosis than those caused by *C. neoformans* [(25)], but in the regions where both species complexes are endemic, mixed infections are not rare. Thus, it is important to maintain the original isolates in long-term storage for future studies on mixed infections and its clinical outcomes.

The freezing at -20 °C method obtained a good total cell recovery, although the recovery of both species *C. gattii* and *C. neoformans* was unequal after one month, indicating *C. neoformans* is more resistant to this method. Freeze-drying showed the highest total cell viability counts and on the maintenance of viability of both species. The ultra-freezing methods (-80 °C and -196 °C) performed equally well in the maintenance of both species, despite the low overall viability counts. In the

present work, skim milk as cryoprotectant presented a better recovery of cells in general (at -20 °C as well as freeze-drying) than glycerol. Viability counting after preservation using glycerol demonstrated a higher loss of cells, this could be related to the amount of cells used in this study, which is smaller than the concentration of at least 10^7 CFU/ml recommended [(26)]. In the CFRVS collection, the cryopreservation methods with glycerol at 15% do not show significant decrease in viability after 7 days preservation, and it is usually without logarithmic loss. Another possible reason for the high cell loss using glycerol is that the mode of action of these different cryoprotectants can influence the cell viability, since the skim milk acts extracellularly and the glycerol intracellularly, with the last one can cause higher cellular damage [(12)].

According to our results, freeze-drying should be preferred for the maintenance of multi-lineages isolates, but it requires specialized and expensive equipment [(10)], which are not often present in the research laboratories. Freezing at –20°C is low-cost and used to keep a few organisms for as long as 1–2 years [(27)]. At this storage temperature the preservation period varies depending on the medium used [(26)]. For example, although the preservation at -20°C did not perform satisfactorily in the present study, adjustments, as the use of combined cryoprotectants, should be evaluated, especially in facilities where more than one methodology can be used. Reductions in labor costs, operation time and the space required for the containment of a large number of cultures are also desirable [(28)]. Nevertheless, such universal method has not been created up to now. Therefore it is highly desirable to develop new or improve the current preservation methods, combining advantages and eliminate disadvantages of individual techniques [(10)].

Cryopreservation at -20 °C favors *C. neoformans* isolates and further studies on long-term storage conditions are necessary. The methods that used skim milk (freezing at -20 °C and freeze-drying) had a smaller loss of the initial suspension, already the ultra-freezing methods had, despite the significant loss over the initial suspension, stable counts for both species and adjustments in the concentration and composition of the cryoprotectant can improve the perfomance of these preservation methods. Finally freeze-drying should be preferred for the maintenance of multi-lineages isolates of isolates of the *C. neoformans* and *C. gattii* species complexes.

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AUTHOR'S CONTRIBUTION

CRSN and LT - conception of the study and writing of the manuscript; CRSN, ASSS and RMG - participated in the design; MSL - writing of the manuscript.

REFERENCES

1. Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM: Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. Aids. 2009; 23:525-530.

2. Desnos-Ollivier M, Patel S, Spaulding AR, Charlier C, Garcia-Hermoso D, Nielsen K, Dromer F: Mixed Infections and In Vivo Evolution in the Human Fungal Pathogen *Cryptococcus neoformans*. mBio. 2010; 18(1):e00091-10-e00091-10.

3. Swift HF. Preservation of stock cultures of bacteria by freezing and drying. J Exp Med. 1921; 33(1):69–75.

4. Nakasone KK, Petersonn SW, Jong S-C. Preservation and distribution of fungal cultures. *In:* Mueller GM, Bills GF, Foster MS, Biodiversity of fungi. Amsterdan: Elsevier; 2004. 37-47.

5. Polo L, Mañes-Lázaro R, Olmeda I, Cruz-Pio LE, Medina A, Ferrer S, Pardo I. Influence of freezing temperatures prior to freeze-drying on viability of yeasts and lactic acid bacteria isolated from wine. J Appl Microbiol. 2017; 122(6):1603–1614.

6. Atkin L, Moses W, Gray PP. The preservation of yeast cultures by lyophilization. J Bacteriol. 1949; 57(6):575.

7. Lodato P, Segovia de Huergo M, Buera MP. Viability and thermal stability of a strain of *Saccharomyces cerevisiae* freeze-dried in different sugar and polymer matrices. Appl Microbiol Biotechnol. 1999; 52(2):215–220.

8. Dumont F, Marechal P-A, Gervais P. Involvement of Two Specific Causes of Cell Mortality in Freeze-Thaw Cycles with Freezing to -196 C. Appl Environ Microbiol. 2006; 72(2):1330–1335.

9. Hubalek Z, Kocková-Kratochvílová A. Liquid nitrogen storage of yeast cultures I. Survival, and literature review of the preservation of fungi at ultralow temperatures. Antonie Van Leeuwenhoek. 1978; 44(2):229–241.

10. Homolka L. Preservation of live cultures of basidiomycetes – Recent methods. Fungal Biol. 2014; 118(2):107–25.

11. Alonso S. Novel Preservation Techniques for Microbial Cultures. *In:* Ojha KS, Tiwari BK, Novel Food Fermentation Technologies [Internet]. Switzerland: Springer International; 2016. 7–33. Available from: [http://link.springer.com/10.1007/978-3-319-42457-6_2]

12. Abadias M, Benabarre A, Teixido N, Usall J, Vinas I. Effect of freeze drying and protectants on viability of the biocontrol yeast *Candida sake*. Int J Food Microbiol. 2001; 65(3):173–182.

13. Coutinho C, Bernardes E, Félix D, Panek AD. Trehalose as cryoprotectant for preservation of yeast strains. J Biotechnol. 1988; 7(1):23–32.

14. Hubálek Z. Protectants used in the cryopreservation of microorganisms. Cryobiology. 2003; 46(3):205–29.

15. Cody WL, Wilson JW, Hendrixson DR, McIver KS, Hagman KE, Ott CM, Nickerson CA, Schurr MJ. Skim milk enhances the preservation of thawed –80 °C bacterial stocks. J Microbiol Methods. 2008; 75(1):135–138.

16. Prakash O, Nimonkar Y, Shouche YS. Practice and prospects of microbial preservation. FEMS Microbiol Lett. 2013; 339(1):1–9.

17. Qiangqiang Z, Jiajun W, Li L. Storage of fungi using sterile distilled water or lyophilization: comparison after 12 years. Mycoses. 1998; 41(5–6):255–257.

18. Voyron S, Roussel S, Munaut F, Varese GC, Ginepro M, Declerck S, Marchisio V. Vitality and genetic fidelity of white-rot fungi mycelia following different methods of preservation. Mycol Res. 2009; 113(10):1027–1038.

19. Smith D, Onions AH. A comparison of some preservation techniques for fungi. Trans Br Mycol Soc. 1983; 81(3):535–540.

20. Fortes ST, Lazéra MS, Nishikawa MM, Macedo RCL, Wanke B: First isolation of *Cryptococcus neoformans* var. *gattii* from a native jungle tree in the Brazilian Amazon rainforest. Mycoses. 2001; 44(5):137–40.

21. Rosas C de O, Brandão MLL, Bricio SML, Medeiros V de M, Bernardo SPC, La Cruz D, Cardarelli-Leite. Desenvolvimento de material de referência para ensaio de proficiência em microbiologia de alimentos. Rev Inst Adolfo Lutz. 2010; 69(1):15–22.

22. Meyer M, Gilgado F, Ngamskulrungroj P, Trilles L, Hagen F. Molecular Typing of the *Cryptococcus neoformans*/*C. gattii* species complex. *In:* Heitman J, Kozel T, Kwon-Chung J, Perfect J, Casadevall, *Cryptococcus*: from human pathogen to model yeast. Washington: ASM Press; 2011. 327–357.

23. Trilles L, Meyer W, Wanke B, Guarro J, Lazéra MS. Correlation of antifungal susceptibility and molecular type within the *Cryptococcus neoformans/C. gattii* species complex. Med Mycol. 2012; 50(3):328–332.

24. Perfect JR, Dismukes WE, Dromer F, Goldman DL, Graybill JR, Hamill RJ, Harrison TS, Larsen RA, Lortholary O, Nguyen MH, Pappas PG, Powderly WG, Singh N, Sobel JD, Sorrell TC. Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the infectious diseases society of America. Clin Infect Dis. 2010; 50(3): 291–322.

25. Sorrell TC. *Cryptococcus neoformans* variety *gattii*. Med Mycol. 2001; 39:155–168.

26. Heckly R. Preservation of Microorganisms. Academic Press; 1978.

27. Tedeschi R, De Paoli P. Collection and Preservation of Frozen Microorganisms. *In:* Dillner J, Methods in Biobanking. Totowa NJ: Humana Press; 2011. 313–326.

28. Challen MP, Elliot TJ. Polypropylene straw ampoules for the storage of microorganisms in liquid nitrogen. J Microbiol Methods. 1986; 5(1):11–22.

Figures:

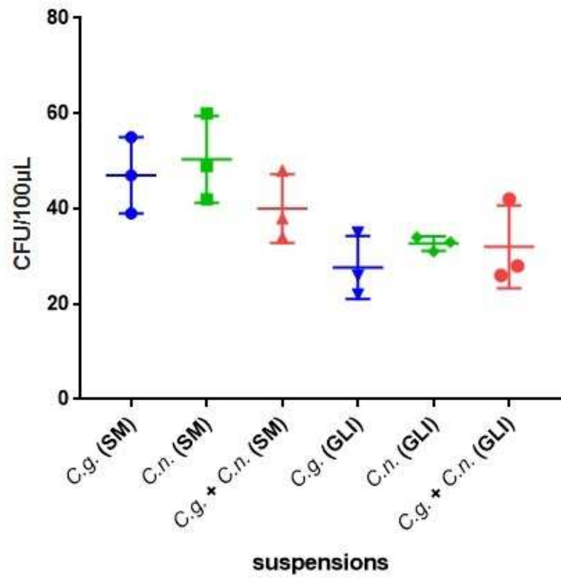

Figure 1: Colony Forming Units of *C. gattii* (C.g.) and *C. neoformans* (C.n.) separately and mixed in 20% skim milk (SM) and 15% glycerol (GLI) before preservation.

