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ESTUDO AMBIENTAL, CARACTERIZAÇÃO MOLECULAR E ESTRATÉGIA DE VIGILÂNCIA DOS AGENTES DA CRIPTOCOCOSE NA MICRORREGIÃO DO RIO NEGRO NO ESTADO DO AMAZONAS

FÁBIO BRITO DOS SANTOS

Rio de Janeiro
Dezembro de 2018

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Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do grau de Doutor em Ciências.

Orientadores: Profª. Drª. Márcia dos Santos Lazéra
Prof. Dr. Wieland Meyer

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Rio de Janeiro, 20 de dezembro de 2018

Dedico essa tese aos verdadeiros
civilizadores do nosso país

“Tudo parece impossível até que seja feito”
(Nelson Mandela)

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RESUMO

TESE DE DOUTORADO EM MEDICINA TROPICAL

Fábio Brito dos Santos

A criptococose é adquirida pela inalação dos propágulos criptocócicos no ambiente. A doença é causada por duas espécies patogênicas do gênero *Cryptococcus*, definidas como *Cryptococcus neoformans* espécie complexa e o *C. gattii* espécie complexa. A criptococose por *Cryptococcus neoformans* é a principal doença oportunista em pacientes infectados pelo HIV, principalmente na África Subsaariana, com mais de 500.000 mortes por ano estimadas devido à meningite criptocócica. No Brasil, além da importância da criptococose em pacientes com HIV/aids, outro problema de saúde pública é a ocorrência da meningite criptocócica por *C. gattii* em crianças e adultos jovens de ambos os sexos no Norte (N) e Nordeste (NE). A compreensão da dinâmica e adaptação dos reservatórios de agentes da criptococose, a identificação de como essa infecção é adquirida pelos seres humanos e quais os meios para evitar ou reduzir seus riscos de infecção são de interesse fundamental. O presente estudo ambiental foi conduzido na microrregião do Rio Negro no estado do Amazonas que é constituída por quatro municípios (Barcelos, Novo Airão, Santa Isabel do Rio Negro e São Gabriel da Cachoeira). Coletamos e analisamos amostras de postes de madeira e principalmente a poeira domiciliar dos três primeiros municípios. A caracterização molecular foi realizada com o auxílio da ferramenta MLST, revelando os principais subtipos de *C. gattii* e *C. neoformans* responsáveis pela criptococose no mundo, além de alguns subtipos de VGII específicos da região Amazônica. A ausência de relatos de casos de criptococose e a presença significativa de um grupo vulnerável de crianças incentiva a realização de estratégia de vigilância dos agentes da criptococose nessa microrregião. Para atender essa demanda foram elaboradas ferramentas de comunicação (folder popular e informe técnico). Como proposta para detectar pacientes oligossintomáticos e assintomáticos nessa área remota e com poucos recursos humanos, otimizamos a utilização do teste rápido do CrAg na urina aumentando a especificidade sem perder a sensibilidade. Esperamos que com os produtos gerados nessa tese seja possível o manejo de futuros casos de criptococose nessa região ou em outras áreas endêmicas remotas do Brasil.

Palavras-chave: 1. Microrregião do Rio Negro. 2. Amostras ambientais. 3. Agentes da criptococose. 4. MLST. 5. Teste CrAg na urina.

Ministério da Saúde

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ENVIRONMENTAL STUDY, MOLECULAR CHARACTERIZATION AND SURVEILLANCE STRATEGY OF CRYPTOCOCCOSIS AGENTS IN THE MICRO-REGION RIO NEGRO IN THE STATE OF AMAZONAS

ABSTRACT

PHD THESIS IN TROPICAL MEDICINE

Fábio Brito dos Santos

Cryptococcosis is acquired by the inhalation of cryptococcal propagules (desiccated yeast cells or basidiospores) from the environment. The disease is caused by two pathogenic members of the genus *Cryptococcus*, the *Cryptococcus neoformans* species complex and the *C. gattii* species complex. Cryptococcosis by *Cryptococcus neoformans* species complex is one of the major opportunistic diseases in HIV-infected patients mainly in Sub-Saharan Africa, where over 500,000 deaths per year are estimated due to cryptococcal meningitis. In Brazil, along with the importance of cryptococcal infection in HIV patients, another public health problem is the endemic occurrence of cryptococcal meningitis by *C. gattii* species complex in children and young adults of both genders in the North (N) and Northeast (NE) regions. The understanding of the dynamics and adaptation of the agents reservoirs, the knowledge of how the infections is acquired by humans, and what are the means to avoid or reduce its risks of infection, are of fundamental interest. The present environmental study was conducted in the Rio Negro micro-region in the state of Amazonas, which consists of 4 municipalities (Barcelos, Novo Airão, Santa Isabel do Rio Negro, and São Gabriel da Cachoeira). We collected and analyzed samples of wood poles and mainly indoor dust from the first three municipalities. The molecular characterization was performed with the aid of the MLST tool, revealing the main subtypes of *C. gattii* VGII and *C. neoformans* VNI responsible for cryptococcosis in the world, in addition to some specific VGII subtypes of the Brazilian Amazon region. The absence of reports of cases of cryptococcosis and the significant presence of a vulnerable group of children encourages the implementation of a surveillance strategy for cryptococcosis agents in this micro-region. To address this demand, we developed communication tools (a popular folder and a technical report). As a proposal to detect oligosymptomatic and asymptomatic patients in this remote area with limited human resources, we optimized the use of rapid CrAg test in the urine increasing specificity without losing sensitivity. We hope that with the products generated in this thesis it will be possible to efficiently manage future cases of cryptococcosis in this region or in other remote endemic areas of Brazil.

Keywords: 1. Rio Negro Micro-region. 2. Environmental samples. 3. Cryptococcosis agentes. 4. MLST. 5. CrAg test-urine.

LISTA DE SIGLAS, ABREVIAÇÕES E SÍMBOLOS.

AFLP	*Amplified Fragment Length Polimorphism*
AM	Amazonas
aids	Síndrome da imunodeficiência adquirida
CrAg	*Cryptococcus Antigen Lateral Flow Assay*
CAP59	Gene da cápsula polissacarídica
CM	Meningite criptocócica
CGB	Canavanina-glicina-azul de bromotimol
DNA	*Deoxyribonucleic Acid*
Fiocruz	Fundação Oswaldo Cruz
GPD1	Gene desidrogenase de gliceraldeído-3-fosfato
HIV	*Human Immunodeficiency Virus*
IGS	*Intergenic Spacer*
IBGE	Instituto Brasileiro de Geografia e Estatística
INI	Instituto Nacional de Infectologia Evandro Chagas
ITS	*Intragenic Spacer*
LCR	Líquido céfalorraquidiano
MALDI-TOF	*Matrix Assisted Laser Desorption/Ionization – Time of flight*
MAT alfa	*Mating Type alfa*
MATα	*Mating Type α*
MEGA	*Molecular Evolutionary Genetics Analysis*
MLST	*Multilocus sequence typing*
PCR	*Polymerase Chain Reaction*
PNW	*Pacific Northwest*
PLB1	*Gene fosfolipase B*
PAS	Ácido periódico de Schiff

PGMT	Pós- graduação de Medicina Tropical
RFLP	*Restriction Fragment Length Polymorphism*
RJ	Rio de Janeiro
RNA	*Ribonucleic Acid*
ST	*Sequence typing*/subtipo
SNC	Sistema nervoso central
SOD1	superóxido dismutase de Cu^{2+}e Zn^{2+}
WGS	*Whole genome sequencing*.
URA5	*Gene Orotidine-5'-phosphate decarboxylase*
VITEK® 2	Sistema automatizado para identificação de fungos e bactérias versão 2.0

SUMÁRIO

1. INTRODUÇÃO

1.1. Histórico da Criptococose e seus agentes

Cryptococcus neoformans foi isolado pela primeira vez de suco de pêssego por Sanfelice em 1894 na Itália e àquela época, foi nomeado *Saccharomyces neoformans*. A doença foi descrita em humanos há pouco mais de 134 anos, em 1884-85, quando dois médicos alemães descrevem em períodos diferentes casos de pacientes com lesões aparentemente tumorais porém com isolamento do agente em cultivo. Busse forneceu a primeira descrição de um caso de criptococose e isolou uma levedura de lesão na tíbia de mulher jovem. Busse nomeou o fungo como *Saccharomyces* e a doença *Saccharomycosis hominis*.

Desde a descoberta desta micose, vários nomes para o fungo e para infecção haviam sido propostos, quando Vuillemin (apud Kwon-Chung & Bennett, 1992) em 1901 reclassificou a levedura isolada por Busse como *Cryptococcus hominis* e a de Sanfelice como *C. neoformans* devido à ausência de formação de ascósporos e à ausência de fermentação de açúcares, presentes no gênero *Saccharomyces.* O neurotropismo característico de *C. neoformans* foi reconhecido pela primeira vez em 1914 por Verse (1914) e dois anos depois por Stoddard & Cutler (1916). No entanto, Stoddard e Cutler denominaram o agente etiológico *Torula histolytica* e a doença "torulosis".

A confusão sobre a nomenclatura do agente de criptococose persistiu até Benham realizar um estudo abrangente com cepas clínicas de *Cryptococcus* spp. que concluiu que todas as cepas de infecções humanas pertenciam a uma espécie com variedades baseadas em diferenças antigênicas. Evans e colaboradores (1949) descrevem sorotipos A, B e C, sendo mais tarde descrito o sorotipo D (Wilson *et al* 1968 apud Casadeval & Perfect 1998).

Em 1950, Benham propôs a denominação criptococose e *Cryptococcus neoformans* como *Nomen conservandum*. Logo esse termo foi absorvido na literatura médica e micológica (Casadevall & Perfect, 1998).

Emmons, em 1951, descreve o primeiro isolamento de *C. neoformans* a partir de fontes ambientais no estado da Virginia, onde isolou o fungo de solo contaminado com excreta de aves, em particular de pombos, sedimentando o conceito de infecção adquirida a partir de ambiente contaminado (Emmons, 1951).

O diagnóstico laboratorial de *C. neoformans* foi drasticamente simplificado no início dos anos 1960 quando Staib descreve meio de cultura indicativo onde somente a levedura de *C. neoformans* produzia pigmento marrom. Tal meio era produzido com sementes de *Guizotia abyssinica*, mais conhecidas como sementes de Níger, grão rico em extratos etéreos (óleos). O óleo vegetal bruto extraído dessa semente é geralmente mais estável do que o óleo refinado correspondente e é rico em tocoferóis (vitamina lipossolúvel), fosfolipídios, fitoesteróis e fenóis que são os mais importantes antioxidantes naturais em óleos brutos (Ramadan et al., 2003). Seeliger e Staib descobriram que *C. neoformans* poderia ser distinguido de outras leveduras brancas por sua atividade de urease (Seeliger 1956) e formação de melanina (Staib 1962b), além da presença de cápsula.

Durante várias décadas, os agentes da criptococose foram organizados em duas variedades que incluíam cinco sorotipos: *C. neoformans* var. *neoformans* para sorotipos A, D e AD (agora reconhecido como um híbrido entre as linhagens A e D) e *C. neoformans* var. *gattii* para os sorotipos B e C (Kwon-Chung et al., 1982). A fase teleomórfica desses agentes inclui duas espécies classificadas no gênero *Filobasidiella* como *Filobasidiella neoformans* (*C. neoformans*) e *Filobasidiella bacillispora* (*C. gattii*) (Kwon-Chung, 1976, 1975). A identificação das espécies *C. neoformans* e *C. gattii* ficou baseada nas diferenças morfológicas da fase teleomórfica e pelas diferenças bioquímicas da fase anamórfica. Apesar da forma dos basidiósporos das duas espécies ser diferente, a morfologia da fase de levedura não possui diferenças marcantes (Kwon-Chung & Varma, 2006). A identificação dos basidiósporos alongados, característicos da espécie *C. gattii*, só pode ser obtida *in vitro* em meios especiais, através da conjugação com cepas padrão de tipo sexuado *MATalfa* e *MATa*, sendo que nem todas são férteis (Kwon-Chung & Varma, 2006). Portanto, esta metodologia utilizada em pesquisa não se aplica à rotina de identificação fenotípica de isolados clínicos ou ambientais.

A criptococose era considerada uma doença rara até o início do uso de terapia com imunossupressores a partir da década de 1970. A importância de entender o patógeno tornou-se ainda mais urgente diante da emergência global da criptococose no início da década de 1980, com advento do vírus da imunodeficiência humana (HIV) e da síndrome da imunodeficiência adquirida (aids). A criptococose é uma das mais importantes infecções fúngicas oportunistas em pacientes vivendo com HIV/aids e uma das principais contribuidoras para mortalidade precoce nesses indivíduos, representando entre 13% e 44% das mortes neste grupo. Segundo Rajasingham e cols., a incidência

global anual de meningite criptocócica está em 223.100 casos, resultando em 181.100 mortes anuais em 2014. A África Subsaariana continua sendo a região responsável pela maior parte dos óbitos de meningite criptocócica no mundo, e recentemente estudos ressaltam que nesse tipo de região ou seja em países de baixa renda, a letalidade pode ser entre 70%-100% dependendo da conduta clínica (Rajasingham et al., 2017).

1.2 AGENTES DA CRIPTOCOCOSE

1.2.1 TAXONOMIA E NOMENCLATURA

O gênero *Cryptococcus* inclui cerca de 70 espécies de leveduras na forma anamórfica. Algumas delas têm a forma teleomórfica conhecida, como por exemplo os gêneros *Filobasidium, Filobasidiella, Cystofilobasidium* e *Kwoniella*, na classe dos *Tremellomycetes* do Filo Basidiomicota. A maioria das espécies de *Cryptococcus* são saprofíticas, mas destacam-se dois agentes, *Cryptococcus neoformans* e *Cryptococcus gattii,* capazes de produzir melanina, apresentar cápsula polissacáride complexa, serem termotolerantes a 37°C e patogênicos para homens e outros mamíferos (Heitman et al., 2010).

A aplicação de métodos moleculares no estudo de *C. neoformans* inicia-se no final da década de 1980 e início da década de 1990, sendo tais métodos intensamente utilizados nas investigações subsequentes (Kwon-Chung and Bennett, 1992). Após implantacão da técnica da reação de polimerase em cadeia (PCR) e do sequenciamento de DNA, foi observada uma ampla diversidade genética dos agentes etiológicos da criptococose. Como exemplo, a divergência na sequência do gene *URA5* de cepas do sorotipo A de *C. neoformans* motivou a criação de variedade separada com o nome de *C. neoformans* var. *grubii* (Franzot et al., 1999). Em 2000, *C. neoformans* var. *gattii* foi considerado uma espécie, *C. gattii* (Kwon-Chung et al., 2002), separado do táxon *C. neoformans* por diferenças filogenéticas. Experimentos de cruzamentos *in vitro* entre cepas de *C. neoformans* e *C. gattii* foram capazes de desenvolver ciclo sexuado entre as duas espécies, mas a progênie em F1 era infértil, evidenciando segregação desses agentes em duas espécies distintas com base no conceito biológico de espécie.

Segundo análise de taxa de evolução genética, estima-se que essas duas espécies divergiram em aproximadamente ~80-100 milhões de anos atrás (Casadevall et al., 2017). Estudo do gene *URA5* revelou que *C. neoformans* e *C. gattii* são compostos por pelo menos quatro subgrupos principais e geneticamente distintos (Hagen et al., 2011).

C. neoformans tem cinco tipos moleculares VNI-VNIV, e o mais recentemente descrito VNB (Litvintseva et al., 2006) e *C. gattii* tem quatro tipos moleculares, VGI-VGIV (Kidd et al., 2004).

Uma proposta recente de nomenclatura sugeriu a criação de 7 espécies separadas, excluindo híbridos diploides/aneuploides formados entre os diferentes tipos moleculares. Essa análise foi realizada com base em dados gerados pelo *multilocus sequence typing* (MLST) e utilizou somente 115 isolados: os resultados gerados sugerem a divisão de *C. neoformans* em duas espécies (*C. neoformans* e *C. deneformans*) e *C. gattii* em cinco espécies (*C. gattii, C. deuterogattii, C. bacillisporus, C. tetragattii* e *C. decagattii*) (Hagen et al., 2015).

Porém um grupo da comunidade cientifica, incluindo um número expressivo de clínicos, se reuniram para apreciar criticamente essa proposta resultando na elaboração de artigo liderado pela Dr June Kwon-Chung, referência fundamental na taxonomia dos agentes da criptococose. Apontamos em seguida os aspectos principais considerados nessa publicação de grande importância para os clínicos e os laboratórios de rotinas que lidam com o desafio de diagnosticar e tratar essa micose (Kwon-Chung et al., 2017):

(i) As designações das espécies filogenéticas certamente irão mudar, já que foram analisados menos de 5% das cepas genotipadas, o que não representa a verdadeira diversidade dentro do complexo de espécies.

(ii) O uso de uma única linhagem para designar espécies (*C. decagattii*), sem características fenotípicas, é altamente controverso e levanta uma questão não resolvida de como diferentes genomas devem ser utilizados no delineamento de uma espécie.

(iii) Utilizar apenas abordagens cladísticas (filogenéticas) para delineação de espécies dos agentes da criptococose é inadequado, uma vez que estes fungos mostram várias taxas de recombinação, clonalidade e hibridização dentro e entre as espécies.

Portanto, além das considerações acima apontadas sobre o trabalho de Hagen e cols (2015), a renomeação de patógenos importantes na área médica requer um consenso dentro da comunidade científica para prevenir a confusão na literatura publicada, bem como evitar confusão na prática clínica e instabilidade taxonômica.

Concluindo, Kwon-Chung e cols (2017) preconizam, que em vez de "espécies" deve-se utilizar, "complexo de espécies", acomodando espécies crípticas já conhecidas e aquelas que podem ser identificadas no futuro. Tipos moleculares dentro de cada complexo de espécies podem ser designados pelo seu tipo molecular por exemplo VNI, VGI e etc, sempre que necessário. No momento, os autores preconizam o uso da nomenclatura "*C. neoformans* espécie complexa" e "*C. gattii* espécie complexa'' (Kwon-Chung et al., 2017).

1.2.2-IDENTIFICAÇÃO FENOTÍPICA

1.2.2.1- TRADICIONAL

Do ponto de vista fenotípico, essas duas espécies só podem ser diferenciadas através do teste canavanina-glicina-azul de bromotimol (CGB) que distingue *C. gattii* por sua capacidade de assimilar glicina como única fonte de carbono e nitrogênio e não ser inibido pela canavanina, resultando em crescimento no meio de cultura, mudança do indicador de pH para cor azul cobalto, resultando em CGB positivo. Por outro lado, *C. neoformans* não cresce no meio, mantêm a cor original, resultando em CGB negativo (Kwon-Chung & Bennett, 1992; Kwon-Chung et al., 2002). O teste CGB é utilizado como critério taxonômico fenotípico para distinção entre as duas espécies, no entanto outros testes como assimilação de ácido málico ou D-prolina também têm sido descrito como ferramenta para distinção destas espécies (Nishikawa et al., 1996; Kurtzman et al 2011).

1.2.3-IDENTIFICAÇÃO MOLECULAR

Extensa variabilidade genética é observada nas espécies *C. neoformans* e *C. gattii*. Análise das sequências de nucleotídeos dos espaços intergênicos (Intergenic Spacer, IGS) e intragênicos (Intragenic Spacer, ITS) dos genes ribossomais de isolados clínicos e ambientais de diferentes regiões geográficas mostraram divergências entre isolados da mesma variedade e espécie (Diaz et al., 2005). Análise dos perfis obtidos por PCR-fingerprinting utilizando como iniciadores oligonucleotídeos obtidos da sequência central do fago M13 e análise de fragmentos de ácidos nucleicos gerados por enzimas de restrição PCR-*URA5* RFLP *"Polymerase Chain Reaction- Restriction Fragment Length Polymorphism"*, discriminam tipos moleculares específicos para *C.*

neoformans denominados VNI e VNII (sorotipo A), VNIII (sorotipo AD), VNIV (sorotipo D) e, para *C. gattii* os tipos moleculares denominados VGI, VGII, VGIII e VGIV, sorotipos B e C, não sendo observada correlação entre os sorotipos e os tipos moleculares em *C. gattii* (Meyer et al., 2003).