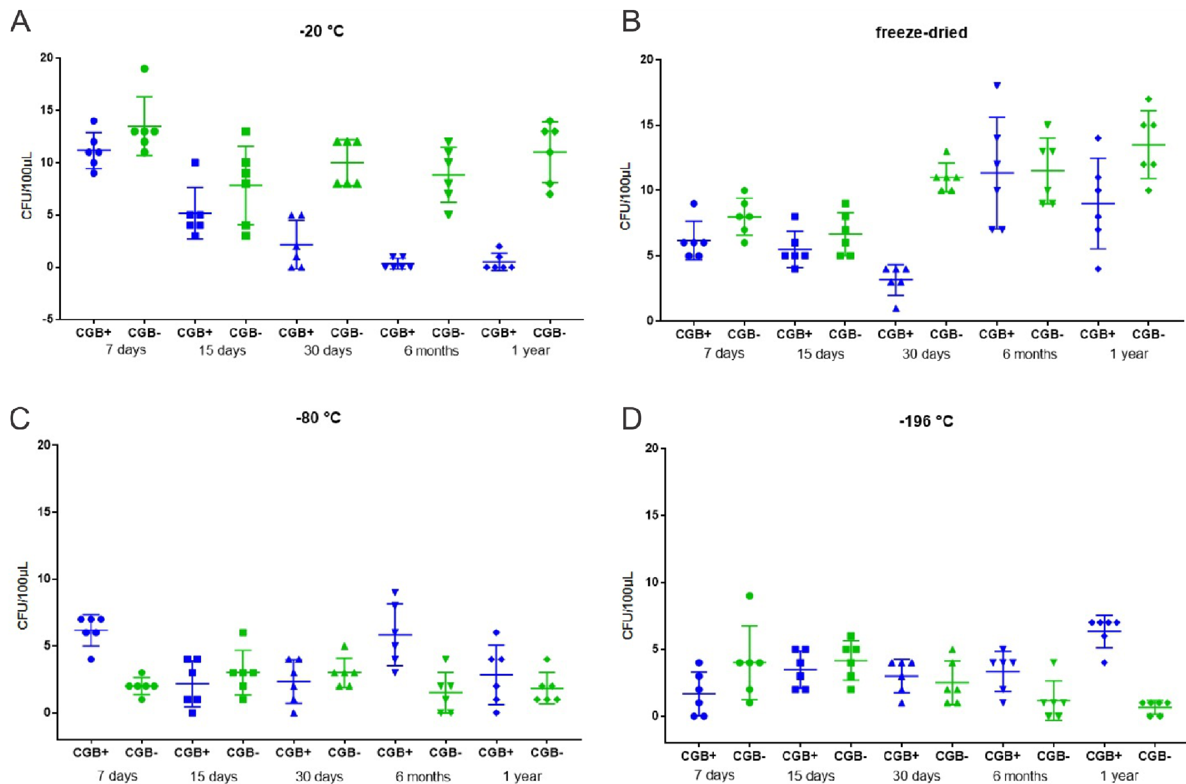

Figure 2: Cell counts of *C. gattii* and *C. neoformans* colonies in different periods and methods of preservation; at -20 °C (A), freeze-dried (B), at -80 °C (C) and at -196 °C (D).

CONCLUSÕES

4. CONCLUSÕES

Uma única colônia isolada pode conter mais de uma espécie, principalmente se essas espécies forem próximas geneticamente e compartilharem o mesmo micro-habitat, como é o caso de *C. neoformans* e *C. gattii*;

Cepa de *C. neoformans* e *C. gattii* oriunda de colônia isolada pode conter grande heterogeneidade fenotípica e genotípica dentre as diferentes células, incluindo importantes fatores de virulência como produção de melanina; espessura de cápsula e termotolerância, porém esta heterogeneidade só é observada se colônias isoladas de mesma origem forem estudadas individualmente;

A heterogeneidade fenotípica e genotípica observada em colônias isoladas obtidas de cepas atípicas - seja *C. neoformans* ou *C. gattii* - pode trazer dificuldades para o diagnóstico etiológico preciso, bem como, para reprodutibilidade em estudos de sensibilidade *in vitro* e de virulência, necessitando devida atenção;

Não houve sinergismo da virulência de colônias mistas no modelo animal proposto, tampouco foi observado ciclo sexuado *in vivo* que justifique a plasticidade fenotípica observada, mas outras cepas e modelos devem ser estudados no futuro;

Atenção deve ser dada aos estudos de termotolerância a 40 °C, pois o não crescimento a esta temperatura pode não causar morte celular, ocorrendo o crescimento de algumas células após reincubação em temperatura ideal de 25 °C, demonstrando os diferentes graus de termotolerância entre as colônias após reincubação;

A preservação adequada de primo-isolados é fundamental para avaliação da sua estrutura populacional em estudos futuros. Neste estudo os métodos de liofilização e criopreservação demonstraram eficiência na preservação de ambas espécies (*C. neoformans* e *C. gattii).*

ASPECTOS ÉTICOS

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O projeto de tese foi apresentado ao Comitê de Ética em Pesquisa (CEP/INI) em 30/04/2014 e pela natureza do projeto, após análise do comitê, evidenciou-se que não há necessidade de sua apreciação pelo CEP, podendo ser submetido à publicação e divulgação dos resultados.

REFERÊNCIAS BIBLIOGRÁFICAS

5. REFERÊNCIAS BIBLIOGRÁFICAS

ABADIAS, M. et al. Effect of freeze drying and protectants on viability of the biocontrol yeast *Candida sake*. **International Journal of Food Microbiology**, v. 65, n. 3, p. 173–182, 2001.

ALMEIDA, A. M. F. et al. Molecular typing and antifungal susceptibility of clinical sequential isolates of *Cryptococcus neoformans* from Sao Paulo State, Brazil: Molecular typing and antifungal susceptibility of *C. neoformans*. **FEMS Yeast Research**, v. 7, n. 1, p. 152–164, jan. 2007.

ALONSO, S. Novel Preservation Techniques for Microbial Cultures. In: OJHA, K. S.; TIWARI, B. K. (Eds.). . **Novel Food Fermentation Technologies**. Cham: Springer International Publishing, 2016. p. 7–33.

ALVES, G. S. B. et al. Molecular typing of environmental *Cryptococcus neoformans* / *C. gattii* species complex isolates from Manaus, Amazonas, Brazil. **Mycoses**, v. 59, n. 8, p. 509–515, ago. 2016.

AMINNEJAD, M. et al. Identification of Novel Hybrids Between *Cryptococcus neoformans* var. *grubii* VNI and *Cryptococcus gattii* VGII. **Mycopathologia**, v. 173, n. 5–6, p. 337–346, jun. 2012.

ATKIN, L.; MOSES, W.; GRAY, P. P. The preservation of yeast cultures by lyophilization. **Journal of bacteriology**, v. 57, n. 6, p. 575, 1949.

AVERY, S. V. Microbial cell individuality and the underlying sources of heterogeneity. **Nature Reviews Microbiology**, v. 4, n. 8, p. 577–587, ago. 2006.

BARBOSA, G. G. et al. *Cryptococcus gattii* VGI and *Cryptococcus neoformans* VNI Associated with Wood Decay in Ficus Hollow Trees in Rio de Janeiro, Brazil. **British Microbiology Research Journal**, v. 3, n. 1, p. 106, 2013.

BARCHIESI, F. Activity of the new antifungal triazole, posaconazole, against *Cryptococcus neoformans*. **Journal of Antimicrobial Chemotherapy**, v. 48, n. 6, p. 769–773, 1 dez. 2001.

BARCHIESI, F. et al. Comparative analysis of pathogenicity of *Cryptococcus neoformans* serotypes A, D and AD in murine cryptococcosis. **Journal of Infection**, v. 51, n. 1, p. 10–16, jul. 2005.

BENNETT, R. J.; FORCHE, A.; BERMAN, J. Rapid Mechanisms for Generating Genome Diversity: Whole Ploidy Shifts, Aneuploidy, and Loss of Heterozygosity. **Cold Spring Harbor Perspectives in Medicine**, v. 4, n. 10, p. a019604–a019604, 1 out. 2014.

BINDER, U.; MAURER, E.; LASS-FLÖRL, C. *Galleria mellonella*: An invertebrate model to study pathogenicity in correctly defined fungal species. **Fungal Biology**, v. 120, n. 2, p. 288–295, fev. 2016.

BLAKE, W. J. et al. Noise in eukaryotic gene expression. **Nature**, v. 422, n. 6932, p. 633–637, abr. 2003.

BOEKHOUT, T. et al. Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. **Microbiology**, v. 147, n. 4, p. 891–907, 2001.

BOVERS, M; HAGEN, F; BOEKHOUT, T. Diversity of the *Cryptococcus neoformans-Cryptococcus gattii* species complex. **Revista Iberoamericana de Micología**, v. 25, n. 1, p. S4-S12, mar. 2008.

BOVERS, M. et al. AIDS Patient Death Caused by Novel *Cryptococcus neoformans* × *C . gattii* Hybrid. **Emerging Infectious Diseases**, v. 14, n. 7, p. 1105–1108, jul. 2008.