A análise da variabilidade genética utilizando Amplified Fragment Lenght Polimorphism (AFLP) que identifica diferentes tipos moleculares como: VNI/AFLP 1 e VNII/AFLP1A e AFLP1B, VNIII/AFLP3, VNIV/AFLP2, VGI/AFLP4, VGII/AFLP6, VGIII/AFLP5 e o genótipo VGIV/AFLP7 também é utilizada (Boekhout et al., 2001).

As diferentes técnicas (PCR-*URA5* RFLP e AFLP) permitem identificar os mesmos tipos molecures havendo correspondência portanto entre as denominações utilizadas: AFLP 1 e 1A, AFLP 2, AFLP 3, AFLP4, AFLP5 e o genótipo AFLP6. Pela simplicidade e baixo custo da técnica PCR-*URA5* RFLP esta tem sido utilizada amplamente em estudos epidemiológicos (Meyer et al., 2003).

Reconhecendo a necessidade urgente de um método de tipagem padronizado e aceitável globalmente, um grupo de trabalho em "Genotipagem de *C. neoformans* e *C. gattii*", foi formado na Sociedade Internacional de Micoses Humanas e Animais (Isham) no início de 2007, no qual participaram todos os principais grupos de pesquisa envolvidos na tipagem molecular de *C. neoformans*. Foi selecionado a Tipagem por Sequência de Múltiplos Locis "*multilocus sequence typing – MLST*" como o método de escolha para a tipagem de cepas em função de seu elevado poder de discriminação, bem como da reprodutibilidade entre diferentes laboratórios. O grupo de trabalho também escolheu cepas de referência padrão que representassem os oito principais tipos moleculares e como conclusão, esse grupo propõe o seguinte conjunto de loci genéticos associados à variabilidade e virulência desses patógenos, como um padrão internacional para tipagem de *C. neoformans* e *C. gattii*: *CAP59*: Gene associado à proteína capsular, *GPD1*: Gliceraldeído-3-fosfato desidrogenase, *LAC1*: lacase, *PLB1*: fosfolipase B, *SOD1*: superóxido dismutase de Cu^{2+}e Zn^{2+}, *URA5*: Orotidina monofosfato pirofosforilase e IGS1: espaços intergênicos RNA Ribosomal (Meyer et al.,2009). Pretendiam com essa ferrementa identificar subtipos (Sts) dentro dos oito tipos moleculares para estudos de distribuição geográfica, expansão e surtos.

O sequenciamento do genoma completo (WGS) é um método abrangente. A informação completa do genoma tem sido fundamental na identificação de mutações hereditárias e no rastreamento de surtos de doenças. A rápida redução dos custos de sequenciamento e a capacidade de produzir grandes volumes de dados com os

sequenciadores atuais, tornam o sequenciamento genômico completo uma ferramenta poderosa para a pesquisa em genômica. Em estudo de populações distintas de *C. gattii* da região do Pacífico na América do Norte (PNW), o sequenciamento do genoma completo foi realizado com intuito de averiguar as fontes naturais e as adaptações genômicas que levaram ao surgimento da infecção nesta região temperada. O WGS revelou um grande número de eventos mutacionais e recombinacionais; no entanto, os três subtipos dominantes do PNW eram de baixa diversidade e completamente clonais. O uso dessa ferramenta forneceu argumentos para uma maior compreensão da evolução de *C. gattii* nas Américas (Engelthaler et al., 2014).

1.3-PATOGENIA

Na criptococose, a infecção ocorre através da inalação de propágulos fúngicos a partir de uma fonte ambiental para o trato respiratório, podendo atingir os pulmões. Este processo no hospedeiro pode resultar em infecção autolimitada, controlada pela própria resposta imune do hospedeiro, e os sintomas podem estar ausentes ou serem inespecíficos. Na primo-infecção, usualmente o fungo entra em latência no foco pulmonar, mantendo-se viável com baixo metabolismo, podendo ser reativado quando o hospedeiro se torna imunocomprometido. A doença pulmonar apresenta-se como processo inflamatório usualmente de apresentação aguda e padrão radiológico inespecífico bem como de nódulos pulmonares periféricos, resultado de uma apresentação tardia (Kishi et al., 2006; Lin and Heitman, 2006). Dependendo da carga fúngica inalada e do estado imune do hospedeiro pode ocorrer disseminação linfohematogênica, com lesão secundária para diferentes órgãos, sendo a infecção do sistema nervoso central (SNC) a principal manisfestação da criptococose no momento do diagnóstico, seja em imunocompetentes ou imunocomprometidos. A infecção do SNC dependerá da capacidade de evasão do fungo das barreiras envolvidas na fase inicial da infecção pulmonar. Curiosamente, Ngamskulrungroj et al. demonstraram que, diferente de *C. gattii*, *C. neoformans* H99 apresenta lesões mais evidentes no SNC do que no pulmão (Ngamskulrungroj et al., 2012), aspectos que demandam futuros estudos.

Os agentes da criptococose vivem em uma relação complexa com o hospedeiro infectado. Durante a primo-infeccão, o patógeno deve ser capaz de se adaptar ao ambiente do hospedeiro e reagir com as mudanças celulares adaptativas. Essas respostas

geralmente incluem a indução de fenótipos específicos que tornam o microrganismo capaz de sobreviver e proliferar dentro desse novo ambiente. Essa plasticidade fenotípica dos fatores de virulência pode evitar que os agentes da criptococose sejam reconhecidos pelo sistema imunológico do hospedeiro (Alspaugh, 2015).

I. Os agentes da criptococose têm alguns fatores de virulência bem caracterizados: Capacidade de crescer à temperatura corporal de mamíferos: *C. neoformans* e *C. gattii* podem crescer e se multiplicar em temperaturas de 37°C a 39ºC, sendo esta característica essencial para a virulência desses patógenos.
II. Produção de cápsula: um polissacarídeo complexo composto de glucuronoxilomanana e galactoxilomanana, forma uma estrutura em volta da superfície da célula, reduz a fagocitose e sua a expressão protege o fungo de estresse oxidativo intracelular.
III. Produção de melanina: tem propriedades antioxidantes e fornece proteção adicional para o fungo.
IV. Outros fatores como urease e fosfolipases são importantes na patogenia do *Cryptococcus* spp. *C. neoformans* também desenvolveu várias estratégias para sobreviver e replicar dentro de células fagocíticas.

No entanto, apesar dessas estratégias bem-sucedidas de virulência, *C. neoformans* não precisa de hospedeiro para completar o seu ciclo de vida, levando assim a designação de "patógeno acidental" (Casadevall and Pirofski, 2007).

Por ser designado patógeno acidental em vários hospedeiros não mamíferos, autores têm utilizado o modelo *Galleria mellonella*, método alternativo, para estudar a virulência e os efeitos dos compostos antifúngicos dos agentes da criptococose. O conceito de "métodos alternativos" é compreendido em um contexto mais amplo considerando, inclusive, os esforços que são realizados pela comunidade científica mundial para diminuir o uso de animais de laboratório para um número mínimo possível (Firacative et al., 2014).

1.4-DIAGNÓSTICO LABORATORIAL

1.4.1 MICROSCOPIA DIRETA

A microscopia direta baseia-se na visualização de levedura capsulada sem hifa ou pseudo-hifa no espécime clínico em preparações com tinta nanquim (tinta da China). Realizada especialmente de espécimes oriundos do trato respiratório, urinário e digestivo, onde outras leveduras capsuladas não patogênicas do gênero *Cryptococcus* podem ser encontradas. No LCR e em materiais obtidos de lesões fechadas o exame direto positivo é de valor diagnóstico (Lacaz et al., 2002).

1.4.2 IDENTIFICAÇÃO EM TECIDO

Em cortes corados ao Mucicarmim de Meyer, *C. neoformans* e *C. gattii* são identificados devido à cápsula que se apresenta na cor vermelha, facilitando o seu reconhecimento, sobretudo nas formas hipo-capsuladas. Coloração pelo ácido periódico de Schiff (PAS) pode ser uma alternativa para demonstração dos agentes. A impregnação argêntea pelo método de Gomori-Grocott evidencia a parede fúngica, mas não permite a identificação desta levedura, pois não discrimina a cápsula. A coloração de Fontana-Masson evidencia o depósito de melanina na parede, auxiliando na sua identificação em tecidos. A hematoxilina-eosina deve ser feita de rotina para localização e análise do padrão reacional das lesões (Kwon-Chung & Bennett, 1992; Lacaz et al., 2002).

1.4.3 IDENTIFICAÇÃO EM CULTIVO

O cultivo em meio de Sabouraud 2% sem actidiona (cicloheximida) deve ser usado de rotina. Atualmente recomenda-se de rotina também o uso do meio de semente de niger com antibiótico (NSA-cloranfenicol), que apresenta excelente rendimento em espécimes clínicos contaminados como escarro, urina e material de lesões cutâneas abertas. Todo material de biópsia deve ser cultivado de rotina e também submetido a estudo histopatológico (Kwon-Chung & Bennett, 1992; Lacaz et al., 2002).

C. neoformans e *C. gattii* apresentam micro-morfologia e um conjunto de características fisiológicas e bioquímicas comuns. Ambos apresentam-se como levedura capsulada, sem hifa ou pseudo-hifa, são termotolerantes a 37°C, produtor de fenol-oxidase em meio NSA ou meio similar contendo compostos fenólicos. Não fermentam açúcares; assimilam como única fonte de carbono a galactose, sacarose, maltose, trealose, melizitose, D-xilose, L-ramnose, sorbitol, manitol, dulcitol, D-manitol, α-metil-d-glucosídeo, salicina, inositol, além da glicose e frutose. Para realização de

metabolismo oxidativo o nitrato não é assimilado como única fonte de nitrogênio inorgânico, também não sofre redução a nitrito. Hidrolisam uréia devido à capacidade de produzirem urease quando cultivados em meio de agar uréia de Christensen, sendo bastante raros os isolados urease-negativos. São sensíveis à cicloheximida, não crescendo nos meios seletivos que contém esta droga nas concentrações de 0,2 a 0,5%, embora alguns isolados possam crescer em concentrações mais baixas da droga (Kwon-Chung & Bennett, 1992; Lacaz et al., 2002). Portanto os testes usuais de assimilação de fontes de carbono e nitrogênio não distinguem as espécies de *Cryptococcus* fenol-oxidase positivas. O teste de CGB (canavanina-glicina-azul de bromotimol) é o teste fisiológico recomendado para diferenciar *C. gattii* de *C. neoformans*. Baseia-se em diferenças na resistência à L-canavanina e assimilação de glicina como única fonte de carbono e nitrogênio, sendo positivo para *C. gattii* e negativo para *C. neoformans.*

1.4.4-METODOS AUTOMATIZADOS

1.4.4.1 SISTEMA VITEK 2

A busca por métodos automatizados capazes de diferenciar os agentes da criptococose sempre foi um desafio, tendo em vista que o diagnóstico rápido e confiável pode implicar diretamente no desfecho da criptococose. No entanto, a identificação de *C. gattii* em sistemas comerciais não é prevista. Observa-se que o sistema automatizado VITEK 2 não distingue entre *C. neoformans* e *C. gattii*, denominando ambos indiscriminadamente como *C. neoformans* (Brito-Santos 2013).

1.4.4.2 MALDI-TOF

Recentemente, a espectrometria de massa por MALDI-TOF (*"Matrix Assisted Laser Desorption/Ionization"*) tem sido utilizada para identificar com sucesso várias espécies de bactérias e fungos. No âmbito da criptococose essa ferramenta foi utilizada para identificação de espécies e subespécies, sendo observada uma boa correlação com os seus respectivos tipos moleculares. É uma técnica simples para a separação dos oito principais tipos moleculares e também para a detecção de cepas híbridas dentro deste complexo de espécies (Firacative et al., 2012; Machado Siqueira et al., 2018; Posteraro et al., 2012). Na comparação com a análise do sequenciamento de DNA, a técnica de MALDI-TOF MS identificou corretamente 100% das espécies de *Cryptococcus*,

distinguindo *C. neoformans* de *C. gattii* (McTaggart et al., 2011). Porém, esta técnica pode apresentar algumas restrições como a limitação do acervo de espectros de referência no banco de dados e o tamanho da cápsula, que pode interferir na diferenciação entre *C. neoformans* e *C. gattii* (Thomaz et al., 2016).

1.4.5-DIAGNÓSTICO IMUNOLÓGICO

O diagnóstico da criptococose baseia-se em exames laboratoriais, sendo o padrão-ouro o isolamento dos agentes em cultivo, mas têm valor diagnóstico também outros três métodos:

I. Detecção de antígeno polissacáride através de técnica de aglutinação em partículas de látex sensibilizadas com anticorpos específicos.
II. Teste imunocromatográfico aplicado a espécimes clínicos *"Cryptococcal Antigen Lateral Flow Assay"* (CrAg LFA). Este teste mais recentemente disponibilizado no mercado permite o diagnóstico da doença criptocócica e apresenta sensibilidade e especificidade (soro e LCR) de quase 100% em população de pacientes infectados pelo HIV (Kambugu et al., 2008; Temfack et al., 2018).

O teste CrAg LFA pode servir como uma ferramenta de rastreio para a doença criptocócica, e a utilização do CrAg LFA como *"Point of care"*, realizado à beira do leito, em pacientes com CD4< 100 células/mm^3 tem sido proposta para diagnóstico precoce da criptococose principalmente em regiões com limitações laboratoriais, visto que esse exame é de fácil execução (Pongsai et al., 2010). Estudos na África revelam prevalência de 4,3 a 12,1% (Jarvis et al., 2009; Wajanga et al., 2011; Osazuwa et al., 2012; Longley et al., 2016). No Brasil a prevalência em pacientes com CD4< 100 células/mm^3 está sendo estudada em diversos centros, sendo que se observou 11,23% em estudo prospectivo de pacientes internados no INI-FIOCRUZ (Ferreira 2016). A prevalência de antigenemia criptocócica em pacientes HIV tem sido estudada em alguns lugares da Ásia (3,05%), África (5,8%) e Brasil (4,81%) (Liechty et al., 2007; Micol et al., 2007,Ferreira 2016). E em recente estudo de meta-análise de efeito aleatório para avaliar a prevalência de positividade de CrAg LFA no sangue (31 estudos; 35.644 participantes) e meningite criptocócica assintomática em indivíduos CrAg LFA positivo,

observou-se 6% (IC 95%: 5-7) de positividade no sangue e dentro deste grupo uma positividade de 33% (IC 95%: 21 - 45) no LCR, indicando meningite assintomática. O rastreamento do antígeno criptocócico CrAg LFA no soro, seguindo de punção lombar nos casos positivos para excluir meningite e o fluconazol preventivo direcionado em adultos infectados pelo HIV, com contagem de CD4 inferior a 100 células/μL, administração de antirretrovirais, parecem promissores para reduzir a carga da meningite criptocócica (CM) (Temfack et al., 2018).

O rastreio de CrAg tem custo benefício favorável em populações cuja prevalência de criptococose seja acima de 3%, com instituição de tratamento preventivo com fluconazol nos casos de antigenemia isolada, podendo reduzir efetivamente a taxa de mortalidade por criptococose (*Guidelines for The Diagnosis, Prevention and Management of Cryptococcal Disease in HIV-Infected Adults, Adolescents and Children*, 2018).

1.5-ECO-EPIDEMIOLOGIA GLOBAL

A criptococose é uma micose de distribuição mundial e seus agentes, *C. neoformans* e *C. gattii*, apresentam oito tipos moleculares com distribuição geográfica heterogênea. Uma análise de 2.755 isolados de *Cryptococcus*, distribuídos em 2.046 isolados clínicos, 68 veterinários e 604 ambientais, demonstrou o predomínio de *C. neoformans* VNI sorotipo A. Esse tipo molecular é responsável por 63% dos isolados clínicos e 41% dos isolados de origem ambiental. A infecção por *C. gattii* e seus tipos moleculares é menos comum, totalizando 20% quando comparado com 80% dos isolados de *C. neoformans* e seus tipos moleculares. Observando isolados de origem ambiental, essa distribuição dentre as espécies é equivalente mundialmente, sendo que *C. gattii* representa 52% e *C. neoformans* 48% dos isolados analisados no estudo (Meyer et al., 2011). Uma diferença notável é observada quanto à distribuição global de *C. neoformans* e *C. gattii*, onde a prevalência de *C. gattii* é maior nas Américas e no hemisfério sul. *C. gattii* é muito pouco isolado tanto em espécimes clínicos quanto em amostras ambientais na Europa (Cogliati et al., 2017), incluindo Rússia e parte da Ásia, especialmente na China, Tailândia e Japão. A maioria das infecções em pacientes imunocompetentes e imunocomprometidos é causada por *C. neoformans* VNI, sorotipo A (Meyer et al., 2011).

Todos os tipos moleculares de *C. neoformans* causam infecção em pacientes imunocomprometidos e os tipos moleculares de *C. gattii* são responsáveis pelas infecções em pacientes imunocompetentes. Essa observação global não inclui a China que apresenta uma casuística significativa de criptococose por *C. neoformans* VNI em pacientes não infectados pelo HIV, sendo que achados similares foram descritos na Coréia do Sul (Woo et al., 2008). Itália, Tailândia e Japão apresentam dados epidemiológicos semelhantes aos citados na literatura (Cogliati et al., 2008; Supparatpinyo et al., 2008; Miyazaki 2008). Uma mudança na distribuição geográfica é observada nos tipos moleculares VNIII (híbrido AD) e VNIV (sorotipo D), que tinham sido descritos anteriormente no sul da Europa, principalmente na Itália e França, e ocorrem também em número relativamente elevado na América Latina (México, Colômbia, Brasil e Chile). Os isolados ambientais na América do Sul têm como predomínio dois tipos moleculares em particular: VNI (46%) e VGII (36%) (Meyer et al., 2011).

Na América Latina, recente estudo analisando 3.486 isolados de diferentes países (Brasil 45,87%, Colômbia 24,99%, México 9,21%, Argentina 8,09%, Cuba 6,05%, Venezuela 2,90%, Peru 1,06%, Equador 0,77%, Chile 0,55%, Guatemala 0,43% assim como Honduras, Paraguai e Uruguai com um isolado cada) revelou que 67,7% dos isolados eram de origem clínica com predomínio do tipo molecular VNI seguido do tipo molecular VGII e 32,2% eram de origem ambiental com mesmo perfil de tipos moleculares dos isolados de origem clínica (Firacative et al., 2018).

Na América do Norte, o tipo molecular VGII é o mais comum, e representa 61% dos isolados ambientais, seguido de VNI (29%). Chamam a atenção os isolados de *C. gattii* VGII relacionados à epidemia de Vancouver, hoje área endêmica, e seus respectivos subtipos denominado VGIIa e VGIIb. Tais subtipos foram isolados de amostras clínicas, veterinárias e ambientais na Ilha de Vancouver e posteriormente ocorre expansão para a costa do pacífico, onde foi isolado um terceiro subtipo VGIIc (Acheson et al., 2017; MacDougall et al., 2007).

Na Oceania, incluindo a Austrália, VGII (70%) é o tipo molecular mais comum em isolados ambientais e na Europa VNI (40%) é o mais comum (Meyer et al., 2011). Na criptococose por *C. neoformans*, três genótipos moleculares são mais frequentemente citados: VNI, VNII e VNB (Meyer et al., 2003). Cepas VNI são globalmente dominantes, e isolados VNII são menos comuns. VNB é um novo

subgrupo molecular descoberto em uma população descrita em Botsuana e também presente na América do Sul (Andrade-Silva et al., 2018; Litvintseva et al., 2006).

Globalmente na criptococose por *C. neoformans,* há o predomínio dos subtipos ST93 e ST5 de VNI. O ST93 é identificado principalmente em isolados de pacientes HIV-positivos em vários países, sendo prevalente na Indonésia, Índia, Uganda e Brasil. Já o subtipo ST5 é mais prevalente entre os isolados clínicos na Europa, Ásia e raramente isolado no Brasil (Ferreira-Paim et al., 2017). Faltam no entanto estudos de isolados de agentes da criptococose em outros países da África e outros em desenvolvimento, para ter um panorama mais amplo.

1.5.1 SURTOS DE CRIPTOCOCOSE

Além do caráter oportunista e endêmico de áreas tropicais e subtropicais, os agentes da criptococose são relacionados também a surtos em diferentes áreas geográficas. Curiosamente, estes relatos apontam *C. gattii* como agente causal desses surtos e até o momento nenhum relato bem documentado tem atribuído a *C. neoformans* esse potencial epidêmico.

Surtos em animais foram descritos, como pneumonia em cabras na Espanha (Baró et al., 1998) e forma disseminada em psitacídeos em aviário no interior de São Paulo (Raso et al., 2004). O maior surto foi registrado na ilha de Vancouver, Canadá, atingindo 38 casos humanos, entre 1999 e 2001, a maioria imunocompetentes, sendo 58% do sexo masculino, 72% com lesão pulmonar, 26% com lesão de SNC e letalidade em torno de 10% (Kidd et al., 2004). A micose foi também diagnosticada em 35 animais, incluindo 18 gatos, 17 cães, 6 golfinhos (*Phocoenidae dalli*), 2 furões e 2 lhamas. Até hoje há relatos de casos relacionados a essa epidemia, que adquiriu um caráter endêmico na região. Essencialmente causada por *C. gattii* VGII com expansão para costa pacifica dos Estados Unidos incluindo também infecção humana e animal (Acheson et al., 2017).

1.6-ECO-EPIDEMIOLOGIA NO BRASIL

Isolados clínicos e ambientais no Brasil mostram considerável diversidade genética de ambas as espécies, demonstrando ocorrência simultânea de diferentes tipos/genótipos (VNI, VNIV e VGII) em ocos de árvores. Estudo retrospectivo dos tipos moleculares de *C. neoformans* e *C. gattii* circulantes no Brasil sugere diferenças regionais na distribuição destes genótipos. Assim, as regiões Sul e Sudeste do Brasil apresentam como tipo molecular predominante VNI, atingindo, sobretudo pacientes imunocomprometidos, principalmente os com aids (Igreja et al., 2004; Matsumoto et al., 2007; Trilles et al., 2008).

Os primeiros casos de criptococose infantil descritos por Corrêa (1999), em Belém, chamaram a atenção para a ocorrência e gravidade da meningoencefalite em crianças HIV negativo nesta região da Amazônia. Estudos mostram o predomínio da criptococose *gattii* sobre a criptococose *neoformans* em centro de referência em Belém do Pará (Santos et al., 2008), onde a maioria absoluta apresenta lesão de SNC. Estudos ambientais evidenciaram a presença dos dois agentes (*C. gattii* e *C. neoformans*) em árvores, na poeira coletada no interior de domicílios e em raspado de madeira em decomposição obtido de tábuas utilizadas na construção de casas da cidade de Belém (Costa et al., 2009).

C. neoformans mostra-se amplamente adaptado a áreas urbanas, sendo capaz de reproduzir-se por expansão clonal e sobreviver em substratos secos, protegido da iluminação direta. Já foi encontrado na poeira domiciliar no Rio de Janeiro, em cerca de 13% dos domicílios investigados e, também em gaiolas de pássaros domésticos (Passoni *et al.*1998). Mostra-se ubíquo e de fácil inalação através de formas dessecadas das leveduras presentes no meio ambiente e, possivelmente, também de basidiósporos. Este estudo no Rio de Janeiro mostrou risco aumentado de adquirir criptococose para moradores com HIV/aids em casas contaminadas por *C.neoformans* sorotipo A (Passoni et al., 1998). Este sorotipo A, que corresponde ao tipo VNI, também é encontrado em ambientes rurais e já foi identificado em cacaueiro e árvores nativas da mata amazônica, indicando habitats naturais em áreas preservadas ou com pouca intervenção humana, provavelmente seu micro habitat primário (Lazera et al., 2000).

Considerando a expansão geográfica de *C. gattii*, um estudo de isolados ambientais investigou ocos de árvores vivas como possível reservatório de *C. gattii* no Rio de Janeiro, sendo que 80 amostras de madeira em decomposição de área urbana e

85 de área silvestre foram coletadas. *C. gattii* VGI foi identificado em 98% das colônias isoladas, seguido de *C. neoformans* VNI (2%). Pela primeira vez no Rio de Janeiro, *C. gattii* VGI foi isolado em oco de árvores (Barbosa et al., 2013).

Trilles e colaboradores (2008) realizaram estudo molecular de 443 de isolados brasileiros de *C. neoformans* e *C. gattii* para determinar sua distribuição geográfica no Brasil. O tipo molecular mais comum encontrado no Brasil foi VNI (64%), seguido por VGII (21%), VNII (5%), VGIII (4%), VNIV e VGI (3% cada), e VNIII (<1%). A criptococose primária causada pelo tipo molecular VGII (sorotipo B, *MATalfa*) prevalece em pacientes imunocompetentes nas regiões Norte e Nordeste, revelando um padrão regional endêmico para este tipo molecular no norte do Brasil Nishikawa et al., 2003; Trilles et al., 2008; Martins et al., 2011).

No Brasil, estudos clínico-epidemiológicos mostram a importância da criptococose *gattii* de SNC sob forma de meningite em adultos jovens de ambos os sexos e crianças nas regiões norte e nordeste, com letalidade de 35% a 40% (Cavalcanti 1995, Correa 1999, Santos 2000, Nishikawa et al., 2003, Martins et al., 2004).

No Brasil, análise de 143 isolados de *C. neoformans* VNI, clínicos e ambientais, por MLST destaca uma estrutura populacional clonal. A alta prevalência do ST93 em isolados clínicos e ambientais e a significativa presença do ST77 em isolados ambientais sugerem uma estrutura populacional altamente clonal no Brasil para este tipo molecular (Ferreira-Paim et al., 2017)

Por outro lado, estudo de 145 isolados *C. gattii* VGII identificou alta variabilidade genética entre isolados, com 81 subtipos diferentes. Os STs mais comuns e frequentes nas amostras clínicas e ambientais foram ST20-VGIIa, ST40 e ST5. O trabalho teve como objetivo avaliar a diversidade genética dentro da população brasileira de *C. gattii* VGII para obter um novo "insight" sobre a origem da dispersão global de *C. gattii*. Os dados apresentados sugerem o surgimento de cepas altamente virulentas de ancestrais nas regiões Norte do Brasil, Amazônia e Nordeste. Numerosos genótipos representam uma ligação entre o Brasil e outras partes do mundo, reforçando a América do Sul como a origem mais provável dos subtipos *C. gattii* VGII e sua subsequente disseminação global, incluindo sua dispersão na América do Norte (Souto et al., 2016).

Foi realizado estudo na região Norte com 57 isolados de pacientes com criptococose em um hospital de atendimento terciário do estado do Amazonas, Brasil, entre 2006 e 2010. A maioria dos isolados de *C. neoformans* (n = 40) foi caracterizada

como membros do tipo molecular VNI (n = 39), um único isolado foi caracterizado como VNII enquanto que todos os isolados de *C. gattii* (n = 17) eram *C. gattii* VGII. Este estudo revelou a prevalência do tipo molecular VNI, a presença do tipo molecular VGII, e foi relatada a primeira observação do tipo molecular VNII na região norte do Brasil (Freire et al., 2012). Alves e col. corroboram com esses achados, demostrando o predomínio de VNI seguido de VGII em isolados ambientais no município de Manaus (Alves et al., 2016).

Considerando a complexidade da criptococose e de seus agentes no Brasil, a expansão geográfica e possibilidade de surtos na extensão da região amazônica, é preciso ampliar os estudos nesta região, buscar o diagnóstico precoce, principalmente em crianças destas regiões, bem como identificar tipos moleculares predominantes em infecções humanas, em animais e no ambiente em nosso país.

2-RACIONAL E JUSTIFICATIVA

O estudo da criptococose dirigido a indivíduos nativos ou moradores de regiões endêmicas em nosso país constitui um desafio considerando que a criptococose, apesar de ser emergente e causar elevada letalidade sob forma de meningite, não é notificada no Brasil, onde sua magnitude é pouco conhecida e os trabalhos publicados indicam diferentes perfis epidemiológicos regionais. Particularmente na Amazônia observa-se elevada ocorrência de criptococose por *C. gattii* em crianças sem HIV apontando um aspecto epidemiológico distinto e não descrito em outras regiões do mundo. Isso sugere precoce exposição ambiental aos agentes desde as fases iniciais da infância. Outro aspecto importante é a relação da expansão geográfica da criptococose com desmatamento e mudanças climáticas. Estariam esses indivíduos mais expostos aos agentes da criptococose em ambientes de colonização na microrregião do Rio Negro/Amazônia, considerando que os agentes da criptococose colonizam substratos vegetais, particularmente madeira em decomposição, favorecida nesta região pela elevada umidade? Por outro lado, o extenso uso da madeira para utensílios e como elemento estrutural do domicílio favoreceria a maior exposição do hospedeiro ao fungo no seu cotidiano?

O primeiro estudo dirigido especificamente a estas questões no Brasil foi realizado em 2013 intitulado "ISOLAMENTO E CARACTERIZAÇÃO MOLECULAR DOS AGENTES DA CRIPTOCOCOSE EM POEIRA DOMICILIAR EM BAIRROS DE SANTA ISABEL DO RIO NEGRO NO ESTADO DO AMAZONAS" pela Pós-graduação da Medicina Tropical do IOC/FIOCRUZ. Amostras de poeira doméstica foram analisadas quanto à presença dos agentes da criptococose, observando-se positividade de 3 dentre 51 casas (5,88%), com isolamento exclusivo de *C. gattii* VGII, tipos sexuados *Mat*-a e *Mat*-alfa, constituindo o primeiro relato de ocorrência ambiental desta espécie e tipo molecular no estado do Amazonas.

Chamou atenção inicialmente que, apesar da exposição intradomiciliar aos agentes da criptococose, não foram encontradas evidências de surto de criptococose nestes ambientes. De fato, é possível que casos de criptococose tenham ocorrido sem diagnóstico, ou tenham sido diagnosticados como meningites inespecíficas em centros distantes. É possível também que a exposição cotidiana à poeira contaminada com diferentes subtipos de VGII cause infecção sub-clínica, quadros respiratórios

regressivos que passam despercebidos ou confundidos com viroses e outras infecções do aparelho respiratório.

Considerando-se que *C. gattii* comporta-se como agente infeccioso primário de expressiva virulência; que apresenta potencial epidêmico e está em franca expansão geográfica mundial, atingindo América do Norte, essencialmente a costa Pacífica; e que estudos filogenéticos indicam a região amazônica como área geográfica de origem do genótipo VGII de *C. gattii,* o achado recente de *C. gattii* tipo molecular VGII em grandes concentrações em poeira domiciliar em residências do município de Santa Isabel do Rio Negro (AM) apresenta implicações importantes: provavelmente não é achado isolado, mas sugere a hipótese de que este agente está presente e adaptado nesta importante região amazônica. Sugere também a exposição cotidiana dos moradores ao agente, dentro dos seus domicílios. Portanto é fundamental estabelecer estudos ambientais nesta região, iniciando pela Bacia do Rio Negro. Será que esse fungo está circulando em outros municípios desta microrregião? Será que esses isolados ambientais apresentam algum potencial de virulência? É possível que ocorram infecções subclínicas, e mesmo criptococose, nestas comunidades, e assim, como podemos detectar esses casos? Todos esses eventos constituem um desafio e têm sido muito pouco estudados no bioma amazônico, particularmente nas áreas urbanizadas onde a perda da biodiversidade original possibilita a adaptação e emergência de variantes virulentos, formação de microfocos associados a construções humanas e contínua exposição de seus moradores. Para estudar a colonização pelos agentes da criptococose nos ambientes urbanos da microrregião do Rio Negro propomos investigar ocorrência dos mesmos em focos ambientais relacionados ao domicílio e ao peridomicílio em outros municípios desta microrregião. Em paralelo, consideramos oportuno sugerir medidas possíveis de vigilância dos agentes da criptococose nessa microrregião.

3-OBJETIVOS

3.1- OBJETIVO GERAL

Investigar a presença dos agentes da criptococose em amostras ambientais intra e peridomiciliares e propor medidas de vigilância para esses agentes na microrregião do Rio Negro no estado do Amazonas.

3.2-OBJETIVOS ESPECÍFICOS

1. Isolar e identificar os agentes da criptococose a partir de fontes ambientais.

2. Caracterizar tipos moleculares e o tipo sexuado (*mating type*) dos agentes da criptococose.

3. Determinar a epidemiologia molecular dos agentes da criptococose na área de estudo.

4. Otimizar técnica para diagnóstico de novos casos de criptococose na Microrregião.

5. Elaborar documento de divulgação dos agentes da criptococose para população da área de estudo.

6. Elaborar informe técnico para as secretarias de saúde da microrregião.

4-CONSIDERAÇÕES ÉTICAS

A presente tese constitui parte do projeto: “Estudo da infecção por *Cryptococcus* spp em crianças e investigação de fontes ambientais de agentes da criptococose na Microrregião do Rio Negro, Amazonas, Brasil”, aprovado pelo Comitê de Ética em Pesquisa do INI/FIOCRUZ em 07/05/15 sob o número CAAE 23238913.3.0000.5262/Versão 4/ número do parecer 1.226.671 (anexo A).

5. CAPITULO 1- EPIDEMIOLOGIA DOS AGENTES DA CRIPTOCOCOSE NA MICRORREGIÃO DO RIO NEGRO.

Neste capítulo, três trabalhos são apresentados.

A criptococose é uma doença cosmopolita e vem sendo relatada como endêmica nas regiões norte e nordeste do Brasil por vários autores. Essa micose não é de notificação compulsória em nosso país e por sua vez temos uma escassez de dados quanto à sua real magnitude. Segundo Prado e col (2009), entre as micoses sistêmicas a criptococose é a primeira causa morte, especialmente em pessoas vivendo com HIV/aids (Prado et al., 2009). A maioria dos dados de mortalidade obtidos por esses autores são relativos tanto à causa primária e à causa associada à morte, esta última refletindo o alto índice de mortalidade da meningite criptocócica (Soares 2015).

O Brasil, junto com países como Austrália, Canadá e EUA são os pioneiros em estudos de fontes ambientais dos agentes da criptococose no mundo. Os estudos clássicos revelam a associação desses agentes a excretas de aves e oco de árvores como habitat natural e fonte primária de infecção. **Existem poucos estudos sobre a exposição domiciliar ou peridomiciliar baseados no conceito "*indoor environment*" que considera que os seres humanos são expostos a microrganismos principalmente pela inalação de partículas em suspensão aérea proveniente da poeira domiciliar. Este é um importante mecanismo de exposição, pois as pessoas gastam mais de 86,9% de suas vidas em ambientes fechados** (Klepeis et al., 2001). Estudos relacionados a esse conceito revelam que o isolamento dos agentes da criptococose no domicílio aumenta 2:1 vezes a chance do paciente adquirir essa micose em sua residência (Passoni et al., 1998).

No primeiro trabalho publicado, alertamos sobre o isolamento de agentes da criptococose associados a casas de madeira no município de Santa Isabel do Rio Negro localizado na Microrregião do alto do Rio Negro. Esses achados iniciais foram publicados na forma de artigo completo na revista científica "*Plos One"*, sendo posteriormente sedimentado através de um segundo relato publicado como documento suplementar na revista "*World Biomedical Frontiers"*, que revelou a presença dos agentes da criptococose em postes de madeira na área antropizada do mesmo município. Essa microrregião mostrou-se assim área de estudo ideal para entender a dinâmica dos agentes da criptococose no ambiente domiciliar. O último trabalho realizado nessa

região, revelou positividade em três de 4 municípios da microrregião do Rio Negro. Também foram avaliadas a epidemiologia molecular e o perfil de virulência *in vivo* dos isolados ambientais. Nesse trabalho confirmamos a ***frequência*** dos agentes da criptococose na microrregião e seu potencial de virulência. O aluno, nesse capítulo, tenta mostrar a importância do estudo das amostras ambientais associadas aos domicílios, reforçando que, baseado no conceito "*indoor environment"*, os habitantes destes 3 municípios podem estar expostos cotidianamente a esses agentes em suas residências.

Mesmo que na microrregião do Rio Negro não se tenha evidências ou relatos de surtos por esses agentes, acreditamos que estes novos relatos contribuirão para o melhor e maior conhecimento acerca da importância do estudo da poeira domiciliar na criptococose, e que de posse destes dados, novos estudos possam aprimorar o olhar clínico-epidemiológico da criptococose nesta região para otimizar o diagnóstico e o tratamento dos pacientes.

PLOS ONE

RESEARCH ARTICLE

Environmental Isolation of *Cryptococcus gattii* VGII from Indoor Dust from Typical Wooden Houses in the Deep Amazonas of the Rio Negro Basin

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Abstract

Cryptococcosis is a human fungal infection of significant mortality and morbidity, especially in the meningoencephalitis form. Cryptococcosis is distributed worldwide and its agents, *C. neoformans* and *C. gattii*, present eight major molecular types—VNI-VNIV and VGI-VGIV respectively. The primary cryptococcosis caused by molecular type VGII (serotype B, MAT alpha) prevails in immunocompetent patients in the North and Northeast of Brazil, revealing an endemic regional pattern to this molecular type. Since 1999, *C. gattii* VGII has been involved in an ongoing outbreak in Canada, and is expanding to the Northwest of the United States, two temperate regions. Exposure to propagules dispersed in the environment, related to various organic substrates, mainly decomposing wood in and around dwellings, initiates the infection process. The present study investigated the presence of the agents of cryptococcosis in dust from dwellings in the upper Rio Negro, municipality of Santa Isabel do Rio Negro in Amazonas state. Indoor dust was collected from 51 houses, diluted and plated on bird seed agar. Dark brown colonies were identified phenotypically, and genotypically by *URA5* restriction fragment length polymorphism analysis and multilocus sequence typing (MLST). The mating type was identified using pheromone-specific primers. Three of the 51 houses were positive for *C. gattii* molecular type VGII, *MATα* and *MATa*, showing a high prevalence of this agent. MLST studies identified eight subtypes, VGIIb (ST7), VGIIa (ST20), (ST5) and 5 new subtypes unique to the region. For the first time in the state of Amazonas, *C. gattii* VGII *MATα* and *MATa* were isolated from the environment and correlates with endemic cryptococcosis in this state. This is the first description of MLST subtypes on environmental isolates in the Brazilian Amazon, indicating domiciliary dust as a potential source for human infection with different subtypes of *C. gattii* VGII *MATα* and *MATa*.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Cryptococcosis is a life-threatening systemic mycosis affecting humans and animals worldwide. The disease is acquired by inhalation of infectious propagules (desiccated yeasts cells or basidiospores) of the members of the *Cryptococcus neoformans*/*C. gattii* species complex from the environment. Its most frequent clinical manifestation is meningoencephalitis [1]. Cryptococcosis by *C. neoformans* is cosmopolitan, affecting mainly immunocompromised individuals, especially patients with AIDS [2]. On the other hand, *C. gattii* causes predominantly a primary infection in immunocompetent individuals, previously associated with tropical and subtropical climates, but now gaining prominence as an important cause of human and veterinary disease in temperate regions of North America [3–7].