BOVERS, M. et al. Unique hybrids between the fungal pathogens *Cryptococcus neoformans* and *Cryptococcus gattii*. **FEMS Yeast Research**, v. 6, n. 4, p. 599–607, jun. 2006.

BRITO, A. M. DE; CASTILHO, E. A. DE; SZWARCWALD, C. L. AIDS e infecção pelo HIV no Brasil: uma epidemia multifacetada. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 34, n. 2, p. 207–217, abr. 2001.

BRITO-SANTOS, F. et al. Environmental Isolation of *Cryptococcus gattii* VGII from Indoor Dust from Typical Wooden Houses in the Deep Amazonas of the Rio Negro Basin. **PLOS ONE**, v. 10, n. 2, p. e0115866, 17 fev. 2015.

BUCHANAN, K. L.; MURPHY, J. W. What makes *Cryptococcus neoformans* a pathogen? **Emerging infectious diseases**, v. 4, n. 1, p. 71, 1998.

BYRNES, E. J. et al. First Reported Case of *Cryptococcus gattii* in the Southeastern USA: Implications for Travel-Associated Acquisition of an Emerging Pathogen. **PLoS ONE**, v. 4, n. 6, p. e5851, 10 jun. 2009.

CALLEJAS, A. et al. First isolation of *Cryptococcus neoformans* var. *gattii*, serotype C, from the environment in Colombia. **Medical Mycology**, v. 36, n. 5, p. 341–344, 9 jan. 1998.

CASADEVALL, A. et al. The Capsule of *Cryptococcus neoformans*. **Virulence**, p. 00–00, 13 fev. 2018.

CASADEVALL, A.; PERFECT, J. R. **Cryptococcus Neoformans**. [s.l.] ASM Press, 1998.

CASALI, A. et al. Molecular typing of clinical and environmental isolates in the Brazilian state Rio Grande do Sul. **FEMS Yeast Research**, v. 3, n. 4, p. 405–415, jun. 2003.

CHALLEN, M. P.; ELLIOT, T. J. Polypropylene straw ampoules for the storage of microorganisms in liquid nitrogen. **Journal of Microbiological Methods**, v. 5, n. 1, p. 11–22, 1986.

CHATURVEDI, S. et al. Direct PCR of *Cryptococcus neoformans* MATα and MATa Pheromones To Determine Mating Type, Ploidy, and Variety: a Tool for Epidemiological and Molecular Pathogenesis Studies. **Journal of Clinical Microbiology**, v. 38, n. 5, p. 2007–2009, maio 2000.

CHEN, Y.-L. et al. Calcineurin Governs Thermotolerance and Virulence of *Cryptococcus gattii*. **G3: Genes|Genomes|Genetics**, v. 3, n. 3, p. 527–539, mar. 2013.

CLEMONS, K. V.; STEVENS, D. A. Cryptococcosis in Experimental Animals: Lessons Learned. In: KWON-CHUNG, K. J. et al. (Eds.). . **Cryptococcus**. [s.l.] American Society of Microbiology, 2011. p. 473–488.

CLINICAL AND LABORATORY STANDARDS INSTITUTE (ED.). **Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard**. 3rd ed ed. Wayne, PA: National Committee for Clinical Laboratory Standards, 2008.

CODY, W. L. et al. Skim milk enhances the preservation of thawed –80 °C bacterial stocks. **Journal of Microbiological Methods**, v. 75, n. 1, p. 135–138, set. 2008.

COUTINHO, C. et al. Trehalose as cryoprotectant for preservation of yeast strains. **Journal of biotechnology**, v. 7, n. 1, p. 23–32, 1988.

DAVIS, J. et al. **A Zebrafish Model of Cryptococcosis**. Open Forum Infectious Diseases. **Anais**...Oxford University Press, 2015Disponível em: <https://academic.oup.com/ofid/article/2633876>. Acesso em: 7 ago. 2017

DESNOS-OLLIVIER, M. et al. Mixed Infections and In Vivo Evolution in the Human Fungal Pathogen *Cryptococcus neoformans*. **mBio**, v. 1, n. 1, p. e00091-10-e00091-10, 18 maio 2010.

DOS SANTOS, W. R. A. et al. Primary endemic Cryptococcosis gattii by molecular type VGII in the state of Pará, Brazil. **Memórias do Instituto Oswaldo Cruz**, v. 103, n. 8, p. 813–818, 2008.

DUMONT, F.; MARECHAL, P.-A.; GERVAIS, P. Involvement of Two Specific Causes of Cell Mortality in Freeze-Thaw Cycles with Freezing to -196 C. **Applied and Environmental Microbiology**, v. 72, n. 2, p. 1330–1335, 1 fev. 2006.

ELLIS, D. H.; PFEIFFER, T. J. Natural habitat of *Cryptococcus neoformans* var. *gattii*. **Journal of Clinical Microbiology**, v. 28, n. 7, p. 1642–1644, 1990.

ESPINEL-INGROFF, A. et al. *Cryptococcus neoformans-Cryptococcus gattii* Species Complex: an International Study of Wild-Type Susceptibility Endpoint Distributions and Epidemiological Cutoff Values for Amphotericin B and Flucytosine. **Antimicrobial Agents and Chemotherapy**, v. 56, n. 6, p. 3107–3113, jun. 2012a.

ESPINEL-INGROFF, A. et al. *Cryptococcus neoformans-Cryptococcus gattii* Species Complex: an International Study of Wild-Type Susceptibility Endpoint Distributions and Epidemiological Cutoff Values for Fluconazole, Itraconazole, Posaconazole, and Voriconazole. **Antimicrobial Agents and Chemotherapy**, v. 56, n. 11, p. 5898–5906, nov. 2012b.

ESPINEL-INGROFF, A.; KIDD, S. Current trends in the prevalence of *Cryptococcus gattii* in the United States and Canada. **Infection and Drug Resistance**, p. 89, maio 2015.

FANG, W.; FA, Z.; LIAO, W. Epidemiology of *Cryptococcus* and cryptococcosis in China. **Fungal Genetics and Biology**, v. 78, p. 7–15, maio 2015.

FERNANDES, K. E. et al. Species in the *Cryptococcus gattii* Complex Differ in Capsule and Cell Size following Growth under Capsule-Inducing Conditions. **mSphere**, v. 1, n. 6, p. e00350-16, 28 dez. 2016.

FIGUEIREDO, T. P. et al. Antifungal susceptibility testing and genotyping characterization of *Cryptococcus neoformans* and *gattii* isolates from hiv-infected patients of Ribeirão Preto, São Paulo, BRAZIL. **Revista do Instituto de Medicina Tropical de São Paulo**, v. 58, n. 0, 2016.

FIRACATIVE, C.; DUAN, S.; MEYER, W. *Galleria mellonella* model identifies highly virulent strains among all major molecular types of *Cryptococcus gattii*. **PLoS ONE**, v. 9, n. 8, p. e105076, 18 ago. 2014.

FORTES, S. T. et al. First isolation of *Cryptococcus neoformans* var. *gattii* from a native jungle tree in the Brazilian Amazon rainforest. **Mycoses**, v. 44, n. 5, p. 137–140, 1 jul. 2001.

FRANZOT, S. P. et al. Microevolution of a standard strain of *Cryptococcus neoformans* resulting in differences in virulence and other phenotypes. **Infection and immunity**, v. 66, n. 1, p. 89–97, 1998.

FRIES, B. C.; COX, G. M. Cryptococcosis in AIDS. In: KWON-CHUNG, K. J. et al. (Eds.). . **Cryptococcus**. [s.l.] American Society of Microbiology, 2011. p. 515–525.

FUCHS, B. B. et al. Methods for using *Galleria mellonella* as a model host to study fungal pathogenesis. **Virulence**, v. 1, n. 6, p. 475–482, nov. 2010.

G. FLORES, V. et al. Meningitis Due to *Cryptococcus neoformans*: Treatment with Posaconazole. **Current HIV Research**, v. 10, n. 7, p. 620–623, 30 set. 2012.

GARCÍA-RODAS, R. et al. *Cryptococcus neoformans* capsular enlargement and cellular gigantism during *Galleria mellonella* infection. **PLoS ONE**, v. 6, n. 9, p. e24485, 7 set. 2011.

HAGEN, F. et al. In vitro antifungal susceptibilities and amplified fragment length polymorphism genotyping of a worldwide collection of 350 clinical, veterinary, and environmental *Cryptococcus gattii* isolates. **Antimicrobial Agents and Chemotherapy**, v. 54, n. 12, p. 5139–5145, 1 dez. 2010.

HAMILTON, A. J.; HOLDOM, M. D. Antioxidant systems in the pathogenic fungi of man and their role in virulence. **Medical Mycology**, v. 37, n. 6, p. 375–389, 1999.

HECKLY, R. **Preservation of Microorganisms**. [s.l.] Academic Press, 1978.

HEWITT, S. K. et al. Phenotypic heterogeneity in fungi: Importance and methodology. **Fungal Biology Reviews**, v. 30, n. 4, p. 176–184, out. 2016.

HOLLAND, S. L. et al. Phenotypic heterogeneity is a selected trait in natural yeast populations subject to environmental stress: Phenotypic heterogeneity is a selected trait. **Environmental Microbiology**, v. 16, n. 6, p. 1729–1740, jun. 2014.

HOMOLKA, L. Preservation of live cultures of basidiomycetes – Recent methods. **Fungal Biology**, v. 118, n. 2, p. 107–125, fev. 2014.

HUBÁLEK, Z. Protectants used in the cryopreservation of microorganisms. **Cryobiology**, v. 46, n. 3, p. 205–229, jun. 2003.