Molecular epidemiological studies have identified eight major molecular types within the *Cryptococcus neoformans*/*C. gattii* species complex. *C. neoformans* is classified into the major molecular types VNI/AFLP1 and VNII/AFLP1A and AFLP1B (serotype A), VNIII/AFLP3 (serotype AD), and VNIV/AFLP2 (serotype D), while *C. gattii* correspond to VGI/AFLP4, VGII/AFLP6, VGIII/AFLP5, and VGIV/AFLP7, all corresponding to serotypes B and/or C [8]. In addition there are inter-specific serotype BD or AB hybrids, corresponding to AFLP8 and AFLP9, respectively [9,10]. To globally standardize genotyping of the *C. neoformans*/*C. gattii* species complex, a MLST scheme was established by the ISHAM working group "Genotyping *C. neoformans* and *C. gattii*" based on variable regions of seven independent genetic loci: *CAP59*, *GPD1*, *LAC1*, *PLB1*, *SOD1*, *URA5* and the IGS1 region, taking advantage of the high discriminatory power and good reproducibility between different laboratories [8]. The allele (AT) and sequence types (ST) of the ISHAM consensus MLST scheme can be determined via the web page at http://mlst.mycologylab.org/.

Cryptococcosis by *C. gattii* in Brazil is endemic and shows a regional pattern, being more common in the North (N) and Northeast (NE) of the country [11]. One hundred thirty four (134) cases have been diagnosed in the Amazon region, including the states of Pará and Amazonas [12–15]. In these states, as well as in other states of the N and NE regions (Roraima, Maranhão and Piauí), noteworthy was the emergence of cryptococcosis in immunocompetent (HIV-negative) children in about one fifth of the reported cases [12, 15–16], suggesting that natural infection occurs early in life. The dynamics of natural infections by *C. gattii* in these regions is not well known and requires more specific environmental studies to detect possible outbreaks. Indeed, environmental sources related to trees colonized by the agents of cryptococcosis have been described in Roraima [17], Pará [18] and Piauí [19] states.

Pioneering studies of cryptococcosis in AIDS in Central Africa and Brazil demonstrated the risk of those patients to acquire cryptococcosis from indoor dust [20, 21]. The current study investigated the presence of *C. neoformans* and *C. gattii* in dwellings of the hinterland of the Brazilian Amazon, and characterized the molecular subtypes and mating types of the obtained isolates.

Methods

Studied Region

The study was conducted in the city of Santa Isabel do Rio Negro, Amazonas state, located 620 km straight or 772 km by waterway away from Manaus, the capital of the Amazonas state, and about 1,000 km far from the Atlantic Ocean coast of the Brazilian Northern region (Fig. 1). The municipality of this city has the following characteristics: land area of 63,127 km², tropical rainforest climate, rainy and humid; maximum temperature 32.6°C, minimum 21.5°C; altitude: 21m above sea level; Cartesian coordinates: 0° 28′ south latitude and 65° 32′ longitude west of

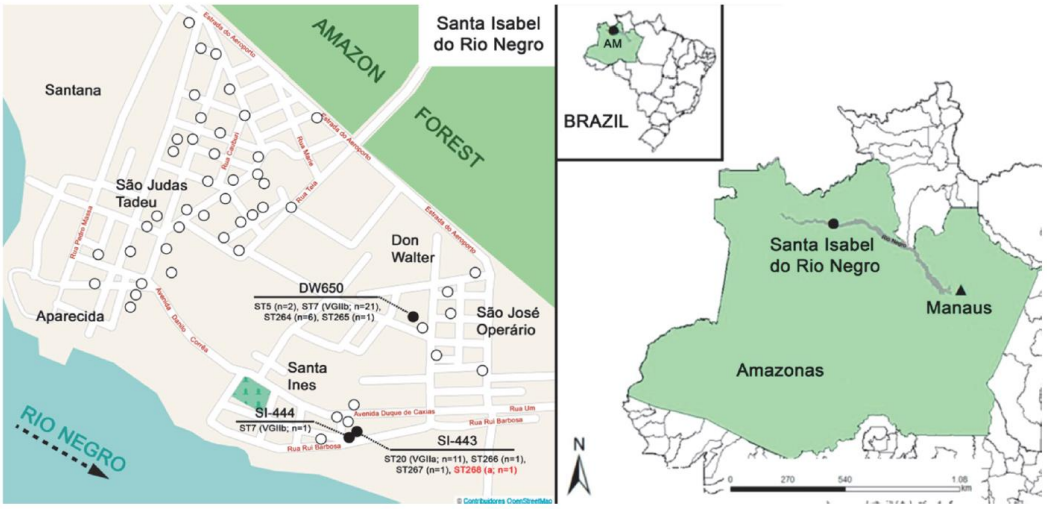

Fig 1. Sampling locations in the city of Santa Isabel do Rio Negro (circles) with the 3 positive houses (dark circles), DW650, SI-444 and SI-443 with the respective sequence types (ST) identified amongst the samples, and location of Santa Isabel do Rio Negro in the Amazonas state, Northern Brazil. The maps were adapted from Bing Maps to reflect the specific study site situation.

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Greenwich. The city settlement occurred approximately one hundred years ago and the population reaches 19,292 inhabitants [22], with the majority of them living in wooden houses. The research was conducted in an urban area (coordinates 65W 01' 08"- 0S 24' 51"), so special permission from authorities was not required. However, the study had the approval of the Municipal Health Department of the city, as well as prior ethical approval in the context of the project "Study of the health conditions of the municipality of Santa Isabel do Rio Negro" (Oswaldo Cruz Foundation Ethical Research Committee, Rio de Janeiro, Brazil, reference n° CAAE 0011.0.009.000–03).

Sampling and Cryptococcal isolation

Dust samples were collected from 51 houses randomly chosen in different neighborhoods of the city of Santa Isabel do Rio Negro, to investigate the presence of the agents of cryptococcosis, *C. neoformans* and *C. gattii*. One sample per house was obtained combining dust from all rooms swept with a different broom per house to prevent cross contamination between houses, and the material was stored in sterile plastic bags (Nasco/WHIRL-PARK). The location of the selected households was obtained using a GPS (Garmin Etrex H). Cryptococcal cells were isolated as described previously [21, 23]. Briefly, 1g of each dust sample was suspended in 50 ml NaCl 0.9% with 0.2 g of chloramphenicol, followed by manual shaking for 5 minutes. After resting 30 minutes, 1 ml of the supernatant was plated onto 10 Niger Seed Agar (NSA) plates (0.1 ml each) [24]. The plates were then incubated at 25°C and checked daily for brown colonies for 5 days. Each phenol oxidase-positive/brown colony was sub-cultured individually on NSA medium for phenotypical and molecular characterization. The isolates were preserved in skim milk at -20°C and in glycerol at -70°C and deposited in the Culture Collection of Pathogenic Fungi (CFP) of the Mycology Laboratory, IPEC/FIOCRUZ. The limit for the detection of phenol oxidase-positive colonies was 50 CFU per gram of sample.

Phenotypic Identification

Brown colonies were purified and tested for urease production on Christensen urea agar [25], and for carbon and nitrogen compound assimilation using VITEK 2-BioMerieux System (VITEK 2, ICB, bioMerieux, Durham, USA). The species *C. neoformans* and *C. gattii* were differentiated on canavanine-glycine-bromothymol blue (CGB) medium [26].

Identification of the Major Molecular Types

After DNA extraction [27], the major molecular types of all studied isolates were identified by *URA5*-RFLP analysis [28]. Amplification of the *URA5* gene was performed in a final volume of 50 μL. Each reaction contained 50 ng of DNA, 1X PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl—Invitrogen), 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Invitrogen), 2 mM magnesium cloride, 1.5 U Taq DNA polymerase (Invitrogen), and 50 ng of each primer URA5 (5' ATGTCCTCCCAAGCCCTCGACTCCG 3') and SJ01 (5' TTAAGACCTCTGAACACCG-TACTC 3'). PCR was performed for 35 cycles in a Eppendorf gradient mastercycler (Hamburg, Germany), using the following cycling conditions: 94°C for 2 min initial denaturation, 45 s of denaturation at 94°C, 1 min annealing at 61°C, and 2 min extension at 72°C, followed by a final extension cycle for 10 min at 72°C. PCR products were double digested with *Sau96*I (10 U/μL) and *Hha*I (20 U/μl) for 3 h, and the DNA fragments were separated via 3% agarose gel electrophoresis at 100 V. *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).

Mating Type identification

The mating type was identified by PCR using specific primers for the pheromone genes: MFalfaU (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** (MAT**a**) [3]. Amplification of both genes was performed independently in a final volume of 50μL containing 50 ng of DNA, 1X PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl—Invitrogen], 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Invitrogen), 2 mM magnesium cloride, 2.5 U Taq DNA polymerase (Invitrogen), and 50 ng of each primer, in a Eppendorf gradient mastercycler (Hamburg, Germany), using the following cycling conditions: 95°C for 3-min initial denaturation, 30 cycles at 94°C for 1 min, annealing at 57.5°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The unique fragment corresponding to each mating type was visualized after 3% agarose gel electrophoresis at 100 V.

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* [8] 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 [8]. 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 [29]. An un-rooted Neighbor-Joining tree was constructed from the combined loci of all environmental isolates studied using the program MEGA version 5. The genetic distance

between isolates was computed using the p-distance, and all positions containing alignment gaps were eliminated in the pairwise sequence comparisons. Bootstrap analysis using 1000 replicates was used to estimate support for clades of the concatenated dataset. The haplotype diversity (Hd) was calculated using DnaSP v5.10 (http://www.ub.edu/dnasp/). Novel genotypes have been submitted to *C. gattii* MLST database (http://mlst.mycologylab.org/).

Results

Fifty one dust samples were obtained from randomly chosen houses in 9 neighborhoods of the city Santa Isabel do Rio Negro, of which three were positive for *C. gattii* (Fig. 1, Table 1). The sample SI-443 accounted for 2.500 CFU/g of dark brown colonies, SI-444 for 50 CFU/g and DW-650 exhibited uncountable number of colonies, with an estimated concentration of >50.000 CFU/g. A total of 144 dark brown colonies from the 3 positive houses grown on birdseed agar were isolated and identified as *C. gattii* molecular type VGII. From those, 45 isolates were randomly selected for further characterization of the mating type and MLST analysis, including the single colony from sample SI-444, 14 colonies from sample SI-443, and 30 colonies from sample DW-650. Mating type analysis demonstrated 44 isolates were MATalpha, and one isolate from sample SI-443 was identified as MAT**a** (Table 1).

Discussion

Publications on the presence of agents of cryptococcosis in indoor dust are scarce. Two studies, originated from index-cases of aids-associated cryptococcosis in South America and Africa, showed similar results: in Rio de Janeiro, southeast of Brazil, the investigation of 77 dwellings revealed the isolation of *C. neoformans* from domestic dust in 11 (14.3%) [21], and some years earlier in Bujumbura, Burundi, *C. neoformans* was isolated from domestic dust in 13 (54%) out of 24 dwellings studied [30]. The principal factor associated with domiciliary contamination by *C. neoformans* in these studies was the presence of avians in the domestic environment or nearby the homes. The present study has no index-case and revealed the exclusive occurrence of *C. gattii* VGII in indoor dust obtained from dwellings in a remote human settlement formed in the heart of the Amazon rainforest in Brazil. A significant positivity, of 5.9%, in indoor dust samples was observed, thus suggesting daily human exposure to these potential disease agents inside those houses. On the contrary to previous reports, no domestic or caged birds were observed in the present study. The houses in Santa Isabel do Rio Negro are rustic, most of them built with planks of wood obtained from the extraction of native species of trees in the surrounding rainforest, especially the Brazil nut tree.

There is much evidence that *C. gattii* VGII is widely distributed in Amazonia, e.g. this genotype has been isolated from several species of trees from Brazil and Colombia [31]. Previous findings in anthropic ecosystems in Brazil suggest that members of the *Cryptococcus neoformans/C. gattii* species complex are not associated with a particular tree, but with a specialized niche resulting from the natural biodegradation of wood [32]. Moreover, the isolation of *C. gattii*, strain LMM645, by Fortes *et al.* 2001 from a native jungle tree (*Guettarda acreana*) on a wild island of the Amazon rainforest, suggests that wild tropical forests may harbour primary sources of this agent. This was recently again emphasised by a global study pointing to the same environmental isolate, LMM645, suggesting an ancient dispersal of the human fungal pathogen *C. gattii* from the Amazon rainforest [33]. Lazéra *et al.* in 1996 suggested already that hollows of living trees may provide environments that are sheltered, damp and probably less exposed to changes in climatic conditions, offering more suitable conditions for the survival, adaptation and reproduction of *C. gattii* [23]. Likely, the wooden houses in Santa Isabel do Rio Negro also may provide comparable conditions for cryptococcal propagules coming from

Table 1. List of isolates used in the study from the positive house samples according to the phenotypic identification (NSA, Urea and CGB) and molecular characterization (*URA5*-RFLP type, Mating type and MLST type).

Positive house	*Strain*	*NSA*	*Urea*	*CGB*	*URA5-RFLP*	*Mating Type*	*MLST Type*
DW650	CFP352	+	+	+	VGIIb	alpha	ST7
	CFP353	+	+	+	VGII	alpha	ST5
	CFP354	+	+	+	VGII	alpha	ST264
	CFP355	+	+	+	VGII	alpha	ST264
	CFP356	+	+	+	VGIIb	alpha	ST7
	CFP380	+	+	+	VGII	alpha	ST264
	CFP381	+	+	+	VGIIb	alpha	ST7
	CFP382	+	+	+	VGIIb	alpha	ST7
	CFP383	+	+	+	VGIIb	alpha	ST7
	CFP384	+	+	+	VGIIb	alpha	ST7
	CFP385	+	+	+	VGIIb	alpha	ST7
	CFP387	+	+	+	VGIIb	alpha	ST7
	CFP388	+	+	+	VGII	alpha	ST265
	CFP389	+	+	+	VGIIb	alpha	ST7
	CFP390	+	+	+	VGIIb	alpha	ST7
	CFP391	+	+	+	VGIIb	alpha	ST7
	CFP392	+	+	+	VGIIb	alpha	ST7
	CFP393	+	+	+	VGIIb	alpha	ST7
	CFP394	+	+	+	VGIIb	alpha	ST7
	CFP395	+	+	+	VGII	alpha	ST264
	CFP396	+	+	+	VGIIb	alpha	ST7
	CFP397	+	+	+	VGII	alpha	ST5
	CFP398	+	+	+	VGIIb	alpha	ST7
	CFP399	+	+	+	VGIIb	alpha	ST7
	CFP400	+	+	+	VGIIb	alpha	ST7
	CFP401	+	+	+	VGIIb	alpha	ST7
	CFP402	+	+	+	VGIIb	alpha	ST7
	CFP403	+	+	+	VGII	alpha	ST264
	CFP404	+	+	+	VGII	alpha	ST264
	CFP405	+	+	+	VGIIb	alpha	ST7
SI443	CFP409	+	+	+	VGII	alpha	ST266
	CFP406	+	+	+	VGIIa	alpha	ST20
	CFP407	+	+	+	VGIIa	alpha	ST20
	CFP408	+	+	+	VGIIa	alpha	ST20
	CFP410	+	+	+	VGII	a	ST268
	CFP411	+	+	+	VGIIa	alpha	ST20
	CFP412	+	+	+	VGIIa	alpha	ST20
	CFP413	+	+	+	VGIIa	alpha	ST20
	CFP414	+	+	+	VGIIa	alpha	ST20
	CFP415	+	+	+	VGIIa	alpha	ST20
	CFP416	+	+	+	VGIIa	alpha	ST20
	CFP417	+	+	+	VGII	alpha	ST267
	CFP418	+	+	+	VGIIa	alpha	ST20
	CFP419	+	+	+	VGIIa	alpha	ST20

(*Continued*)

Table 1. (*Continued*)

Positive house	*Strain*	*NSA*	*Urea*	*CGB*	*URA5-RFLP*	*Mating Type*	*MLST Type*
S444	CFP420	+	+	+	VGIIb	alpha	ST7

MLST analysis revealed a total of 8 sequence types among the 45 VGII environmental isolates (Fig. 2, Table 1), and the DNA polymorphism analysis showed extensive haplotype diversity (H_d = 0.695). The most common sequence type was ST7 (VGIIb), representing 48.9% of the isolates (n = 22) analyzed. The second most common was ST20 (VGIIa), representing 24.5% of the studied isolates (n = 11). The ST264 is exclusive for Brazil and represented 13.3% of the isolates (n = 6). ST5 was identified in 2 isolates. The sequence types ST265, ST266, ST267 and ST268 were unique to this region and to Brazil and represented by one isolate each (2.2%). The MLST data have been deposited in the International MLST database for *C. neoformans* and *C. gattii*, and the corresponding sequences can be obtained in the webpage http://mlst.mycologylab.org/.

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sources of *C. gattii* VGII in the native rainforest, thus forming a microfocus in human dwellings.

Studies on the population structure of the *Cryptococcus neoformans/C. gattii* species complex can help the understanding of the geographic expansion and the reproductive strategies of VGII adapted to human dwellings in the Amazon rainforest. The present study revealed an unexpected high genetic diversity amongst the obtained VGII isolates, with eight MLST sequence types (ST20 (= VGIIa), ST7 (= VGIIb), ST5, ST264, ST265, ST266, ST267, and ST268) being present amongst 45 environmental strains from three houses, compared with Australia where only 6 MLST sequence types (ST20 (VGIIa), ST7 (VGIIb), ST5, ST21, ST33, ST38, and ST48) were found amongst 54 clinical, veterinary and environmental isolates from the entire Australian continent [34], and in the USA, where only 7 MLST sequence types, including VGIIa (ST20), VGIIb (ST7) and VGIIc (ST6) were present amongst 212 clinical and veterinary isolates, with most of them originating from the Pacific Northwest [7]. Both studies revealed the presence of a predominant clonal structure amongst most of the VGII populations in Australia and the USA.

The haplotype diversity has till now only been described for *C. neoformans*, where it ranged from 0.20 in Asia, 0.40 in South America, 0.46 in Italy, 0.75 in North America and 0.79 in Africa [35]. Concerning *C. gattii*, no previous similar analysis has been reported. The VGII haplotype diversity index observed in the present study (HD = 0.695) is impressive, and was based on the analysis of all the seven studied MLST loci, amongst all 45 investigated strains, suggesting a high genetic diversity, especially when considering the very restricted area studied (4 km^2). The major genotype found in Santa Isabel do Rio Negro, ST7 (= VGIIb), is also the most common one found around the world especially in Australia, Thailand, and caused the minority of the Vancouver Island outbreak in British Columbia, Canada and the Pacific Northwest of the USA (PNW) [4–6, 33,34]. The sequence type ST5, first identified in Australia, was also found in one dwelling (DW650) closely related to ST7 (= VGIIb) and two other new subtypes (ST264, ST265).

In the positive house SI443, approximately 1 km away from the house DW650, the occurrence and distribution of the identified subtypes was totally different. Here the sequence type ST20 (= VGIIa) was predominant, which corresponds to the main agent of the Vancouver Island and Pacific Northwest outbreaks [4,6]. In the same house this sequence type was associated to three more new subtypes (ST266, ST267, ST268). On the other hand, the house SI444, located very close to the house SI443, showed only a single sequence type, ST7 (= VGIIb), which is the same as the one found in the house DW650, located 1km away. The different distribution of subtypes amongst the houses cannot be explained only by mechanical dispersal factors, such as air flow, small animals, wood debris or by shoes. Probably the contamination of the houses by cryptococcal propagules was a past event and most likely resulted from the

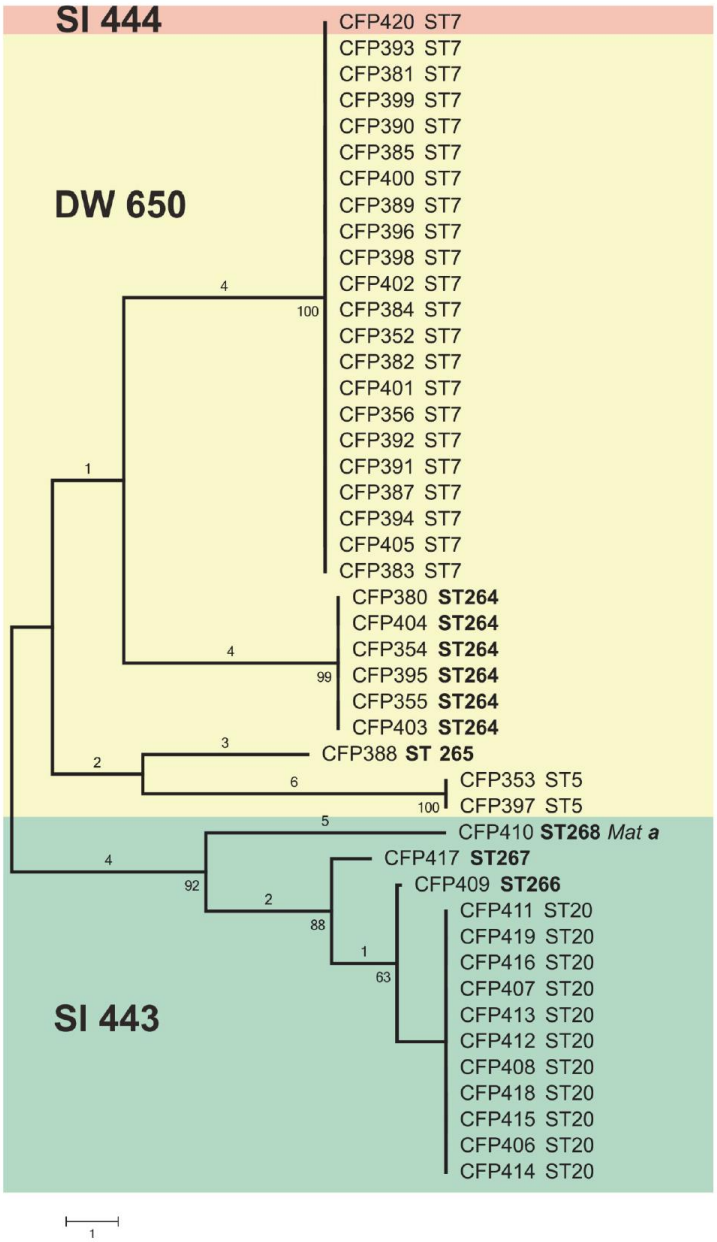

Fig 2. Unrooted neighbor-joining tree inferred from the combined MLST sequences of *CAP59*, *GPD1*, *LAC1*, *SOD1*, *URA5*, *PLB1* genes and the IGS1 region of the 45 strains investigated in this study obtained with the program MEGA version 5. Numbers above the branches are nucleotides differences and below the branches are bootstrap values obtained from 1,000 pseudoreplicates. Sequence types in bold are unique to Brazil.

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forest wood of which the houses were constructed. This initiated the process of adaptation of selected strains inside the houses, where they undergo reproduction. The predominance of sequence type ST7 (= VGIIb) in one of the houses, with more than 50.000 CFUs, and only MATalpha suggests an overwhelming clonal, mitotic reproduction mode. On the other hand, the house with the lower density of cryptococcal propagules (2.500 CFU/g), in which both mating types, MATalpha and MATa, were identified, suggest a possible event of alpha-a recombination in nature. Further analysis of dwellings in other areas of the Amazon region, as well as in the same area should allow for a better understanding of the dynamics of the colonization and possible changes in the population structure of *C. gattii* VGII.

Our results emphasise that indoor exposure risks to the subtypes of VGII must be considered as a possible infection source, although in the current study no evidence of a disease outbreak, based on the data available, has been observed amongst the residents of Santa Isabel do Rio Negro. Suboptimal diagnosis of cryptococcosis and the absence of surveillance may contribute to the underreporting of cryptococcosis. On the other hand, the frequent exposure to *C. gattii* propagules may lead to subclinical infection with regressive pulmonary lesions and acquired natural immunity to cryptococcal infection, warranting a detailed clinical surveillance in the region. Evidence for a direct impact of continuous exposure to infectious propagules from VGII strains in domestic environment is given by the previous reports of a high proportion of cryptococcosis cases due to *C. gattii* VGII in the Amazon region of Brazil, in HIV negative children [13, 15].

Over the past two decades, several cryptococcal outbreaks have occurred, including the high-profile 'Vancouver Island' and 'Pacific Northwest' outbreaks, caused by *C. gattii* VGII, which have affected hundreds of otherwise healthy humans and animals [3–7]. To understand the origins of those outbreaks a number of studies have been performed. Fraser *et al.* (2005) initially suggested same-sex mating between to MAT alpha strains involving a low virulent Australian strain (genotype ST7 (= VGIIb) leading to the raise of the high virulent ST20 (= VGIIa) genotype strains and the expansion of the ecological niche of this species. Later studies showed a high genetic diversity among strains from the Amazon rainforest and the possible occurrence of mating between strains of opposite mating types as the source of a global dispersal of this species [33].

In conclusion, the herein described results provide evidence for the later perspective and showed clearly the presence of two different ways of propagation, clonal and recombination, which can lead to the establishment of environmental populations present in indoor dust in urban houses within remote settlements in the Amazon rainforest. These yeast populations are most likely associated with the original sources of the building materials obtained from the surrounding forests. The current study established the basis for future studies investigating the impact of early and continuous exposure to the development of infections and the prevalence of subclinical and clinical infections amongst the inhabitants of houses colonized by *C. gattii* VGII.

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Author Contributions

Conceived and designed the experiments: MSL BW FACC FBS. Performed the experiments: FBS GGB LT MMN. Analyzed the data: FBS BW WM FACC LT. Contributed reagents/materials/analysis tools: BW WM FACC MSL. Wrote the paper: FBS GGB LT BW WM FACC MMN MSL.

References

1. Perfect JR (2006) Cryptococcus neoformans: the yeast that likes it hot. FEMS Yeast 4: 463–468. PMID: 16696642
2. Kwon-Chung KJ, Bennett JE (1992) Cryptococcosis. In: Kwon-Chung KJ, Bennett JE, editors. Medical Mycology. pp 397–446.
3. Fraser JA, Subaran RL, Nichols CB, Heitman J (2003) Recapitulation of the sexual cycle of the primary fungal pathogen Cryptococcus neoformans var. gattii: implications for an outbreak on Vancouver Island, Canada. Eukaryot Cell 2(5):1036–1045. PMID: 14555486
4. Kidd SE, Hagen F, Tscharke RL, Huynh M, Bartlett KH, et al. (2004) A rare genotype of Cryptococcus gattii caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). Proc Natl Acad Sci U S A 101: 17258–17263. PMID: 15572442
5. Fraser JA, Giles SS, Wenink EC, Geunes-Boyer SG, Wright JR, et al. (2005) Same-sex mating and the origin of the Vancouver Island Cryptococcus gattii outbreak. Nature 437:1360–1364. PMID: 16222245
6. Byrnes EJ, Heitman J (2009) Cryptococcus gattii outbreak expands into the Northwestern United States with fatal consequences. F1000 Biol Rep 1:62. doi: 10.3410/B1-62 PMID: 20150950
7. Lockhart SR, Iqbal N, Harris JR, Grossman NT, DeBess E, et al. (2013) Cryptococcus gattii in the United States: genotypic diversity of human and veterinary isolates. PLoS ONE 8(9):e74737. doi: 10.1371/journal.pone.0074737 PMID: 24019979
8. Meyer W, Aanensen DM, Boekhout T, Cogliati M, Diaz MR, Esposto MC, et al. (2009) Consensus multi-locus sequence typing scheme for Cryptococcus neoformans and Cryptococcus gattii. Med Mycol 47:561–570. doi: 10.1080/13693780902953886 PMID: 19462334
9. Bovers M, Hagen F, Kuramae EE, Diaz MR, Spanjaard L, et al.(2006) Unique hybrids between the fungal pathogens Cryptococcus neoformans and Cryptococcus gattii. FEMS Yeast Res 6:599–607. PMID: 16696655
10. Bovers M., Hagen F, Kuramae EE, Hoogveld HL, Dromer F, et al. (2008) AIDS Patient Death Caused by Novel Cryptococcus neoformans × C. gattii Hybrid. Emerg. Infect Dis 7:1105–1108. doi: 10.3201/eid1407.080122 PMID: 18598632
11. Trilles L, Lazéra Mdos S, Wanke B, Oliveira RV, Barbosa GG, et al. (2008) Regional pattern of the molecular types of Cryptococcus neoformans and Cryptococcus gattii in Brazil. Mem Inst Oswaldo Cruz 103:455–462. PMID: 18797758
12. Correa Mdo P, Oliveira EC, Duarte RR, Pardal PP, Oliveira Fde M, et al. (1999) Cryptococcosis in children in the State of Pará, Brazil Rev Soc Bras Med Trop 32:505–508. PMID: 10881083
13. Santos WR, Meyer W, Wanke B, Costa SP, Trilles L, et al.(2008) Primary endemic Cryptococcosis gattii by molecular type VGII in the state of Pará, Brazil. Mem Inst Oswaldo Cruz 8: 813–818. PMID: 19148422
14. Gomes FS, Sarmento DN, Santo EPTE (2010) Chemotype determination and phenotypic characterization of isolated Cryptococcus from Belém, Pará State, Brazil. Rev Pan-Amaz Saúde 4:43–49. doi: 10.1016/j.rbr.2014.04.003 PMID: 25628262
15. Freire AK, dos Santos Bentes A, de Lima Sampaio I, Matsuura AB, Ogusku MM, et al. (2012) Molecular characterization of the causative agents of Cryptococcosis in patients of a tertiary healthcare facility in the state of Amazonas-Brazil. Mycoses 55:145–150.
16. Martins LM, Wanke B, Lazéra M, Trilles L, Barbosa GG, et al. (2011) Genotypes of Cryptococcus neoformans and Cryptococcus gattii as agents of endemic cryptococcosis in Teresina, Piauí (northeastern Brazil). Mem Inst Oswaldo Cruz 106:725–730. PMID: 22012227
17. Fortes ST, Lazéra MS, Nishikawa MM, Macedo RC, Wanke B (2001) First isolation of Cryptococcus neoformans var. gattii from a native jungle tree in the Brazilian Amazon rainforest. Mycoses 44:137–140. PMID: 11486449

18. Costa SP, Lazéra M, Santos WR, Morales BP, Bezerra Cde C et al. (2009) First isolation of Cryptococcus gattii molecular type VGII and Cryptococcus neoformans molecular type VNI from environmental sources in the city of Belém, Pará, Brazil. Mem Inst Oswaldo Cruz 104:662–664. PMID: 19722095
19. Lazéra MS, Cavalcanti MA, Trilles L, Nishikawa MM, Wanke B (1998) Cryptococcus neoformans var. gattii evidence for a natural habitat related to decaying wood in a pottery tree hollow. Medical Mycology 36:119–122. PMID: 9988500
20. Swinne D, Deppner M, Laroche R, Floch JJ, Kadende P (1989) Isolation of Cryptococcus neoformans from houses of AIDS-associated cryptococcosis patients in Bujumbura (Burundi). AIDS 3:389–390. PMID: 2502153
21. Passoni LF, Wanke B, Nishikawa MM, Lazéra MS (1998) Cryptococcus neoformans isolated from human dwellings in Rio de Janeiro, Brazil: An analysis of domestic environment of AIDS patients with and without cryptococcosis. J Med Vet Mycol 36:305–311.
22. Amazon Association of Municipalities; Available: http://www.aam.org.br/prefeituras/364-prefeitura-municipal-de-santa-isabel-do-rio-negro/ Accessed 17 December 2014.
23. Lazéra MS, Pires FDA, Camillo-Coura L, Nishikawa MM, Bezerra CCF, et al. (1996) Natural habitat of Cryptococcus neoformans var. neoformans in decaying wood forming hollows in living trees. J Med Vet Mycol 34:127–131. PMID: 8732358
24. Staib F, Seeliger HP (1966) A new selective medium for the isolation of C. neoformans from fecal material and from soil. Ann Inst Pasteur (Paris) 110:792–793. PMID: 5909624
25. Christensen WB (1946) Urea decomposition as a means of differentiating Proteus and paracolon cultures from each other and from Salmonella and Shigella. J Bacteriol 52:461–466. PMID: 16561200
26. Klein KR, Hall L, Deml SM, Rysavy JM, Wohlfiel SL, Wengenack NL (2009) Identification of Cryptococcus gattii by use of L-canavanine glycine bromothymol blue medium and DNA sequencing. J Clin Microbiol 47:3669–3672. doi: 10.1128/JCM.01072-09 PMID: 19794048
27. Ferrer C, Colom F, Frases S (2001) Detection and identification of fungal pathogens by PCR and by ITS2 and 5.8S ribosomal DNA typing in ocular infections. J Clin Microbiol 39:2873–2879. PMID: 11474006
28. Meyer W, Castañeda A, Jackson S, Huynh M, Castañeda E (2003) IberoAmerican Cryptococcal Study Group. Molecular typing of IberoAmerican Cryptococcus neoformans isolates. Emerg Infect Dis 9:189–195. PMID: 12603989
29. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 10:2731–2739. doi: 10.1093/molbev/msr121 PMID: 21546353
30. Swinne D, Deppner M, Maniratunga S, Laroche R, Floch JJ, et al. (1991) Aids-associated cryptococcosis in Bujumbura, Burundi: an epidemiological study. J Med Vet Mycol 29:25–30. PMID: 2061793
31. Mitchell T.G, Castañeda E, Kirsten N, Wanke B. Lazéra M.S (2011) Environmental Niches for Cryptococcus neoformans and Cryptococcus gattii. In Cryptococcus: From Humans Pathogen to Model Yeast. Edited by Heitman J. et al. ASM press, Washington, DC. pp 237–251.
32. Lazéra MS, Cavalcanti MAS, Londero AT, Trilles L, Nishikawa MM, et al. (2000) Possible primary ecologycal niche of Cryptococcus neoformans. Med Mycol 38:379–383. PMID: 11092385
33. Hagen F, Ceresini PC, Polacheck I, Ma H, van Nieuwerburgh F, et al.(2013) Ancient dispersal of the human fungal pathogen Cryptococcus gattii from the Amazon rainforest.PLoS ONE 8(8):e71148. doi: 10.1371/journal.pone.0071148 PMID: 23940707
34. Carriconde F, Gilgado F, Arthur I, Ellis D, Malik R, et al. (2011) Clonality and α-a recombination in the Australian Cryptococcus gattii VGII population-an emerging outbreak in Australia. PLoS ONE 6(2): e16936. doi: 10.1371/journal.pone.0016936 PMID: 21383989
35. Cogliati M, Zamfirova RR, Tortorano AM, Viviani MA, Fimua Cryptococcosis Network (2013) Molecular epidemiology of Italian clinical Cryptococcus neoformans var. grubii isolates. Med Mycol 51:499–506. doi: 10.3109/13693786.2012.751642 PMID: 23286351

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Environmental isolation of *Cryptococcus gattii* VGII from indoor dust associate to typical wooden houses in the deep Amazon of the Rio Negro basin

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Abstract

Cryptococcosis is a human fungal infection of significant mortality and morbidity, especially when causing meningoencephalitis. The agents of cryptococcosis, *C. neoformans* and *C. gattii*, are found worldwide, and can be classified into eight major molecular types: VNI-VNIV and VGI-VGIV, respectively. Primary cryptococcosis caused by molecular type VGII (serotype B, MATalpha) prevails in immunocompetent patients in the North and Northeast of Brazil, revealing an endemic regional pattern. Since 1999, *C. gattii* VGII has been involved in an ongoing outbreak in Vancouver, Canada, which expanded subsequently into the Northwest of the United States, two temperate regions. Exposure to infectious propagules (desiccated yeasts or basidiospores), dispersed in the environment, related to various organic substrates, mainly decomposing wood in and around dwellings, initiates the infection process. The present study aimed to investigate the presence of the agents of cryptococcosis in dust from dwellings in the upper Rio Negro, in the municipality of Santa Isabel do Rio Negro, in the Brazilian state Amazonas. Indoor dust was collected from 51 houses, diluted and plated on bird-seed agar. Dark brown colonies were identified phenotypically and genotypically by *URA5* restriction fragment length polymorphism analysis and multilocus sequence typing (MLST). The mating type was identified using pheromone-specific primers. Three of the 51 houses were positive for *C. gattii* molecular type VGII, MATα and MATa, showing a high prevalence of this agent. MLST studies identified eight subtypes, VGIIb (ST7), VGIIa (ST20), (ST5) and 5 new subtypes unique to the region. For the first time in the state of Amazonas, *C. gattii* VGII MATα and MATa were isolated from the environment, indicating *Cryptococcus* is endemic in this state. This is the first description of MLST subtypes on environmental isolates in the Brazilian Amazon, indicating domiciliary dust as a potential source for human infection with different subtypes of *C. gattii* VGII MATα and MATa.

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Supplement:

Cryptococcosis is an important infectious disease globally and, as an AIDS-associated disease, is the leading cause of death along with tuberculosis. Inhalation of airborne cryptococci initiates pulmonary infection, which may be asymptomatic or subclinical, unspecific and almost always regressive or may spread to other organs mainly the central nervous system (CNS) and cause meningitis. Disease is caused by the two sibling species *Cryptococcus neoformans* (molecular types VNI-VNIV) and *Cryptococcus gattii* (molecular types VGI-VGIV). The agents of cryptococcosis are found in decaying wood, trees, bird excreta, soil, water, and air. Exposure to those exogenous sources can lead to colonization and subsequent infection of humans and animals. Infection due to *C. neoformans* is distributed worldwide and attains mainly immunosuppressed hosts. Fatal infections like meningoencephalitis in apparently immunocompetent hosts are mostly caused by *C. gattii*, which has progressively expanded its natural range from tropical to temperate areas around the world, showing a great potential to generate outbreaks due to different virulent variants. Both species are endemic in the North and Northeastern Brazil, as indicated by the high numbers of infected children and adolescents (20 up to 30 %), which suggest early infection in native hosts (1).

Figure 1. (1a) Wooden houses in Santa Isabel do Rio Negro from which *C. gattii* was collected. **(1b)** Sample collection from wooden electricity poles in the same locality from which *C. gattii* and *C. neoformans* have been isolated.