HUBALEK, Z.; KOCKOVÁ-KRATOCHVÍLOVÁ, A. Liquid nitrogen storage of yeast cultures I. Survival, and literature review of the preservation of fungi at ultralow temperatures. **Antonie van Leeuwenhoek**, v. 44, n. 2, p. 229–241, 1978.

HULL, C. M.; HEITMAN, J. Genetics of *Cryptococcus neoformans*. **Annual Review of Genetics**, v. 36, n. 1, p. 557–615, dez. 2002.

IGREJA, R. P. et al. Molecular epidemiology of *Cryptococcus neoformans* isolates from AIDS patients of the Brazilian city, Rio de Janeiro. **Medical Mycology**, v. 42, n. 3, p. 229–238, jan. 2004.

IROKANULO, E. A. O.; AKUESHI, C. O. Virulence of *Cryptococcus neoformans* serotypes A, B, C and D for four mouse strains. **Journal of medical microbiology**, v. 43, n. 4, p. 289–293, 1995.

JACOBSON, E. S.; EMERY, H. S. Temperature regulation of the cryptococcal phenoloxidase. **Journal of Medical and Veterinary Mycology**, v. 29, n. 2, p. 121–124, 1 mar. 1991.

JAIN, N.; FRIES, B. C. Phenotypic Switching of *Cryptococcus neoformans* and *Cryptococcus gattii*. **Mycopathologia**, v. 166, n. 4, p. 181–188, out. 2008.

KASSI, F. K. et al. First case of mixed infection with *Cryptococcus deuterogattii* and *Cryptococcus neoformans* VNI in an Ivorian HIV-positive patient. **JMM Case Reports**, v. 3, n. 4, 11 jul. 2016.

KIDD, S. E. et al. A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). **Proceedings of the National Academy of Sciences of the United States of America**, v. 101, n. 49, p. 17258–17263, 7 dez. 2004.

KON, A. S. et al. Guidelines In Cryptococcosis-2008 [consenso Em Criptococose-2008]. **Revista da Sociedade Brasileira de Medicina Tropical**, 2008.

KWON-CHUNG, K. J. Chapter 114 - *Filobasidiella* Kwon-Chung (1975) A2 - Boekhout, Cletus P. KurtzmanJack W. FellTeun. In: **The Yeasts (Fifth Edition)**. London: Elsevier, 2011. p. 1443–1455.

KWON-CHUNG, K. J. et al. The case for adopting the “species complex” nomenclature for the etiologic agents of cryptococcosis. **mSphere**, v. 2, n. 1, p. e00357-16, 22 fev. 2017.

KWON-CHUNG, K. J.; BENNETT, J. E. Cryptococcosis. In: **Medical Mycology**. [s.l.] Lea & Febiger, 1992. p. 392–446.

KWON-CHUNG, K. J.; POLACHECK, I.; POPKIN, T. J. Melanin-lacking mutants of *Cryptococcus neoformans* and their virulence for mice. **Journal of bacteriology**, v. 150, n. 3, p. 1414–1421, 1982.

KWON-CHUNG, K. J.; RHODES, J. C. Encapsulation and melanin formation as indicators of virulence in *Cryptococcus neoformans*. **Infection and Immunity**, v. 51, n. 1, p. 218–223, 1986.

LAZÉRA, M. S. et al. *Cryptococcus neoformans* var. *gattii* — evidence for a natural habitat related to decaying wood in a pottery tree hollow. **Medical Mycology**, v. 36, n. 2, p. 119–122, 3 jan. 1998.

LAZERA, M. S. et al. Possible primary ecological niche of *Cryptococcus neoformans*. **Medical Mycology**, v. 38, n. 5, p. 379–383, 9 jan. 2000.

LEAL, A. L. et al. Epidemiological profile of cryptococcal meningitis patients in Rio Grande do Sul, Brazil. **Mycopathologia**, v. 166, n. 2, p. 71, 29 abr. 2008.

LEIMANN, B. C. Q.; KOIFMAN, R. J. Cryptococcal meningitis in Rio de Janeiro State, Brazil, 1994-2004. **Cadernos de Saúde Pública**, v. 24, n. 11, p. 2582–2592, nov. 2008.

LENGELER, K. B.; COX, G. M.; HEITMAN, J. Serotype AD strains of *Cryptococcus neoformans* are diploid or aneuploid and are heterozygous at the mating-type locus. **Infection and Immunity**, v. 69, n. 1, p. 115–122, 1 jan. 2001.

LEV, S. et al. Phospholipase C of *Cryptococcus neoformans* regulates homeostasis and virulence by providing inositol trisphosphate as a substrate for Arg1 kinase. **Infection and Immunity**, v. 81, n. 4, p. 1245–1255, abr. 2013.

LIN, X. *Cryptococcus neoformans*: Morphogenesis, infection, and evolution. **Infection, Genetics and Evolution**, v. 9, n. 4, p. 401–416, jul. 2009.

LIN, X. et al. Diploids in the *Cryptococcus neoformans* serotype A population homozygous for the α mating type originate via unisexual mating. **PLoS Pathogens**, v. 5, n. 1, p. e1000283, 30 jan. 2009.

LIN, X. et al. αADα Hybrids of *Cryptococcus neoformans*: Evidence of same-sex mating in nature and hybrid fitness. **PLoS Genetics**, v. 3, n. 10, p. e186, 2007.

LINDBERG, J. et al. *Cryptococcus gattii* risk for tourists visiting Vancouver Island, Canada. **Emerging Infectious Diseases**, v. 13, n. 1, p. 178–179, jan. 2007.

LINDENBERG, A. D. S. C. et al. Clinical and epidemiological features of 123 cases of cryptococcosis in Mato Grosso do Sul, Brazil. **Revista do Instituto de Medicina Tropical de São Paulo**, v. 50, n. 2, p. 75–78, abr. 2008.

LITVINTSEVA, A. P. Multilocus Sequence Typing Reveals Three Genetic Subpopulations of *Cryptococcus neoformans* var. *grubii* (Serotype A), including a unique population in Botswana. **Genetics**, v. 172, n. 4, p. 2223–2238, 15 dez. 2005.

LOCKHART, S. R.; GHANNOUM, M. A.; ALEXANDER, B. D. Establishment and use of epidemiological cutoff values for molds and yeasts by use of the Clinical and Laboratory Standards Institute M57 Standard. **Journal of Clinical Microbiology**, v. 55, n. 5, p. 1262–1268, maio 2017.

LODATO, P.; SEGOVIA DE HUERGO, M.; BUERA, M. P. Viability and thermal stability of a strain of *Saccharomyces cerevisiae* freeze-dried in different sugar and polymer matrices. **Applied Microbiology and Biotechnology**, v. 52, n. 2, p. 215–220, 23 ago. 1999.

MANDAL, P. et al. Dual infections with pigmented and albino strains of *Cryptococcus neoformans* in patients with or without human immunodeficiency virus infection in India. **Journal of Clinical Microbiology**, v. 43, n. 9, p. 4766–4772, 1 set. 2005.

MARTINEZ, L. R.; GARCIA-RIVERA, J.; CASADEVALL, A. *Cryptococcus neoformans* var. *neoformans* (Serotype D) strains are more susceptible to heat than *C. neoformans* var. *grubii* (serotype A) strains. **Journal of Clinical Microbiology**, v. 39, n. 9, p. 3365–3367, 1 set. 2001.

MARTINS, L. M. S. et al. Genotypes of *Cryptococcus neoformans* and *Cryptococcus gattii* as agents of endemic cryptococcosis in Teresina, Piauí (northeastern Brazil). **Memorias do Instituto Oswaldo Cruz**, v. 106, n. 6, p. 725–730, 2011.

MAZIARZ, E. K.; PERFECT, J. R. Cryptococcosis. **Infectious Disease Clinics of North America**, v. 30, n. 1, p. 179–206, mar. 2016.

MEYER, W. et al. Consensus multi-locus sequence typing scheme for *Cryptococcus neoformans* and *Cryptococcus gattii*. **Medical Mycology**, v. 47, n. 6, p. 561–570, jan. 2009.

MEYER, W. et al. Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. **Journal of Clinical Microbiology**, v. 31, n. 9, p. 2274–2280, set. 1993.
MEYER, W. et al. Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. **Journal of Clinical Microbiology**, v. 31, n. 9, p. 2274–2280, set. 1993.
MEYER, W. et al. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. **Emerging infectious diseases**, p. 189–195, 2003.

MOLLOY, S. F. et al. Antifungal combinations for treatment of cryptococcal meningitis in Africa. **New England Journal of Medicine**, v. 378, n. 11, p. 1004–1017, 15 mar. 2018.

MONTENEGRO, H.; PAULA, C. R. Environmental isolation of *Cryptococcus neoformans* var. *gattii* and *C. neoformans* var. *neoformans* in the city of São Paulo, Brazil. **Medical Mycology**, v. 38, n. 5, p. 385–390, 2000.

MORA, D. J. et al. Genotype and mating type distribution within clinical *Cryptococcus neoformans* and *Cryptococcus gattii* isolates from patients with cryptococcal meningitis in Uberaba, Minas Gerais, Brazil. **Medical Mycology**, v. 48, n. 4, p. 561–569, jun. 2010.

MUHAMMED, M. et al. Mouse models for the study of fungal pneumonia: A collection of detailed experimental protocols for the study of *Coccidioides*, *Cryptococcus*, *Fusarium*, *Histoplasma* and combined infection due to *Aspergillus-Rhizopus*. **Virulence**, v. 3, n. 3, p. 329–338, maio 2012.

MYLONAKIS, E. et al. *Galleria mellonella* as a model system to study *Cryptococcus neoformans* pathogenesis. **Infection and Immunity**, v. 73, n. 7, p. 3842–3850, 1 jul. 2005.