We hypothesize humans are exposed daily to the infectious propagules in the Brazilian Amazon region. To demonstrate this, we conducted an environmental study analyzing indoor dust from the houses **(Figure 1a)** of inhabitants and wooden electricity poles **(Figure 1b)** of the city Santa Isabel of Rio Negro, located in the upper Rio Negro river in the Amazonas state, Brazil. Indoor dust was collected from 51 typical wooden houses. Three (5.9%) houses were positive, yielding uncountable colonies of *C. gattii* molecular type VGII **(Figure 2)**. From the obtained colonies, 45 were selected for further molecular analysis. Surprisingly eight MLST subtypes of VGII (ST5, ST7, ST20, ST264, ST265, ST266, ST267 and ST268) were identified in the small area studied (approximately 5 km^2 in Amazonia), the highest genetic diversity described to date in environmental studies worldwide (2). The diversity of *C. gattii* VGII found inside the wooden houses in a countryside town in the amazon is an important finding since it may point to a much broader impact on human health, considering the human occupation with the construction of rustic wooden houses in recently deforested areas in the last decades. Interestingly around the positive houses, all the native trees have been cut down during and after the settlement. Later on trunks of native trees were used as wooden poles throughout the city to support the electric wires. From 10 wooden poles, 2 decomposing wood samples were positive for the agents of cryptococcosis, molecular types VGII (*C. gattii*) and VNI (*C. neoformans*). Our findings show that the residents of Santa Isabel do Rio Negro live daily with the presence of the agents of cryptococcosis, *C. gattii* VGII or *C. neoformans* VNI. We believe that the same happens in other cities with a similar occupation profile throughout Amazonia. Both species responsible for cryptococcosis seem to be well adapted to environmental conditions found in Amazonia, which is drastically transformed by human environmental intervention, exposing people to potential pathogens. Destroyed environments lead to the loss of microbial biodiversity and new competition, favoring overgrowth of the more adapted species. In this specific case, *C. gattii* and *C. neoformans*, being wood decomposers, are very well adapted to environmental conditions in the villages, which are basically built with woods from the rainforest. A study in the wild tropical forest without anthropic action in Amazonia demonstrated the presence of *C. gattii* inhabiting a *Guettarda* tree, suggesting the original ecological niche of the species (3). In urban areas, the prevalence of both species is much higher, increasing the potential risk of human exposure to those pathogens. Due to their proximity to human dwellings, it is highly possible that a phase transition and adaptation of these agents may occur in the wooden electricity poles, thus allowing a secondary dispersion to the home environments and, as such, they function as true biological corridors. Interestingly, some of the in Santa Isabel do Rio Negro identified subtypes (ST7 (VGIIb), ST20 (VGIIa)) are also responsible for the ongoing outbreak in the Pacific Northwest (Canada and United States) (4, 5). Recent studies of coalescence gene genealogy and whole genome analysis on the origin of the geographically expanding *C. gattii* VGII population have demonstrate that these outbreak strains have arisen from a highly-recombining *C. gattii* population in the pristine environment of the native rainforest of Northern Brazil (4, 5).

The importance of this study.

This study, for the first time, found VGIIa and VGIIb in the state of Amazonas, where they were isolated from the environment, specifically from indoor dust collected in homes and, from debris isolated from wooden electricity poles (VGII and VNI) in the town of Santa Isabel do Rio Negro, located in deforested areas of the Amazon rainforest at the Rio Negro banks. The types of houses and the wooden poles of the city, with a predominance of wooden structures, are related to the generation of suitable substrates for recombination and expansion of the agents of cryptococcosis. Inhabitants should be informed about the risks associated with the use of untreated wood as construction material. Decomposed wood in and around the homes should be replaced with new pre-treated ones, as a simple measure that can prevent the spread of this fungus in the environment and future outbreaks in the region.

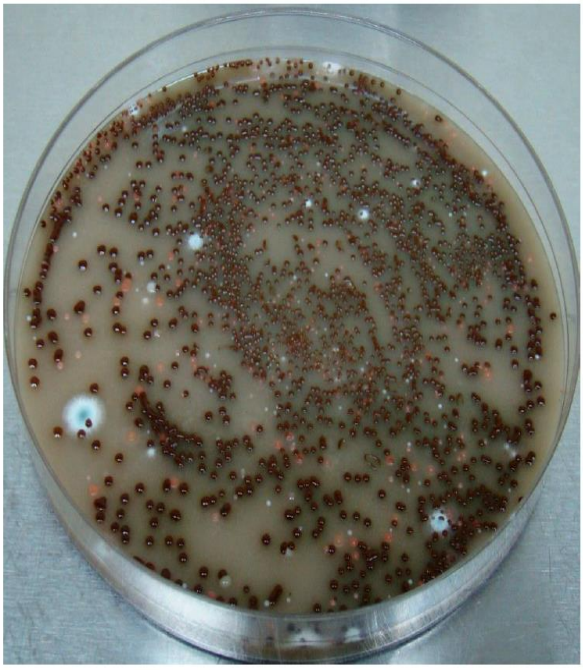

Figure 2. Dust indoor sample DW-650 after 48 h incubation at 25 ºC plated on bird-seed agar, which resulted in uncountable dark brown colonies (suspected colonies of *Cryptococcus* spp.).

References:

1. **Trilles L, Lazéra Mdos S, Wanke B, Oliveira RV, Barbosa GG, Nishikawa MM, Morales BP, Meyer W.** 2008. Regional pattern of the molecular types of *Cryptococcus neoformans* and *Cryptococcus gattii* in Brazil. Mem Inst Oswaldo Cruz. 5:455-462.
2. **Brito Santos-F, Barbosa GG, Trilles L, Nishikawa MM, Wanke B, Meyer W, Carvalho-Costa FA, Lazera MS.** 2015. Environmental isolation of *Cryptococcus gattii* VGII from indoor dust associate to typical wooden houses in the deep Amazon of the Rio Negro basin PLoS One.Feb 17;10(2): e0115866.
3. **Fortes ST, Lazéra MS, Nishikawa MM, Macêdo RCL, Wanke B.** 2001.First isolation of *Cryptococcus neoformans* var. *gattii* from a native jungle tree in the Brazilian Amazon rainforest. Mycosis 44:137-140.
4. **Hagen F, Ceresini PC, Polacheck I, Ma H, vanNieuwerburgh F, Gabaldón T, Kagan S, Pursall ER, Hoogveld HL, vanersel LJ, Klau GW, Kelk SM, Stougie L, Bartlett KH, Voelz K, Pryszcz LP, Castañeda E, Lazera M, Meyer W, force D, Meis JF, de Maio RC, Klaassen CH, Boekhout T**. 2014. Ancient dispersal of the human fungal pathogen *Cryptococcus gattii* from the Amazon rainforest. PLoS One. 8(8):e71148.
5. **Engelthaler DM, Hicks ND, Gillece JD, Roe CC, Schupp JM, Driebe EM, Gilgado F, Carriconde F, Trilles L, Firacative C, Ngamskulrungroj P, Castañeda E, dos Santos Lazera M, Melhem MSC, Pérez-Bercoff Å, Huttlley G, Sorrell TC, Voelz K, May RC, Fisher MC, Thompson GR, III, Lockhart SR, Keim P, Meyer W.** 2014. *Cryptococcus gattii* in North American Pacific Northwest: whole-population genome analysis provides insights into species evolution and dispersal. mBio, 5(4):e01464-14.

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Virulent strains of the agents of cryptococcosis inhabit indoor dust from a micro-region of Rio Negro, Amazonas, Brazil - New evidences and big challenges.

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Abstract

Cryptococcal infection is acquired by exposure to exogenous sources. The understanding of the dynamics and adaptation of the agents reservoirs, identification of how the infection is acquired by humans, and what are the means to avoid or reduce its risks of infection, are of fundamental interest. A number of studies have found high levels of emerging contaminants in indoor dust worldwide, but those studies usually identify the fungal agent only at genus level, missing the opportunity to detect pathogenic species/strains. The present study analyzed the frequency, genetic diversity and virulence of the agents of cryptococcosis isolated from indoor dust in a micro-region of the Brazilian Amazon. We found 8.9% of the studied houses positive for *C. gattii* and *C. neoformans*, revealing an endemic pattern and fungal adaptation to domestic microenvironments. Sixteen *C. gattii* VGII and nine *C. neoformans* VNI isolates were genotyped using the ISHAM consensus MLST scheme for the *C. neoformans/C. gattii* species complexes. The most common *C. gattii* subtypes were ST7 (Vancouver Island Outbreak subtype VGIIb) and ST20 (Vancouver Island Outbreak subtype VGIIa), ST5, found in Australia, and ST264, ST265, ST266, ST267, ST268, ST445, unique to this region, were also identified. Amongst the nine *C. neoformans* (VNI) isolates ST93 and ST5 were identified, showing a clonal population. Comparison of the degree of virulence using the *Galleria mellonella* model showed that five subtypes of *C. gattii* VGII and one subtype of *C. neoformans* VNI, presented a similar pathogenic potential to CDR265 (VGIIa) from Vancouver Island, Canada. This study showed that humans are frequently exposed to virulent agents of cryptococcosis in house dust, with the main subtypes described worldwide being highly present in the Rio Negro micro-region of the Brazilian Amazon. Future studies are necessary to analyze

the impact of early and continuous exposure to dust indoor to the development of subclinical or clinical infections amongst the inhabitants of those houses.

1. Introduction

Cryptococcosis is a potentially fatal respiratory and neurological mycoses affecting humans and animals worldwide. The disease is caused by two pathogenic members of the genus *Cryptococcus*, the *Cryptococcus neoformans* species complex and the *C. gattii* species complex (Kwon-Chung et al., 2017). Cryptococcosis caused by *C. neoformans* is cosmopolitan, attaining mainly immunocompromised individuals, especially HIV-infected patients, with an estimation of 223,000 new cases of cryptococcal meningitis each year in this group of patients (Rajasingham et al., 2017). On the other hand, *C. gattii,* which was previously associated with tropical and subtropical climates, causes predominantly a primary infection in immunocompetent individuals. It is to notice that endemic cryptococcosis by *C. gattii* shows a regional pattern in Brazil, being more common in the North (N) and Northeast (NE) of the country, where cryptococcosis occurs mainly as isolated cases in immunocompetent hosts, including children and young adults (Trilles et al., 2008). From 1999 to 2015, 393 cases of cryptococcosis were reported in British Columbia, Canada, and currently, Vancouver Island has one of the highest annual incidences of *C. gattii* infections among humans in the world, making this region an important temperate endemic area of human and veterinary cases of the disease (Phillips et al., 2015).

Cryptococcosis is acquired by inhalation of cryptococcal propagules (desiccated yeasts cells or basidiospores) from the environment (Kwon-Chung et al., 2014). As such, the search for the ecological niche of the agents of cryptococcosis is a challenge, even though some studies have shown the presence of the yeasts in different environmental sources (Litvintseva et al., 2005)**.** However, while *C. neoformans* can

readily be isolated from pigeon guano and has been shown to grow and mate on medium containing this substrate (Sorrell et al., 1996), *C. gattii* has not been isolated from pigeon excreta, but *C. neoformans* and mainly *C. gattii* have instead been recovered from various tree species (Lazera et al., 2000).

Pioneering studies on cryptococcosis in AIDS patients in Central Africa and Brazil demonstrated the risk of these patients to acquire cryptococcosis from indoor dust (Passoni et al., 1998; Swinne et al., 1989). The first study describing the presence of *C. neoformans* and *C. gattii* in dwellings of the hinterland of the Brazilian Amazon, identified the genotypes ST7/VGIIb and ST20/VGIIa, which have been involved as causative agents of outbreaks elsewhere. Residents of Santa Isabel do Rio Negro city who live in wooden houses could be daily exposed to the agents of cryptococcosis, which could also be happening in other cities with similar living styles in the Amazon region (Brito-Santos et al., 2015). The present study in the micro-region Rio Negro, which is composed of four municipalities in the Amazon region in Brazil, analyzes the frequency, genetic diversity and virulence traits of the agents of cryptococcosis isolated from indoor dust.

2. Methods

2.1 *Studied region* - The study was conducted in the micro-region Rio Negro in the Brazilian Amazon, which is composed of four municipalities, Barcelos, Novo Airão, Santa Isabel do Rio Negro and São Gabriel da Cachoeira **(Figure 1)**. The Rio Negro is the largest left tributary of the Amazon River and largest blackwater river in the world, which runs crosses all those cities on the way to the capital of the Amazonas state, Manaus. This micro-region has a population of 110,602 inhabitants and an area of 332,278.183 km² according to the last Brazilian census. From the four cities in the Rio

Negro micro-region, three were included in this study, Santa Isabel, Barcelos, and Novo Airão. The area encompasses the tropical rainforest climate, with a maximum temperature of 32.6ºC, and a minimum temperature of 21.5°C, and is located 21-40 m above the sea level. The cities settlement occurred approximately one hundred years ago and the majority of the population lives in houses of wood or wood with masonry (Source: IBGE 2017).

2.2 ***Sampling and isolation of cryptococcal strains*** – Indoor dust was collected from houses in the three municipalities in different neighborhoods of each city, to investigate the presence of *C. neoformans* and *C. gattii*. After sweeping the house with a broom from each residence, one sample per household was obtained. The study had the approval of each City Health Department, as well as ethical approval by the National Institute of Infectology Ethical Research Committee, Rio de Janeiro, Brazil (Reference Nº CAAE 23238913.3.0000.5262). Seventy-nine samples of indoor dust were obtained from the three municipalities of the micro-region of Rio Negro, 51 from Santa Isabel do Rio Negro, 12 from Barcelos and 16 from Novo Airão. Cryptococcal isolates were recovered as described previously (Lazera et al., 1996; Passoni et al., 1998). Briefly, 1 g of each dust sample was suspended in 50 ml NaCl 0.9% with 0.2 g of chloramphenicol, followed by manual shaking for 5 minutes. After resting 30 minutes, 1 ml of the supernatant was plated onto 10 Niger seed agar (NSA) plates (0.1 ml each). The plates were then incubated at 25°C and checked daily during 5 days for growth of brown colonies. Phenol oxidase-positive or brown colonies were sub-cultivated for phenotypic and molecular identification. The limit for the detection of phenol oxidase-positive colonies was 50 CFU per gram of sample.

2.3 ***Phenotypic identification*** **-** Brown colonies were recovered and tested for urease production on Christensen urea agar and for carbon and nitrogen compound assimilation

using VITEK 2-BioMerieux System (VITEK 2, ICB, bioMerieux, Durham, USA). The species *C. neoformans* and *C. gattii* were distinguished on canavanine-glycine-bromothymol blue (CGB) medium (Brito-Santos et al., 2015).

2.4 *Molecular characterization* - After DNA extraction (Ferrer et al., 2001), the mating type was determined by PCR using specific primers for the pheromone genes as described previously (Chaturvedi et al., 2000). Genotyping was performed according to the ISHAM consensus multilocus sequence typing (MLST) scheme for the *C. neoformans*/*C. gattii* species complexes, including seven unlinked genetic loci, which are the genes: *CAP59*, *GPD1*, *LAC1*, *PLB1*, *SOD1*, *URA5*, and the IGS1 region (Meyer et al., 2009). The sequences were manually edited using the software Sequencher 5.3 (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/), and the new ATs and STs were deposited to the online MLST database.

2.5 *Phylogenetic analyses* – The genetic relationships of the 7 concatenate MLST loci were shown using the software Splitstree4 v. 4.14.5 (http://www.splitstree.org/). Unrooted phylogenetic network analysis using the Neighbor-net algorithm was performed for comparison of the subtypes (STs) identified in this study with 42 STs identified in different countries previously published (Souto et al., 2016).

2.6 Galleria mellonella *model* – Larvae of the moth *G. mellonella* were used to evaluate the virulence of selected cryptococcal isolates recovered in this study. Larvae were obtained after the oviposition of adult moths reared and preserved at 26°C and 60% relative humidity in the insectarium of the Westmead Hospital Animal Care Facility, Sydney, Australia. Ten larvae of similar size (about 3 g each) were selected, placed in a 90 mm plastic Petri dishes, weighed, and used for inoculation. Before

inoculation, each fungal strain was grown on Sabouraud agar for 48 h at 27°C. After cell counting using a Neubauer Chamber, an inoculum of 10^8 yeast cells/ml was prepared in phosphate buffered saline (PBS), from which 10 µl were inoculated into the hemocoel of each larva by injection into the last left pro-leg, using a 50U insulin syringe with a 29-gauge needle. To monitor potential effects on survival due to physical injury, a group of 10 larvae was also inoculated with PBS, while another 10 larvae were not inoculated as a non-infected control. Two groups of larvae, inoculated separately with the well-characterized highly virulent strains *C. neoformans* H99 and *C. gattii* CDCR265, were included, as reference to determine the degree of virulence of the isolates. After injection, the larvae were incubated in Petri dishes at 37°C for 10 days and checked daily for any mortality (Firacative et al., 2014).

2.7 *Statistical analysis* – Per strain, survival curves were graphed, median survival times were calculated and the estimation of differences in survival was analyzed by the Log-rank (Mantel-Cox) test. Median survival times were not determined (ND) when more than five larvae (50%) were alive at the end of the experiment.

3. Results

From the 79 samples obtained, seven (8.9%) were positive for the agents of cryptococcosis, 3 from Santa Isabel do Rio Negro, 2 from Barcelos and 2 from Novo Airão **(Supplementary Table 1)**.

From the positive samples, 16 *C. gattii* VGII and nine *C. neoformans* VNI isolates were genotyped using the ISHAM consensus MLST scheme for the *C. neoformans/C. gattii* species complexes (Meyer et al., 2009). The most common *C. gattii* subtypes were ST7 (5 out of 16) (Vancouver Island Outbreak subtype VGIIb) and ST20 (2 out of 16) (Vancouver Island Outbreak subtype VGIIa). In addition ST5 (1/16),

found in Australia, and ST264 (2/16), ST265 (1/16), ST266 (1/16), ST267 (1/16), ST268 (1/16), ST445 (2/16), unique to this region, were also identified. The analysis of the combined MLST loci showing the placement of the 16 isolated strains from the micro-region of Rio Negro in context with the STs obtained from the global *C. gattii* VGII population and identifying the genetic diversity in this Amazon region, is shown **(Figure 2)**. Amongst the 9 *C. neoformans* (VNI) isolates ST93 (6/9) and ST5 (3/9) were identified, showing a clonal population. Mating type analysis demonstrated mostly *MATα* and only one *MAT*a amongst the *C. gattii* isolates, which is ST268 (VGII), strain SI443-17.

The death rate of *G. mellonella* larvae infected with the *C. neoformans* VNI was more rapid compared to that one of the larvae infected with *C. gattii* VGII isolates. However, no statistical difference was observed between the survival curves of *C. gattii* VGII and *C. neoformans* VNI infected-larvae (*p*-value = 0.6764) **(Figure 3)**. Amongst the VGII strains, 5 genotypes demonstrated a higher *in vivo* virulence, which was comparable (*p*-value <0.05) to the Vancouver Island outbreak strain CDCR265: one strain ST7 (VGIIb), both of the ST20 (VGIIa) strains, and the strains with STs 266, 267 and 445. All *C. neoformans* strains tested were less virulent than the strain H99 (*p*-value < 0.05 - data not shown), but 3 of them (BAR10-16, BAR10-19, BAR08-1) and all ST 93, were as virulent as the VGII Vancouver Island outbreak strain CDCR265 (*p*-value < 0.05) **(Table 1)**.

4. Discussion

Cryptococcal infection is acquired by human and animal exposure to exogenous sources, as such the understanding of the dynamics and adaptation of the agents reservoirs is of fundamental interest, as well as to seek answers to the main questions:

how humans get infected and what are the means to avoid or reduce risks of infection (Lazera et al., 2000). Humans are primarily exposed to organic indoor air inhalation, and indoor dust, important mechanisms of exposure, as people spend over 86.9% of their lives in indoor environments (Klepeis et al., 2001). Studies have found high levels of emerging contaminants in indoor dust worldwide, but those studies usually identify the fungal agent at genus level, missing the opportunity to detect pathogenic species/strains (Dannemiller et al., 2016; Hanson et al., 2016; Lemons et al., 2017).

Studies on emerging contaminants in indoor dust and the resulting levels of human exposure to pathogenic *Cryptococcus* species/strains are scarce. Pioneering studies in Central Africa in the late 1980s detected a large number of *C. neoformans* positive indoor dust samples in households of patients with AIDS-associated cryptococcosis (Swinne et al., 1994, 1989). Another similar study was carried out in the city of Rio de Janeiro, Brazil, by Passoni et al. (1998). The authors analyzed households of AIDS patients from the metropolitan area and found 13% of positivity for *C. neoformans*. Besides, the authors observed that cryptococcosis was twice more frequent among AIDS patients residing in positive dwellings, thus suggesting an important role of positive indoor dust samples in the acquisition of cryptococcal infection in HIV/AIDS patients (Passoni et al., 1998).

An initial and local study in one municipality of the Rio Negro micro-region (Amazonas state, Brazil) revealed *C. gattii* isolates in indoor dust associated with wooden houses (Brito-Santos et al., 2015), suggesting the possibility of cryptococcal infection by *C. gattii* acquired from the domestic environment. In the present study, a larger area including two more municipalities (Barcelos and Novo Airão) in the Amazon region showed that 8.9% of the studied houses were positive for *C. gattii* and

C. neoformans, revealing an endemic pattern and adaptation to domestic microenvironments in this region of Amazonia.

An indoor microbial study revealed special concerns for vulnerable groups, such as children, for the risk of indoor-acquired infection (Malliari and Kalantzi, 2017). In Brazil, cryptococcosis by *C. gattii* manifested as CNS infections in immunocompetent young adults and children of both sexes in the Amazon and northeast regions of Brazil is common, with the associated lethality ranging from 35% to 40% (Corrêa et al., 1999; Freire et al., 2012; Martins et al., 2011; Santos et al., 2008). The high incidence of *C. gattii* in indoor dust in the Rio Negro river micro-region could explain the endemic pattern of cryptococcosis by VGII in children and young adults and thus indicate the need for future studies in other endemic areas to understand the role the agents of cryptococcosis in indoor environments.

The subtypes ST20 (VGIIa) and ST7 (VGIIb) related to the outbreak of Vancouver Island were also found in the micro-region of Rio Negro, as well as new subtypes of VGII. The genetic diversity of VGII in house dust correlates with the very diverse *C. gattii* VGII Brazilian population previously described, and strongly supports the emergence of virulent strains from ancestors in the northern region of Brazil (Souto et al., 2016).

The globally most common *C. neoformans* subtypes, ST93 and ST5, were also identified in the Rio Negro micro-region. The ST93 has been associated with infections in individuals with HIV in the Amazonia state and comprise the majority of clinical isolates in southeastern Brazil (Rocha et al., 2018). The ST5 is one of the most prevalent STs amongst the clinical isolates from Europe and Asia, but was only rarely identified in clinical and environmental isolates from Brazil (Ferreira-Paim et al., 2017).

During the present study, there was no evidence of cryptococcosis cases in the inhabitants of the positive dwellings, such as the outbreaks related to VGII described in other geographic areas. However, the STs of *C. neoformans* and *C. gattii* from the indoor dust analyzed in present study were all virulent using the *G. mellonella* model. Comparison of the degree of virulence using the *G. mellonella* model showed that five subtypes of *C. gattii* VGII (ST7, ST20, ST266, ST267 and ST445) and one subtype of *C. neoformans* VNI (ST93), presented similar pathogenic potential to that of strain CDR265 (VGIIa, ST20) from Vancouver Island, Canada (Galanis, 2010) .

Microbiome studies of indoor dust and outdoor air samples in Boston and California (USA) detected a moderate percentage of the genus *Cryptococcus*, being the third most abundant genus in such samples (Adams et al., 2013; Hanson et al., 2016). These authors reinforce the importance of the fact that the great majority of *Cryptococcus* spp. are non-pathogenic to humans. The present study shows that pathogenic *Cryptococcus* spp. can also be found on indoor dust regardless of cryptococcal infection in the dwellings inhabitants.

In summary, we point out a possible frequent exposure of humans to virulent agents of cryptococcosis in house dust in the micro-region Rio Negro of the Brazilian Amazon, where the main subtypes described worldwide are present and shown virulence potential. Future studies are now warranted to analyze the impact of early and continuous exposure to indoor dust to the development of subclinical or clinical infections amongst the inhabitants of those houses.

5. REFERENCES

Adams, R.I., Miletto, M., Taylor, J.W., Bruns, T.D., 2013. Dispersal in microbes: fungi in indoor air are dominated by outdoor air and show dispersal limitation at short distances. ISME J 7, 1262–1273. https://doi.org/10.1038/ismej.2013.28

Brito-Santos, F., Barbosa, G.G., Trilles, L., Nishikawa, M.M., Wanke, B., Meyer, W., Carvalho-Costa, F.A., Lazéra, M. dos S., 2015. Environmental Isolation of *Cryptococcus gattii* VGII from Indoor Dust from Typical Wooden Houses in the Deep Amazonas of the Rio Negro Basin. PLOS ONE 10, e0115866. https://doi.org/10.1371/journal.pone.0115866

Chaturvedi, S., Rodeghier, B., Fan, J., McClelland, C.M., Wickes, B.L., Chaturvedi, V., 2000. Direct PCR of *Cryptococcus neoformans* MATalpha and MATa pheromones to determine mating type, ploidy, and variety: a tool for epidemiological and molecular pathogenesis studies. J. Clin. Microbiol. 38, 2007–2009.

Corrêa, M. do P., Oliveira, E.C., Duarte, R.R., Pardal, P.P., Oliveira, F. de M., Severo, L.C., 1999. [Cryptococcosis in children in the State of Pará, Brazil]. Rev. Soc. Bras. Med. Trop. 32, 505–508.

Dannemiller, K.C., Gent, J.F., Leaderer, B.P., Peccia, J., 2016. Indoor microbial communities: Influence on asthma severity in atopic and nonatopic children. J. Allergy Clin. Immunol. 138, 76-83.e1. https://doi.org/10.1016/j.jaci.2015.11.027

Ferreira-Paim, K., Andrade-Silva, L., Fonseca, F.M., Ferreira, T.B., Mora, D.J., Andrade-Silva, J., Khan, A., Dao, A., Reis, E.C., Almeida, M.T.G., Maltos, A., Junior, V.R., Trilles, L., Rickerts, V., Chindamporn, A., Sykes, J.E., Cogliati, M., Nielsen, K., Boekhout, T., Fisher, M., Kwon-Chung, J., Engelthaler, D.M., Lazéra, M., Meyer, W., Silva-Vergara, M.L., 2017. MLST-Based Population Genetic Analysis in a Global Context Reveals Clonality amongst *Cryptococcus neoformans* var. *grubii* VNI Isolates from HIV Patients in Southeastern Brazil. PLoS Negl Trop Dis 11, e0005223. https://doi.org/10.1371/journal.pntd.0005223

Ferrer, C., Colom, F., Frasés, S., Mulet, E., Abad, J.L., Alió, J.L., 2001. Detection and identification of fungal pathogens by PCR and by ITS2 and 5.8S ribosomal DNA typing in ocular infections. J. Clin. Microbiol. 39, 2873–2879. https://doi.org/10.1128/JCM.39.8.2873-2879.2001

Firacative, C., Duan, S., Meyer, W., 2014. *Galleria mellonella* Model Identifies Highly Virulent Strains among All Major Molecular Types of *Cryptococcus gattii*. PLoS ONE 9, e105076. https://doi.org/10.1371/journal.pone.0105076

Freire, A.K.L., dos Santos Bentes, A., de Lima Sampaio, I., Matsuura, A.B.J., Ogusku, M.M., Salem, J.I., Wanke, B., de Souza, J.V.B., 2012. Molecular characterisation of the causative agents of Cryptococcosis in patients of a tertiary healthcare facility in the state of Amazonas-Brazil: Cryptococcosis in the state of Amazonas-Brazil. Mycoses 55, e145–e150. https://doi.org/10.1111/j.1439-0507.2012.02173.x

Galanis, E., 2010. Epidemiology of *Cryptococcus gattii*, British Columbia, Canada, 1999–2007. Emerging Infectious Diseases. https://doi.org/10.3201/eid1601.090900

Hanson, B., Zhou, Y., Bautista, E.J., Urch, B., Speck, M., Silverman, F., Muilenberg, M., Phipatanakul, W., Weinstock, G., Sodergren, E., Gold, D.R., Sordillo, J.E., 2016. Characterization of the bacterial and fungal microbiome in indoor dust

and outdoor air samples: a pilot study. Environ Sci Process Impacts 18, 713–724. https://doi.org/10.1039/c5em00639b

Klepeis, N.E., Nelson, W.C., Ott, W.R., Robinson, J.P., Tsang, A.M., Switzer, P., Behar, J.V., Hern, S.C., Engelmann, W.H., 2001. The National Human Activity Pattern Survey (NHAPS): a resource for assessing exposure to environmental pollutants. J Expo Anal Environ Epidemiol 11, 231–252. https://doi.org/10.1038/sj.jea.7500165

Kwon-Chung, K.J., Bennett, J.E., Wickes, B.L., Meyer, W., Cuomo, C.A., Wollenburg, K.R., Bicanic, T.A., Castañeda, E., Chang, Y.C., Chen, J., Cogliati, M., Dromer, F., Ellis, D., Filler, S.G., Fisher, M.C., Harrison, T.S., Holland, S.M., Kohno, S., Kronstad, J.W., Lazera, M., Levitz, S.M., Lionakis, M.S., May, R.C., Ngamskulrongroj, P., Pappas, P.G., Perfect, J.R., Rickerts, V., Sorrell, T.C., Walsh, T.J., Williamson, P.R., Xu, J., Zelazny, A.M., Casadevall, A., 2017. The Case for Adopting the "Species Complex" Nomenclature for the Etiologic Agents of Cryptococcosis. mSphere 2. https://doi.org/10.1128/mSphere.00357-16

Kwon-Chung, K.J., Fraser, J.A., Doering, T.L., Wang, Z.A., Janbon, G., Idnurm, A., Bahn, Y.-S., 2014. *Cryptococcus neoformans* and *Cryptococcus gattii*, the Etiologic Agents of Cryptococcosis. Cold Spring Harbor Perspectives in Medicine 4, a019760–a019760. https://doi.org/10.1101/cshperspect.a019760

Lazera, M.S., Cavalcanti, M.S., Londero, A.T., Trilles, L., Nishikawa, M.M., Wanke, B., 2000. Possible primary ecological niche of *Cryptococcus neoformans*. Medical Mycology 38, 379–383.

Lazera, M.S., Pires, F.D.A., Camillo-Coura, L., Nishikawa, M.M., Bezerra, C.C.F., Trilles, L., Wanke, B., 1996. Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees. Journal of medical and veterinary mycology 34, 127–131.

Lemons, A.R., Hogan, M.B., Gault, R.A., Holland, K., Sobek, E., Olsen-Wilson, K.A., Park, Y., Park, J.-H., Gu, J.K., Kashon, M.L., Green, B.J., 2017. Microbial rRNA sequencing analysis of evaporative cooler indoor environments located in the Great Basin Desert region of the United States. Environ Sci Process Impacts 19, 101–110. https://doi.org/10.1039/c6em00413j

Litvintseva, A.P., Kestenbaum, L., Vilgalys, R., Mitchell, T.G., 2005. Comparative Analysis of Environmental and Clinical Populations of *Cryptococcus neoformans*. Journal of Clinical Microbiology 43, 556–564. https://doi.org/10.1128/JCM.43.2.556-564.2005

Malliari, E., Kalantzi, O.-I., 2017. Children's exposure to brominated flame retardants in indoor environments - A review. Environ Int 108, 146–169. https://doi.org/10.1016/j.envint.2017.08.011

Martins, D.B., Zanette, R.A., França, R.T., Howes, F., Azevedo, M.I., Botton, S.A., Mazzanti, C., Lopes, S.T.A., Santurio, J.M., 2011. Massive cryptococcal disseminated infection in an immunocompetent cat: Letter to the Editor. Veterinary Dermatology 22, 232–234. https://doi.org/10.1111/j.1365-3164.2010.00948.x

Meyer, W., Aanensen, D.M., Boekhout, T., Cogliati, M., Diaz, M.R., Esposto, M.C., Fisher, M., Gilgado, F., Hagen, F., Kaocharoen, S., Litvintseva, A.P., Mitchell, T.G., Simwami, S.P., Trilles, L., Viviani, M.A., Kwon-Chung, J., 2009. Consensus multi-locus sequence typing scheme for *Cryptococcus neoformans* and *Cryptococcus gattii*. Medical Mycology 47, 561–570. https://doi.org/10.1080/13693780902953886

Passoni, L.F.C., Wanke, B., Nishikawa, M.M., Lazéra, M.S., 1998. *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 36, 305–311.

Phillips, P., Galanis, E., MacDougall, L., Chong, M.Y., Balshaw, R., Cook, V.J., Bowie, W., Steiner, T., Hoang, L., Morshed, M., Ghesquiere, W., Forrest, D.M., Roscoe, D., Doyle, P., Kibsey, P.C., Connolly, T., Mirzanejad, Y., Thompson, D., for the British Columbia *Crypotococcus gattii* Study Group, 2015. Longitudinal Clinical Findings and Outcome among *Cryptococcus gattii* Patients in British Columbia. Clinical Infectious Diseases. https://doi.org/10.1093/cid/civ041

Rajasingham, R., Smith, R.M., Park, B.J., Jarvis, J.N., Govender, N.P., Chiller, T.M., Denning, D.W., Loyse, A., Boulware, D.R., 2017. Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis. The Lancet Infectious Diseases 17, 873–881. https://doi.org/10.1016/S1473-3099(17)30243-8

Rocha, D.F.S., Cruz, K.S., Santos, C.S. da S., Menescal, L.S.F., Neto, J.R. da S., Pinheiro, S.B., Silva, L.M., Trilles, L., Braga de Souza, J.V., 2018. MLST reveals a clonal population structure for *Cryptococcus neoformans* molecular type VNI isolates from clinical sources in Amazonas, Northern-Brazil. PLoS ONE 13, e0197841. https://doi.org/10.1371/journal.pone.0197841

Santos, W.R.A. dos, Meyer, W., Wanke, B., Costa, S.P.S.E., Trilles, L., Nascimento, J.L.M. do, Medeiros, R., Morales, B.P., Bezerra, C. de C.F., Macêdo, R.C.L. de, Ferreira, S.O., Barbosa, G.G., Perez, M.A., Nishikawa, M.M., Lazéra, M. dos S., 2008. Primary endemic *Cryptococcosis gattii* by molecular type VGII in the state of Pará, Brazil. Mem. Inst. Oswaldo Cruz 103, 813–818.

Sorrell, T.C., Chen, S.C., Ruma, P., Meyer, W., Pfeiffer, T.J., Ellis, D.H., Brownlee, A.G., 1996. Concordance of clinical and environmental isolates of *Cryptococcus neoformans* var. *gattii* by random amplification of polymorphic DNA analysis and PCR fingerprinting. J. Clin. Microbiol. 34, 1253–1260.

Souto, A.C.P., Bonfietti, L.X., Ferreira-Paim, K., Trilles, L., Martins, M., Ribeiro-Alves, M., Pham, C.D., Martins, L., dos Santos, W., Chang, M., Brito-Santos, F., Santos, D.C.S., Fortes, S., Lockhart, S.R., Wanke, B., Melhem, M.S.C., Lazéra, M.S., Meyer, W., 2016. Population Genetic Analysis Reveals a High Genetic Diversity in the Brazilian *Cryptococcus gattii* VGII Population and Shifts the Global Origin from the Amazon Rainforest to the Semi-arid Desert in the Northeast of Brazil. PLOS Neglected Tropical Diseases 10, e0004885. https://doi.org/10.1371/journal.pntd.0004885

Swinne, D., Deppner, M., Laroche, R., Floch, J.J., Kadende, P., 1989. Isolation of *Cryptococcus neoformans* from houses of AIDS-associated cryptococcosis patients in Bujumbura (Burundi). AIDS 3, 389–390.

Swinne, D., Taelman, H., Batungwanayo, J., Bigirankana, A., Bogaerts, J., 1994. [Ecology of *Cryptococcus neoformans* in central Africa]. Med Trop (Mars) 54, 53–55.

Trilles, L., Lazéra, M. dos S., Wanke, B., Oliveira, R.V., Barbosa, G.G., Nishikawa, M.M., Morales, B.P., Meyer, W., 2008. Regional pattern of the molecular types of *Cryptococcus neoformans* and *Cryptococcus gattii* in Brazil. Memórias do Instituto Oswaldo Cruz 103, 455–462.

Table 1. Median survival times (MST) of *Galleria mellonella* larvae after being inoculated with different *Cryptococcus* strains recovered in this study. Values of each survival curve from *Cryptococcus neoformans* isolates (n=9) and *C. gattii* isolates (n=16) were compared with the highly virulent strain CDCR265 (VGIIa, ST20) to determine the degree of virulence.