NGAMSKULRUNGROJ, P. et al. *Cryptococcus gattii* virulence composite: Candidate genes revealed by microarray analysis of high and less virulent Vancouver Island outbreak strains. **PLoS ONE**, v. 6, n. 1, p. e16076, 13 jan. 2011.

NIELSEN, K.; KWON-CHUNG, K. J. A Role for mating in cryptococcal virulence. In: KWON-CHUNG, K. J. et al. (Eds.). . **Cryptococcus**. [s.l.] American Society of Microbiology, 2011. p. 167–174.

NISHIKAWA, M. M. et al. Serotyping of 467 *Cryptococcus neoformans* isolates from clinical and environmental sources in Brazil: Analysis of host and regional patterns. **Journal of Clinical Microbiology**, v. 41, n. 1, p. 73–77, 1 jan. 2003.

NUNES, J. DE O. et al. The simultaneous occurrence of histoplasmosis and cryptococcal fungemia: A case report and review of the literature. **Mycopathologia**, v. 181, n. 11, p. 891–897, 1 dez. 2016.

NURUDEEN, T. A.; AHEARN, D. G. Regulation of melanin production by *Cryptococcus neoformans*. **Journal of clinical Microbiology**, v. 10, n. 5, p. 724–729, 1979.

OLIVEIRA-NETTO, I. C. et al. Meio século de criptococose no Brasil: revisão de 308 casos (1941-1992). **Âmbito Hosp**, v. 7, p. 5–16, 1993.

PAPPALARDO, M. C.; MELHEM, M. S. Cryptococcosis: a review of the Brazilian experience for the disease. **Revista do Instituto de Medicina Tropical de São Paulo**, v. 45, n. 6, p. 299–305, 2003.

PARK, B. J. et al. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. **Aids**, v. 23, p. 525-530, 2009.

PASSONI, LFC et al. *Cryptococcus neoformans* isolated from human dwellings in Rio de Janeiro, Brazil: an analysis of the domestic environment of AIDS patients with and without cryptococcosis. **Medical Mycology**, v. 36, p. 305–311, 1998.

PERFECT, J. R. *Cryptococcus neoformans*: A sugar-coated killer with designer genes. **FEMS Immunology & Medical Microbiology**, v. 45, n. 3, p. 395–404, set. 2005.

PERFECT, J. R. *Cryptococcus neoformans*: the yeast that likes it hot. **FEMS Yeast Research**, v. 6, n. 4, p. 463–468, jun. 2006.

PERFECT, J. R. et al. Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the Infectious Diseases Society of America. **Clinical Infectious Diseases**, v. 50, n. 3, p. 291–322, fev. 2010.

PFALLER, M. A. Epidemiology of fungal infections: the promise of molecular typing. **Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America**, v. 20, n. 6, p. 1535–1539, jun. 1995.

POLACHECK, I.; LEBENS, G.; HICKS, J. B. Development of DNA probes for early diagnosis and epidemiological study of cryptococcosis in AIDS patients. **Journal of Clinical Microbiology**, v. 30, n. 4, p. 925–930, abr. 1992.

POLO, L. et al. Influence of freezing temperatures prior to freeze-drying on viability of yeasts and lactic acid bacteria isolated from wine. **Journal of Applied Microbiology**, v. 122, n. 6, p. 1603–1614, jun. 2017.

PRADO, M. et al. Mortality due to systemic mycoses as a primary cause of death or in association with AIDS in Brazil: a review from 1996 to 2006. **Memórias do Instituto Oswaldo Cruz**, v. 104, n. 3, p. 513–521, 2009.

PRAKASH, O.; NIMONKAR, Y.; SHOUCHE, Y. S. Practice and prospects of microbial preservation. **FEMS Microbiology Letters**, v. 339, n. 1, p. 1–9, fev. 2013.

QIANGQIANG, Z.; JIAJUN, W.; LI, L. Storage of fungi using sterile distilled water or lyophilization: comparison after 12 years. **Mycoses**, v. 41, n. 5–6, p. 255–257, 1998.

RAJASINGHAM, R. et al. Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis. **The Lancet Infectious Diseases**, v. 17, n. 8, p. 873–881, ago. 2017.

RHODES, J. C.; POLACHECK, I.; KWON-CHUNG, K. J. Phenoloxidase activity and virulence in isogenic strains of *Cryptococcus neoformans*. **Infection and Immunity**, v. 36, n. 3, p. 1175–1184, 1982.

ROSARIO, I. et al. Isolation of *Cryptococcus* species including *C. neoformans* from cloaca of pigeons. **Mycoses**, v. 48, n. 6, p. 421–424, 2005.

ROSAS, C. DE O. et al. Desenvolvimento de material de referência para ensaio de proficiência em microbiologia de alimentos. **Revista do Instituto Adolfo Lutz (Impresso)**, v. 69, n. 1, p. 15–22, 2010.

ROZENBAUM, R. et al. *Cryptococcus neoformans* varieties as agents of cryptococcosis in Brazil. **Mycopathologia**, v. 119, n. 3, p. 133–136, 1 set. 1992.

ROZENBAUM, R.; GONÇALVES, A. J. Clinical epidemiological study of 171 cases of cryptococcosis. **Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America**, v. 18, n. 3, p. 369–380, mar. 1994.

SAAG, M. S. et al. Practice Guidelines for the Management of Cryptococcal Disease. **Clinical Infectious Diseases**, v. 30, p. 9, 2000.

SABIITI, W.; MAY, R. C.; PURSALL, E. R. Experimental Models of Cryptococcosis. **International Journal of Microbiology**, v. 2012, p. 1–10, 2012.

SEVERO, L. C.; OLIVEIRA, F. DE M.; LONDERO, A. T. Cryptococcosis due to *Cryptococcus neoformans* var. *gattii* in Brazilian patients with AIDS. Report of three cases. **Revista Iberoamericana de Micología**, v. 16, p. 3, 1999.

SIA, R. A.; LENGELER, K. B.; HEITMAN, J. Diploid strains of the pathogenic basidiomycete *Cryptococcus neoformans* are thermally dimorphic. **Fungal Genetics and Biology**, v. 29, n. 3, p. 153–163, abr. 2000.

SINGH, A. et al. Factors required for activation of urease as a virulence determinant in *Cryptococcus neoformans*. **mBio**, v. 4, n. 3, p. e00220-13-e00220-13, 7 maio 2013.

SIONOV, E. et al. Heteroresistance to fluconazole in *Cryptococcus neoformans* is intrinsic and associated with virulence. **Antimicrobial Agents and Chemotherapy**, v. 53, n. 7, p. 2804–2815, 1 jul. 2009.

SMITH, D.; ONIONS, A. H. A comparison of some preservation techniques for fungi. **Transactions of the British Mycological Society**, v. 81, n. 3, p. 535–540, 1983.

SMITH, I. M. et al. Cryptococcosis due to *Cryptococcus gattii* in Germany from 2004–2013. **International Journal of Medical Microbiology**, v. 305, n. 7, p. 719–723, out. 2015.

SORRELL, T. C. *Cryptococcus neoformans* variety *gattii*. **Medical mycology**, v. 39, n. 2, p. 155–168, 2001.

SPINA-TENSINI, T. et al. Geographic distribution of patients affected by *Cryptococcus neoformans* / *Cryptococcus gattii* species complexes meningitis, pigeon and tree populations in Southern Brazil. **Mycoses**, v. 60, n. 1, p. 51–58, jan. 2017.

STEPHEN, C. et al. British Columbia: Multispecies outbreak of cryptococcosis on southern Vancouver Island, British Columbia. **The Canadian Veterinary Journal**, v. 43, n. 10, p. 792–794, out. 2002.

SUMNER, E. R.; AVERY, S. V. Phenotypic heterogeneity: differential stress resistance among individual cells of the yeast *Saccharomyces cerevisiae*. **Microbiology**, v. 148, n. 2, p. 345–351, 1 fev. 2002.

TANAKA, R. et al. Determination of ploidy in *Cryptococcus neoformans* by flow cytometry. **Journal of medical and veterinary mycology**, v. 34, n. 5, p. 299–301, 1996.

TEDESCHI, R.; DE PAOLI, P. Collection and preservation of frozen microorganisms. In: DILLNER, J. (Ed.). . **Methods in Biobanking**. Totowa, NJ: Humana Press, 2011. v. 675p. 313–326.

THOMPSON, G. R. et al. Phenotypic differences of *Cryptococcus* molecular types and their implications for virulence in a *Drosophila* model of infection. **Infection and Immunity**, v. 82, n. 7, p. 3058–3065, 1 jul. 2014.

TRILLES, L. et al. Correlation of antifungal susceptibility and molecular type within the *Cryptococcus neoformans/C. gattii* species complex. **Medical Mycology**, v. 50, n. 3, p. 328–332, abr. 2012.

TRILLES, L. et al. Genetic characterization of environmental isolates of the *Cryptococcus neoformans* species complex from Brazil. **Medical Mycology**, v. 41, n. 5, p. 383–390, 10 jan. 2003.

TRILLES, L. et al. Regional pattern of the molecular types of *Cryptococcus neoformans* and *Cryptococcus gattii* in Brazil. **Memórias do Instituto Oswaldo Cruz**, v. 103, n. 5, p. 455–462, 2008.

TSCHARKE, R. L. et al. Haploid fruiting in *Cryptococcus neoformans* is not mating type α-specific. **Fungal Genetics and Biology**, v. 39, n. 3, p. 230–237, ago. 2003.

VAN DUIN, D. et al. Effects of Voriconazole on *Cryptococcus neoformans*. **Antimicrobial Agents and Chemotherapy**, v. 48, n. 6, p. 2014–2020, 1 jun. 2004.

VOYRON, S. et al. Vitality and genetic fidelity of white-rot fungi mycelia following different methods of preservation. **Mycological Research**, v. 113, n. 10, p. 1027–1038, out. 2009.

YAO, Y. et al. Voriconazole: a novel treatment option for cryptococcal meningitis. **Infectious Diseases**, v. 47, n. 10, p. 694–700, 3 out. 2015.

ZARAGOZA, O.; CASADEVALL, A. Experimental modulation of capsule size in *Cryptococcus neoformans*. **Biological Procedures Online**, v. 6, p. 10–15, 3 mar. 2004.

APÊNDICES

APÊNDICES

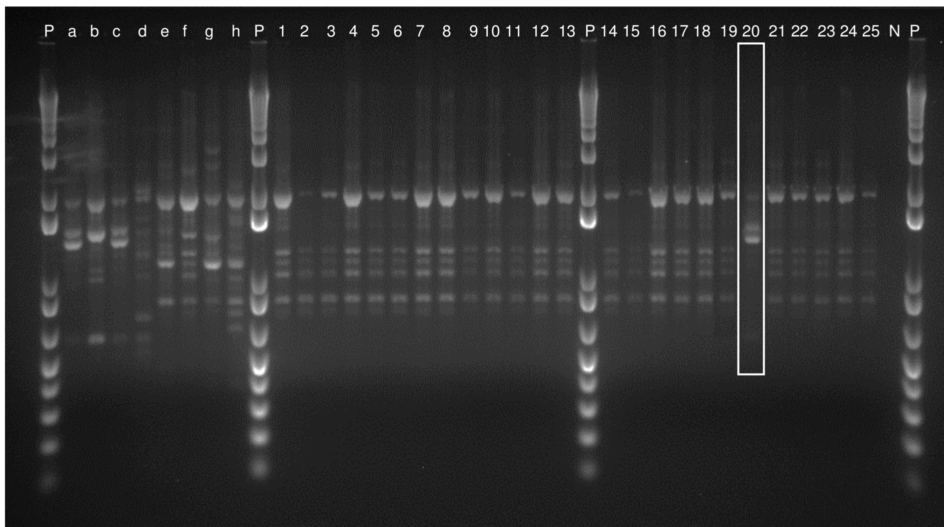
APÊNDICE A: Figuras que não foram diponibilizadas no Capítulo 1

Figura A.1.1: PCR *fingerprinting* das colônias isoladas da população 78MC1. Todas as colônias tiveram perfis compatíveis com VGII, exceto 78MC1-42 (em destaque) que apresentou perfil compatível com VNI.

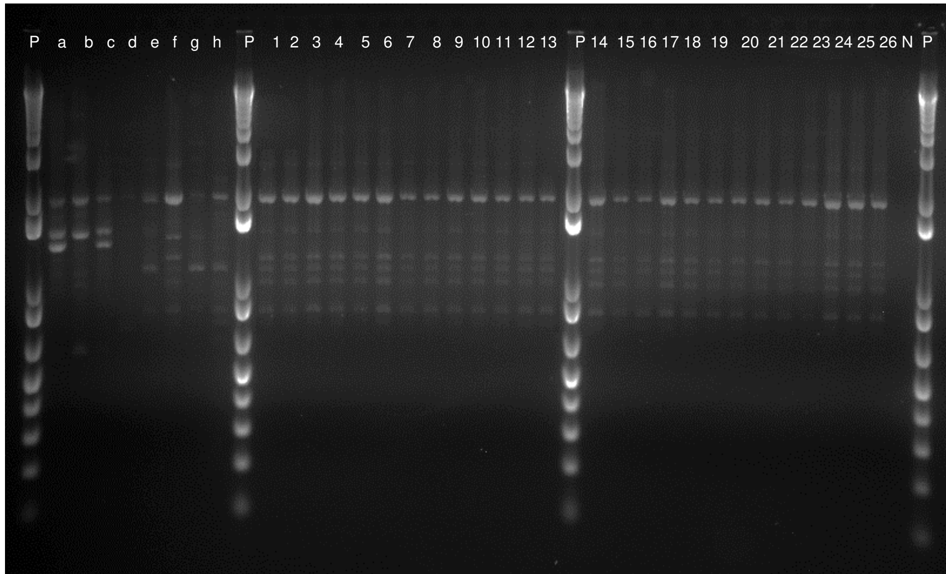
a. 1 – 78MC1-21, 2 – 78MC1-22, 3 – 78MC1-23, 4 – 78MC1-24, 5 – 78MC1-25, 6 – 78MC1-26, 7 – 78MC1-27, 8 - 78MC1-28, 9 – 78MC1-29, 10 – 78MC1-30, 11 – 78MC1-31, 12 – 78MC1-32, 13 – 78MC1-33, 14 – 78MC1-34, 15 – 78MC1-35, 16 – 78MC1-36, 17 – 78MC1-37, 18 – 78MC1-38, 19 – 78MC1-39, 20 – 78MC1-42, 21 – 78MC1-40, 22 – 78MC1-43, 23 – 78MC1-57, 24 – 78MC1-58, 25 – 78MC1-59.

b. 1 – 78MC1-60, 2 – 78MC1-61, 3 – 78MC1-62, 4 – 78MC1-63, 5 – 78MC1-64, 6 – 78MC1-65, 7 – 78MC1-66, 8 - 78MC1-67, 9 – 78MC1-68, 10 – 78MC1-69, 11 – 78MC1-70, 12 – 78MC1-71, 13 – 78MC1-72, 14 – 78MC1-73, 15 – 78MC1-74, 16 – 78MC1-75, 17 – 78MC1-76, 18 – 78MC1-77, 19 – 78MC1-78, 20 – 78MC1-79, 21 – 78MC1-80, 22 – 78MC1-81, 23 – 78MC1-82, 24 – 78MC1-83, 25 e 26 – 78MC1-B.

a.



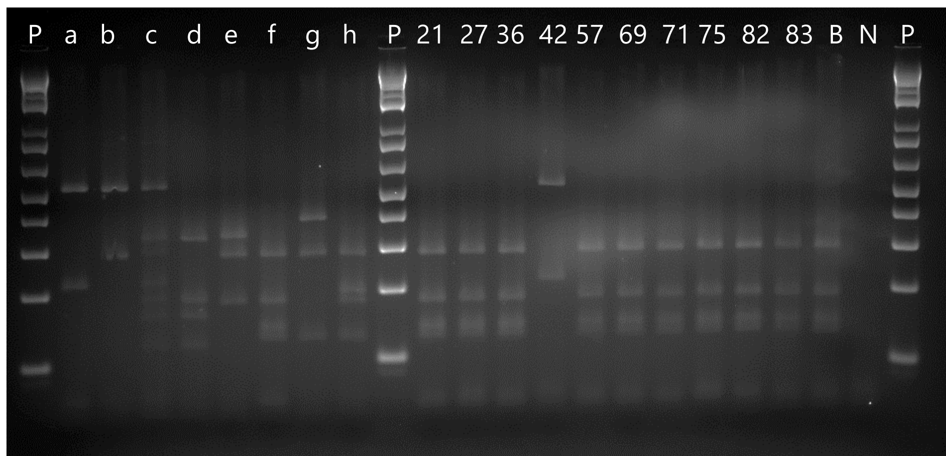
b.



Fonte: Do autor, 2018.

Legenda: P – peso molecular 1 Kb Plus, a – padrão VNI, b – padrão VNII, c - padrão VNIII, d - padrão VNIV, e - padrão VGI, f - padrão VGII, g - padrão VGIII, h - padrão VGIV, N – controle negativo

Figura A.1.2: RFLP das colônias isoladas da população 78MC1. Todas as colônias mantiveram perfis compatíveis com VGII, bem como 78MC1-42 que apresentou perfil compatível com VNI.
1 – 78MC1-21, 2 - 78MC1-27, 3 - 78MC1-36, 4 - 78MC1-42, 5 - 78MC1-59, 6 -78MC1-63, 7 - 78MC1-66, 8 - 78MC1-71, 9 - 78MC1-77, 10 - 78MC1-82, 11 - 78MC1-B, N – controle negativo.

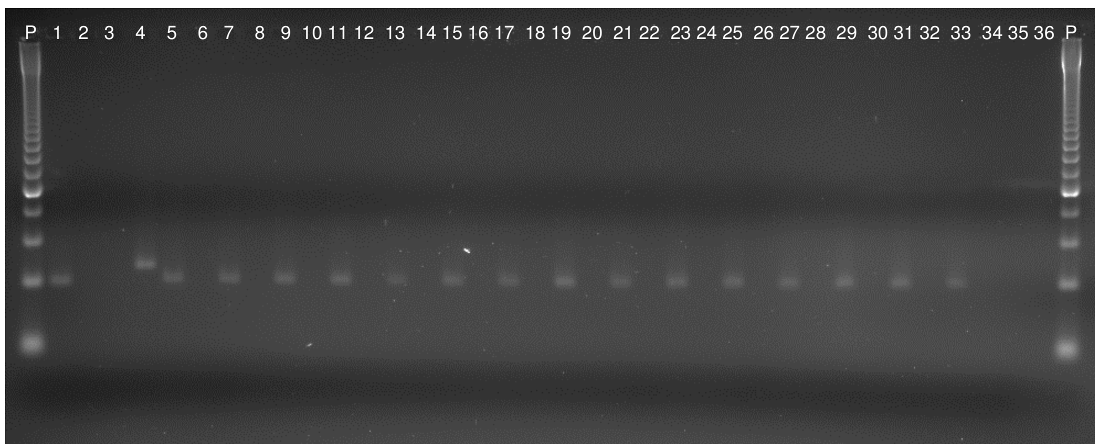


Fonte: Do autor, 2018.

Legenda: P – peso molecular 1 Kb Plus, a – padrão VNI, b – padrão VNII, c - padrão VNIII, d - padrão VNIV, e - padrão VGI, f - padrão VGII, g - padrão VGIII, h - padrão VGIV

Figura A.1.3: *Mating type* das colônias isoladas da população 78MC1. Todas as colônias testadas foram *mating type* alpha (α).

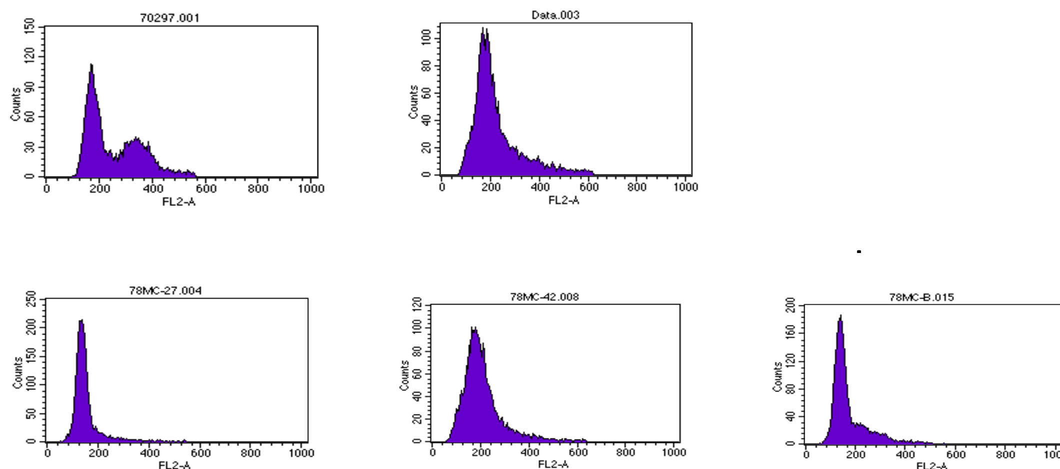
1 – CFRVS 40113 (α), 2 - CFRVS 40113 (a), 3 - CFRVS 40142 (α), 4 - CFRVS 40142 (a), 5 - 78MC1-36 (α), 6 - 78MC1-36 (a), 7 - 78MC1-37 (α), 8 - 78MC1-37 (a), 9 - 78MC1-38 (α), 10 - 78MC1-38 (a), 11 - 78MC1-39 (α), 12 - 78MC1-39 (a), 13 - 78MC1-42 (α), 14 - 78MC1-42 (a), 15 - 78MC1-56 (α), 16 - 78MC1-56 (a), 17 - 78MC1-57 (α), 18 - 78MC1-57 (a), 19 - 78MC1-58 (α), 20 - 78MC1-58 (a), 21 - 78MC1-59 (α), 22 - 78MC1-59 (a), 23 - 78MC1-60 (α), 24 - 78MC1-60 (a), 25 - 78MC1-61 (α), 26 - 78MC1-61 (a), 27 - 78MC1-62 (α), 28 - 78MC1-62 (a), 29 - 78MC1-63 (α), 30 - 78MC1-63 (a), 31 - 78MC1-64 (α), 32 - 78MC1-64 (a), 33 - 78MC1-65 (α), 34 - 78MC1-65 (a), 35 - controle negativo (α), 36 - controle negativo (a).



Fonte: Do autor, 2018.

Legenda: P – peso molecular 50 bp, CFRVS – Coleção de Fungos de Referência em Vigilância Sanitária

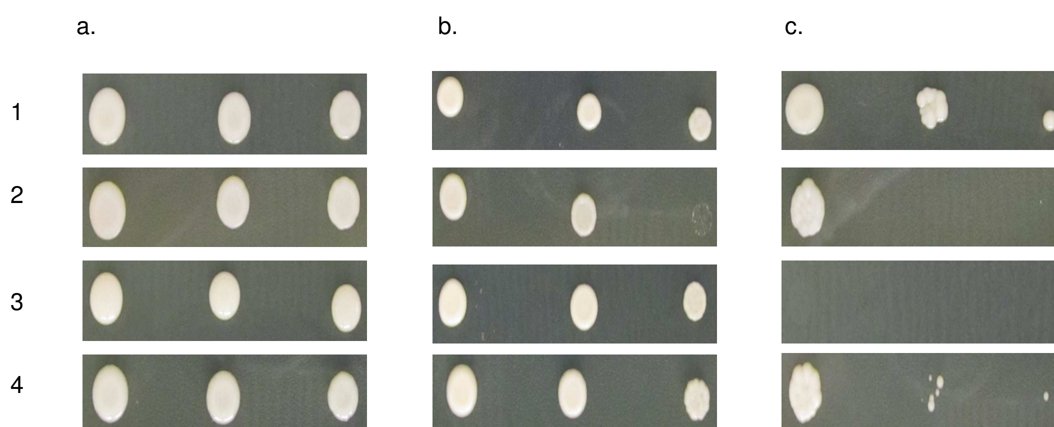
Figura A.1.4: Citometria de fluxo de algumas colônias isoladas da população 78MC1, todas as 25 colônias testadas apresentaram o mesmo padrão haploide, onde: a. controle positivo diploide (CFRVS 70297), b. controle positivo haploide (CFRVS 70302 – "Data.003"), c. 78MC1-27, d. 78MC1-47, 78MC1-B.



Fonte: Do autor, 2018.

Legenda: CFRVS – Coleção de Fungos de Referência em Vigilância Sanitária

Figura A.1.5: Termotolerância das colônias da população 78MC1. A 25 °C todas as colônias testadas apresentaram crescimento nas três diluições inoculadas. A 37 °C todas as colônias de C. gatti também apresentaram crescimento nas três diluições testadas, apenas C. neoformans (78MC1-42) apresentou crescimento em duas diluições (10^6 e 10^5 UFCXmL^{-1}). A 40 °C nenhuma das colônias testadas apresentaram crescimento após 48h, no entanto após a reincubação das placas a 25ºC ocorreu variação de crescimento entre as colônias analisadas.
a. 25ºC; b. 37ºC; c. reincubação a 25ºC após incubação a 40ºC. 1- 78MC1-21, 2- 78MC1-42; 3- 78MC1-B; 4- controle positivo CFP411.



Fonte: Do autor, 2018.

Legenda: CFP – Coleção de Fungos Patogênicos

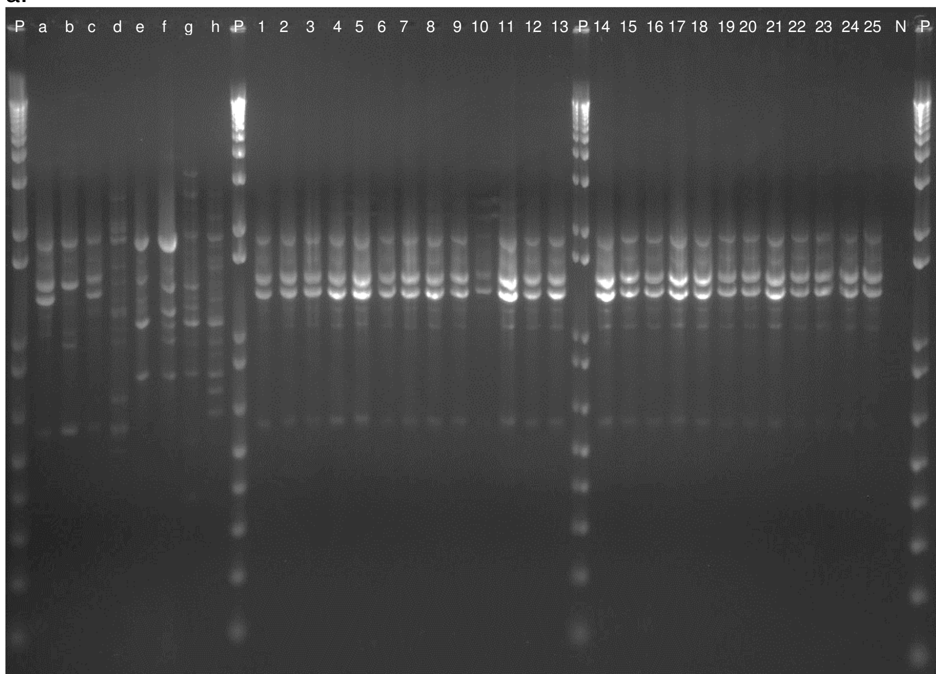
APÊNDICE B: Figuras que não foram disponibilizadas no Capítulo 2

Figura A.2.1: PCR *fingerprinting* das colônias isoladas da população LMM21. Todas as colônias testadas apresentaram perfil compatível com VN1.

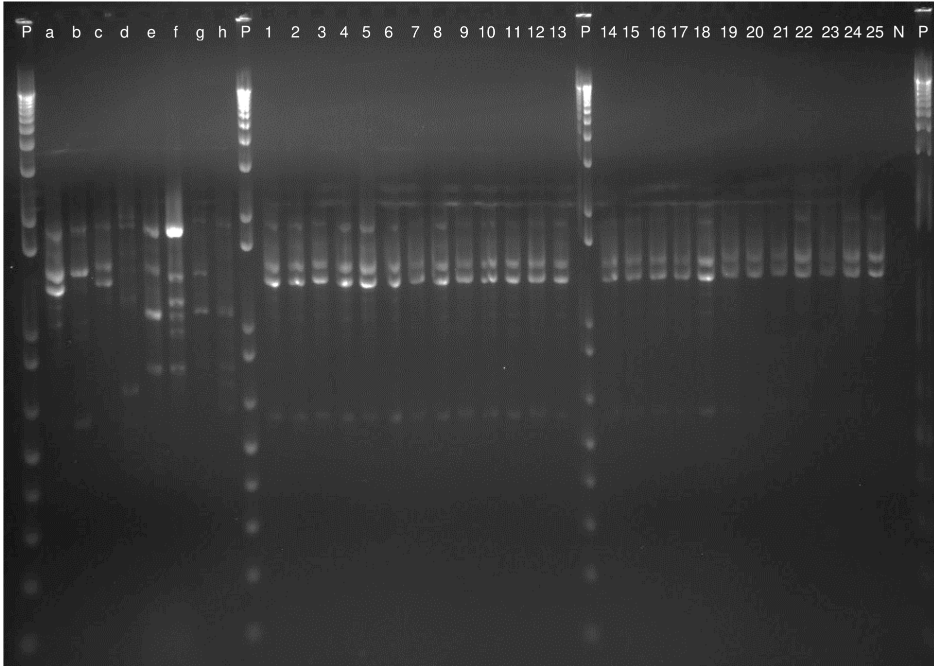
a. 1 – LMM21-1, 2 – LMM21-2, 3 – LMM21-3, 4 – LMM21-4, 5 – LMM21-5, 6 – LMM21-6, 7 – LMM21-7, 8 - LMM21-8, 9 – LMM21-9, 10 – LMM21-10, 11 – LMM21-11, 12 – LMM21-12, 13 – LMM21-13, 14 – LMM21-14, 15 – LMM21-15, 16 – LMM21-16, 17 – LMM21-17, 18 – LMM21-18, 19 – LMM21-19, 20 – LMM21-20, 21 – LMM21-21, 22 – LMM21-22, 23 – LMM21-23, 24 – LMM21-24, 25 – LMM21-25.

b. 1 – LMM21-26, 2 – LMM21-27, 3 – LMM21-28, 4 – LMM21-29, 5 – LMM21-30, 6 – LMM21-31, 7 – LMM21-32, 8 - LMM21-33, 9 – LMM21-34, 10 – LMM21-35, 11 – LMM21-36, 12 – LMM21-37, 13 – LMM21-38, 14 – LMM21-39, 15 – LMM21-40, 16 – LMM21-41, 17 – LMM21-42, 18 – LMM21-43, 19 – LMM21-44, 20 – LMM21-45, 21 – LMM21-46, 22 – LMM21-47, 23 – LMM21-48, 24 – LMM21-49, 25 – LMM21-50.

a.



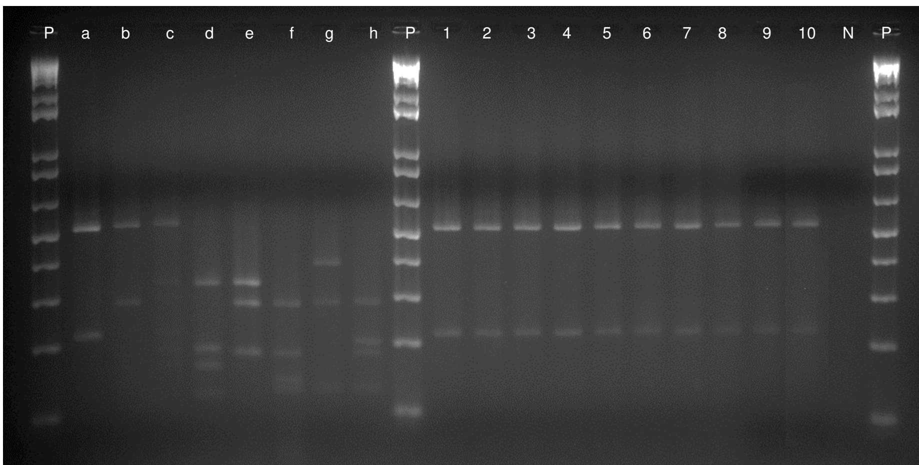
b.



Fonte: Do autor, 2018.

Legenda: P – peso molecular 1 Kb Plus, a – padrão VNI, b – padrão VNII, c - padrão VNIII, d - padrão VNIV, e - padrão VGI, f - padrão VGII, g - padrão VGIII, h - padrão VGIV, N – controle negativo

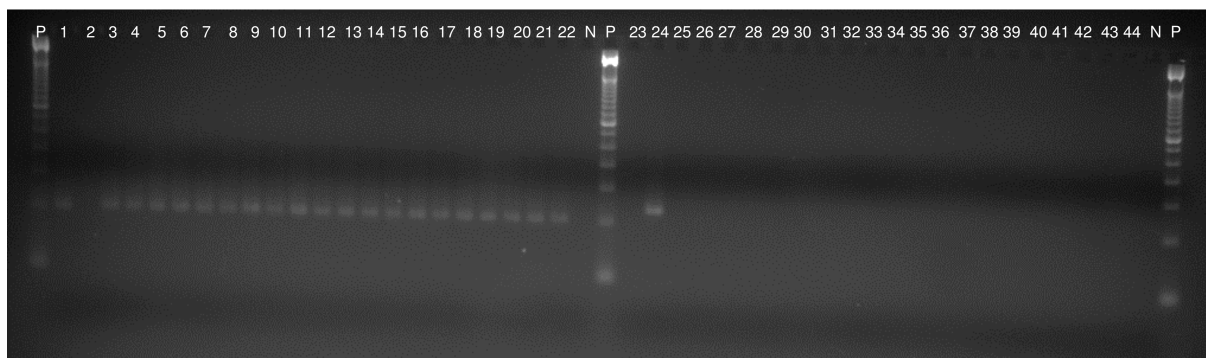
Figura A.2.2: RFLP das colônias isoladas da população LMM21. Todas as colônias testadas apresentaram perfil compatível com VN1.
1 - LMM21-1, 2 - LMM21-6, 3 - LMM21-15, 4 - LMM21-18, 5 - LMM21-22, 6 - LMM21-27, 7 - LMM21-30, 8 - LMM21-34, 9 - LMM21-44, 10 - LMM21-48, N – controle negativo.



Fonte: Do autor, 2018.

Legenda: P – peso molecular 1 Kb Plus, a – padrão VNI, b – padrão VNII, c - padrão VNIII, d - padrão VNIV, e - padrão VGI, f - padrão VGII, g - padrão VGIII, h - padrão VGIV

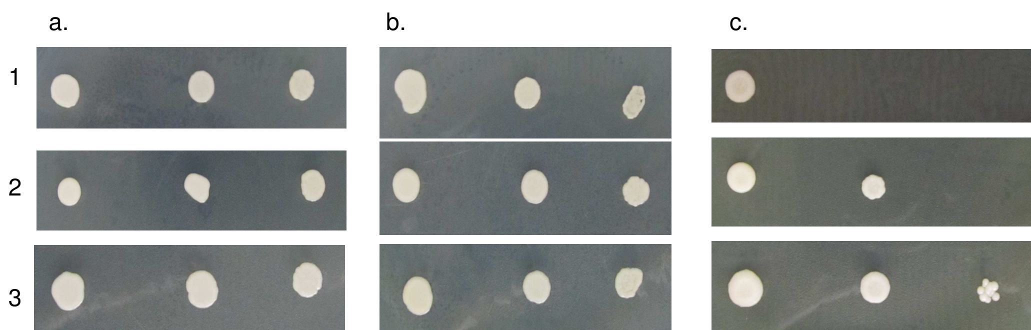
Figura A.2.3: *Mating type* das colônias isoladas da população LMM21. Todas as colônias testadas foram *mating type* alpha (α).
1 - CFRVS 40113 (α), 2 - CFRVS 40142 (α), 3 - LMM21-1 (α), 4 - LMM21-2 (α), 5 - LMM21-3 (α), 6 - LMM21-4 (α), 7 - LMM21-5 (α), 8 - LMM21-6 (α), 9 - LMM21-7 (α), 10 - LMM21-8 (α), 11 - LMM21-9 (α), 12 - LMM21-10 (α), 13 - LMM21-11 (α), 14 - LMM21-12 (α), 15 - LMM21-13 (α), 16 - LMM21-14 (α), 17 - LMM21-15 (α), 18 - LMM21-16 (α), 19 - LMM21-17 (α), 20 - LMM21-18 (α), 21 - LMM21-19 (α), 22 - LMM21-20 (α).
23 - CFRVS 40113 (a), 24 - CFRVS 40142 (a), 25 - LMM21-1 (a), 26 - LMM21-2 (a), 27 - LMM21-3 (a), 28 - LMM21-4 (a), 29 - LMM21-5 (a), 30 - LMM21-6 (a), 31 - LMM21-7 (a), 32 - LMM21-8 (a), 33 - LMM21-9 (a), 34 - LMM21-10 (a), 35 - LMM21-11 (a), 36 - LMM21-12 (a), 37 - LMM21-13 (a), 38 - LMM21-14 (a), 39 - LMM21-15 (a), 40 - LMM21-16 (a), 41 - LMM21-17 (a), 42 - LMM21-18 (a), 43 - LMM21-19 (a), 44 - LMM21-20 (a).



Fonte: Do autor, 2018.

Legenda: P – peso molecular 50 bp, CFRVS – Coleção de Fungos de Referência em Vigilância Sanitária, N – controle negativo

Figura A.2.4: Termotolerância das colônias da população LMM21. Nas temperaturas de 25 °C e 37 °C todas as colônias testadas apresentaram crescimento nas três diluições inoculadas. A 40 °C nenhuma das colônias testadas apresentaram crescimento após 48h, no entanto após a reincubação das placas a 25ºC ocorreu variação de crescimento entre as colônias analisadas.
a. 25ºC; b. 37ºC; c. reincubação a 25ºC após incubação a 40ºC. 1- LMM21-1, 2- LMM21-23; 3- LMM21-34.



Fonte: Do autor, 2018.