Species	Strain	Place of isolation	Mating type	ST	Number of deaths	Median survival time (h)	*p*-value	Significance
C. neoformans	ARA-P15-3	Novo Airão	alfa	5	8	192	0.0040	**
	BAR10-07	Barcelos	alfa	93	9	180	0.0374	*
	BAR10-13	Barcelos	alfa	93	10	144	0.0340	*
	BAR10-16	Barcelos	alfa	93	10	144	0.1089	ns
	BAR10-19	Barcelos	alfa	93	10	144	0.9461	ns
	BAR10-21	Barcelos	alfa	93	10	180	0.0065	**
	BAR08-1	Barcelos	alfa	93	10	156	0.1050	ns
	BAR08-4	Barcelos	alfa	5	10	168	0.0320	*
	BAR08-17	Barcelos	alfa	5	9	156	0.0477	*
C. gattii	DW650-1	Santa Isabel do Rio Negro	alfa	7	9	240	<0.0001	****
	DW650-2	Santa Isabel do Rio Negro	alfa	5	7	192	<0.0001	****
	DW650-3	Santa Isabel do Rio Negro	alfa	264	8	204	<0.0001	****
	DW650-4	Santa Isabel do Rio Negro	alfa	264	7	192	0.0067	**
	DW650-5	Santa Isabel do Rio Negro	alfa	7	7	216	0.0002	***
	DW650-14	Santa Isabel do Rio Negro	alfa	265	10	144	0.5853	ns
	DW650-24	Santa Isabel do Rio Negro	alfa	7	10	168	0.0041	**

	SI443-13	Santa Isabel do Rio Negro	alfa	266	10	132	0.6349	ns
	SI443-14	Santa Isabel do Rio Negro	alfa	20	10	144	0.8420	ns
	SI443-15	Santa Isabel do Rio Negro	alfa	20	10	156	0.1337	ns
	SI443-17	Santa Isabel do Rio Negro	**a**	268	9	180	0.0097	**
	SI443-24	Santa Isabel do Rio Negro	alfa	267	9	156	0.0880	ns
	SI444-1	Santa Isabel do Rio Negro	alfa	7	9	192	0.0004	***
	ARA-P9A	Novo Airão	alfa	445	8	204	<0.0001	****
	ARA-P15-1	Novo Airão	alfa	445	5	228	0.0621	ns
	ARA-P15-2	Novo Airão	alfa	7	10	192	0.0005	***
	CDCR265	Canada	alfa	20	10	132	NA	NA

(*): More asterisks indicate that the strains are less virulent compared to CDCR265.
ns: Not significant, which means that the virulence of those strains is statistically equal to CDCR265
NA: not applicable

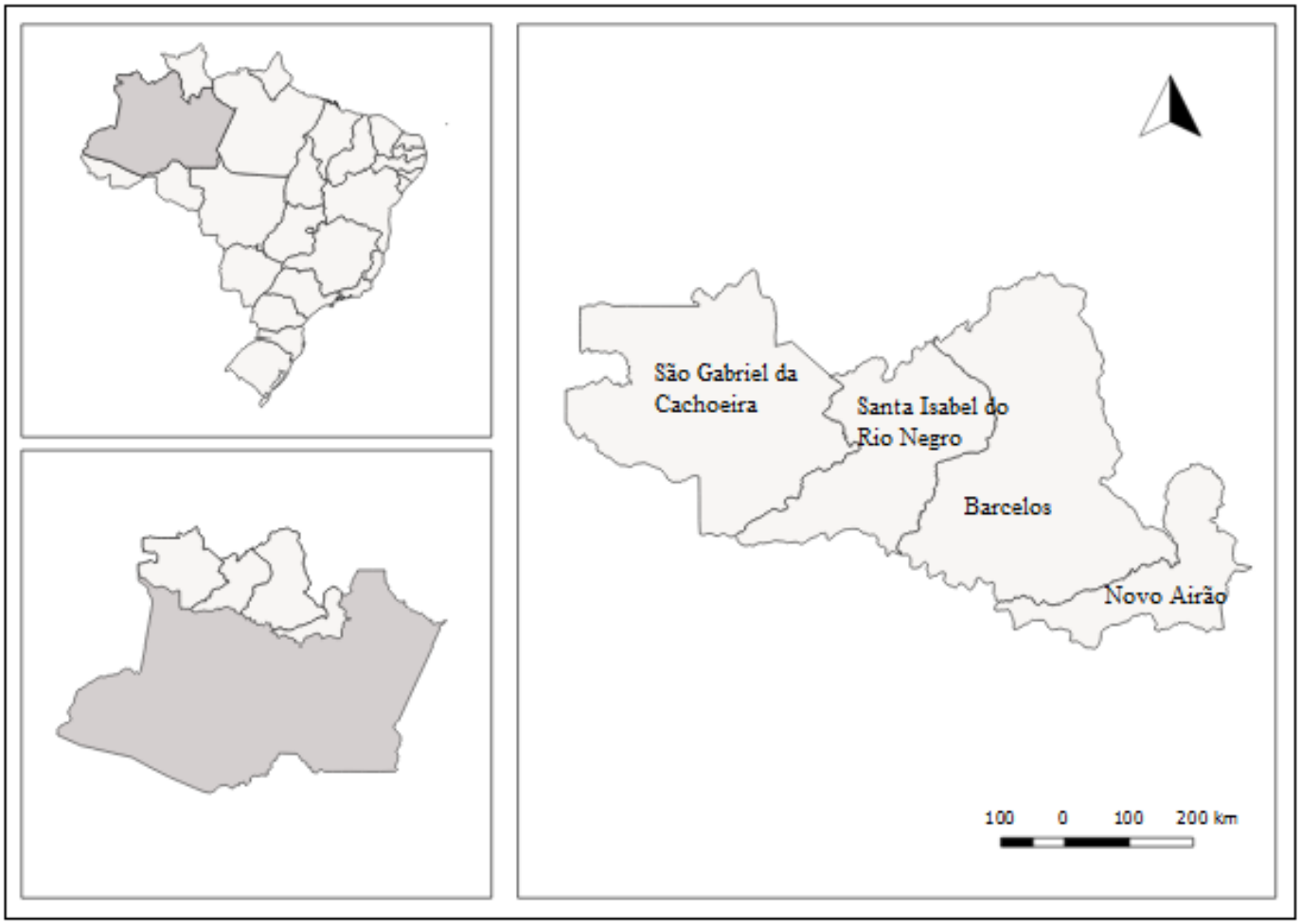
Supplementary Table 1: Data about cryptococcal isolation from indoor dust collected from the micro-region of Rio Negro, Brazilian Amazon, and molecular characterization (URA5-RFLP and MLST type) of positive environmental samples.

Municipalities	Collected samples	Positive samples	Frequency of positivity (%)	Range of CFU/g*	Molecular type isolated	MLST profiles
Santa Isabel do Rio Negro	51	3	5.9	2.500 - >50.000	VGII	ST20 (VGIIa), ST7 (VGIIb), ST5 and new STs 264, 266-268 (VGII)
Barcelos	12	2	16.7	600 - 1.300	VNI	ST5 and ST93 (VNI)
Novo Airão	16	2	12.5	200 - 300	VGII	ST7 (VGIIb) and new ST445 (VGII)
					VNI	ST5 (VNI)
All municipalities	79	7	8.9	200->50.00	VNI and VGII	All above mentioned

*CFU/g: colony-forming unit per gram

NA: not applicable

Figure 1. Map of the micro-region of Rio Negro, Brazilian Amazon, which is composed of four municipalities.



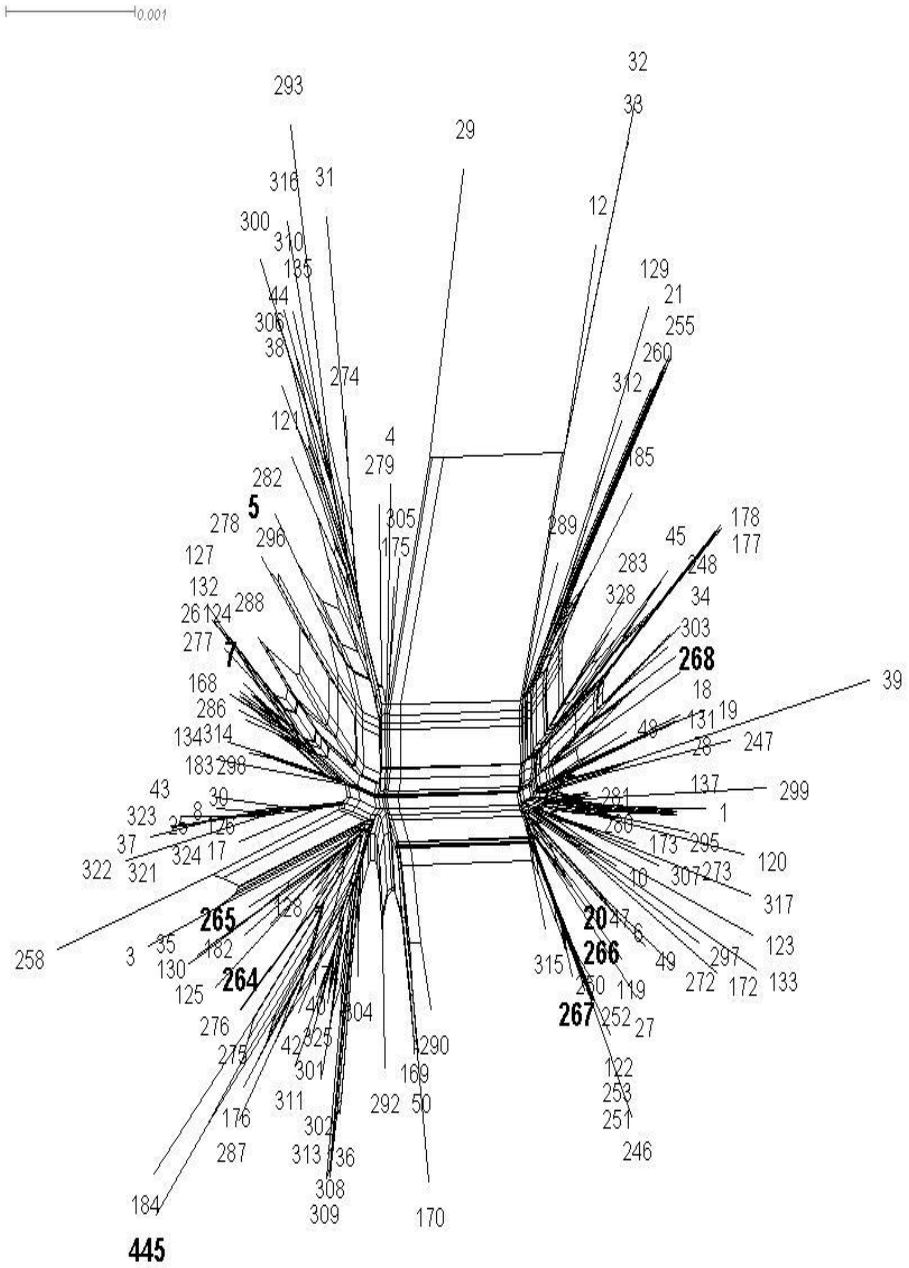
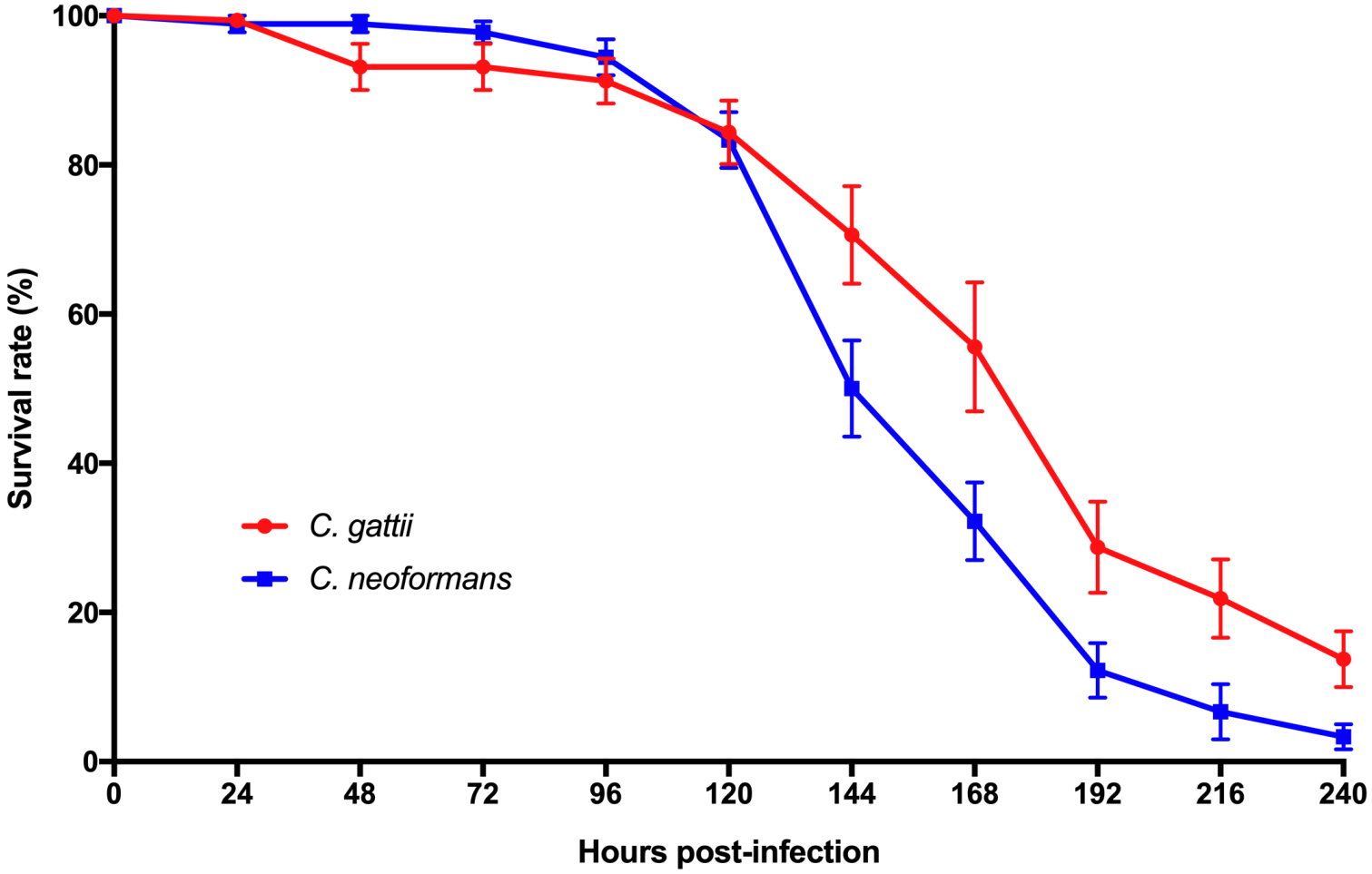


Figure 2

SplitsTree analysis of the combined MLST loci showing the placement of the STs (numbers in bold) from the micro-region of Rio Negro, Brazilian Amazon, in context with the sequence types obtained from the global *C. gattii* VGII population (obtained from published data from the website at mlst.mycologylab.org).

Figure 3: Survival curves of *Galleria mellonella* larvae inoculated with the *Cryptococcus neoformans* (n=9) and *C. gattii* (n=16) isolates recovered in this study. Death of *G. mellonella* larvae infected with *C. neoformans* VNI isolates resulted in a more rapid rate compared to those infected with *C. gattii* VGII, although the difference was not statistically significant (*p*-value 0.6764).



6. CAPÍTULO 2– VIGILÂNCIA DOS AGENTES DA CRIPTOCOCOSE NA MICRORREGIÃO DO RIO NEGRO.

Neste capítulo, um trabalho e dois produtos são apresentados.

Baseado nos achados citados no capítulo 1, que mostraram um possivel padrão endêmico dos agentes da criptococose e a prevalência de *C. gattii* na poeira domiciliar, o que gera um risco de criprococose primária para população, o doutorando foi incentivado a propor um modelo de medidas de vigilância para os agentes da criptococose na microrregião do Rio Negro. Dada a necessidade de aplicar uma ferrementa para o diagnóstico rápido da criptococose em áreas remotas ou de difícil acesso, o primeiro trabalho do capítulo de vigilância reforça a utilização da urina pré-aquecida como um espécime clínico útil para realização de diagnóstico e/ou inquéritos nessas áreas. Quanto aos produtos voltados para a comunidade, o primeiro desafio foi propor um projeto de divulgação científica mediante os achados da microrregião do Rio Negro. Esse projeto foi elaborado com o intuito de dar um retorno para a comunidade de estudo em questão. Mediante a elaboração desse produto sentiu-se a necessidade de confeccionar no primeiro momento um folder educativo voltado para os moradores com uma linguagem de fácil compreensão explicando "O que é a criptococose". A elaboração de um informativo técnico também foi incentivada como uma proposta de medidas preventivas que devem ser tomadas pelas secretarias de saúde de cada município, sendo sugerida nessa nota a cooperação conjunta com a Fundação de Vigilância em Saúde e por fim foi proposto um treinamento dos profissionais de saúde para o diagnóstico da criptococose na área de estudo. Essse capítulo de vigilância é de fato um dos principais pontos para o doutorando no programa de pós-graduação em Medicina Tropical e não por menos uma resposta concreta para a população em estudo. As propostas e ferramentas geradas podem auxiliar na investigação de futuros casos individuais ou surtos de criptococose nessa microrregião da Amazônia.

PLOS | NEGLECTED TROPICAL DISEASES

FROM INNOVATION TO APPLICATION

Preheating of urine improves the specificity of urinary cryptococcal antigen testing using the lateral flow assay

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Overview

A delay in laboratory diagnosis is related to sequelae and death. Thus, early diagnosis is the key to decrease the high lethality rate due to cryptococcosis. The cryptococcal antigen lateral flow assay (CrAg LFA) Immy test is standardized for a fast screening point-of-care test for cryptococcosis, detecting cryptococcal antigen in serum and cerebrospinal fluid (CSF). Urine screening would be ideal as a noninvasive approach, but previous studies have shown that fresh urine present false positive results. For this reason, we introduced just one simple physical procedure (i.e, heating of fresh urine prior to CrAg LFA Immy testing) to increase the specificity without compromising test sensitivity.

Introduction

Cryptococcosis by *Cryptococcus neoformans* is a major opportunistic infection in HIV patients, responsible for a high lethality (13%–44%), mainly in resource-limited countries. In sub-Saharan Africa, over 500,000 deaths due to cryptococcal meningitis are estimated to occur each year [1]. In Brazil, besides cryptococcosis associated with HIV, another public health problem is the endemic occurrence of cryptococcal meningitis by *C. gattii* in north and northeast regions with a lethality rate of 35%–40% [2]. A delay in laboratory diagnosis is related to sequelae and death. Thus, early diagnosis is the key to decrease the high lethality rate due to cryptococcosis.

Previous studies that used a cryptococcal antigen lateral flow assay (CrAg LFA) Immy in serum, plasma, finger stick whole blood, and cerebrospinal fluid (CSF) demonstrated its high sensitivity and specificity for screening cryptococcosis [3,4]. Recently, a review on diagnostic accuracy of the CrAg LFA showed a sensitivity of 85.0% (95% CI, 78.7%–90.1%) in urine. However, the specificity was not estimated [3]. A urine sample is easier to obtain than blood or CSF, becoming a promising methodology for early diagnosis of cryptococcosis, especially in developing countries [3,4]. Considering that the *Cryptococcus* target molecule glucuronoxylomannan (GXM) is thermostable, we included a heating step before the CrAg LFA Immy test to overcome the false positive results in urine, as shown in previous studies.

Methods

A prospective cohort study performed from April 2014 to April 2015 included all HIV-positive patients over 18 years of age with $CD4^+$ T cell counts $\leq$200 cells/mm^3 admitted at the Evandro Chagas National Institute of Infectious Diseases (INI), FIOCRUZ, Rio de Janeiro, Brazil. Healthy individuals were included as a negative control and patients presenting proven cryptococcosis as a positive control. All patients were invited to participate in this study and provided written informed consent. The study was conducted with the approval of the INI Ethics Committee (CAAE: 3248151400005262).

Cryptococcal antigen testing was performed in blood serum and urine from each volunteer using the CrAg LFA Immy test (Immuno-Mycologics, Norman, Oklahoma, USA), following the manufacturer´s instructions. The CrAg LFA in serum samples was considered as the gold standard. Each fresh urine sample was tested under two conditions: unheated (untreated) and heated (treated) by five minutes incubation at 100˚C. Clinical specimens such as blood, CSF, and urine were subsequently cultivated to investigate cryptococcal infection. Sensitivity, specificity, positive predictive values (PPVs), negative predictive values (NPVs), and Kappa statistic were determined at 95% CI by SPSS version 18.0.2 (IBM).

Results

The study was performed on a prospective cohort of 77 volunteers: 53 HIV-positive ($CD4^+$ T cell <200) patients, 18 healthy individuals (negative controls), and 6 HIV-positive patients with active proven cryptococcosis (positive controls).

Twenty-four out of 53 HIV-positive volunteers had a CrAg LFA–positive profile (42.3%) when untreated fresh urine was tested. When heated, only eight samples were positive (15%), presenting 100% of agreement with the positive results obtained from serum samples submitted to CrAg LFA Immy assay. Out of those eight positive patients, five had proven cryptococcosis (positive culture for *C. neoformans* in CSF and/or blood culture) and three patients had cryptococcal antigenemia (negative for *Cryptococcus* in blood and CSF cultures). The untreated fresh urine has shown 16 false positive results (30.2%). After treatment, those urine samples were negative, as confirmed by negative results in the serum.

The positive control group was positive in serum samples, in untreated and treated urine. Furthermore, *C. neoformans* was isolated from the clinical specimens in all cases in this group.

Thirteen out of 18 samples from the negative control group had the untreated urine positive (72%), representing false positive results, because after heating those urine samples were negative, resulting in 100% agreement with the CrAg LFA results obtained in serum.

Comparing to the serum as the gold standard, the CrAg LFA using untreated urine had a sensitivity of 100% (14/14), a specificity of 41% (26/63), a PPV of 27% (14 /51), and an NPV of 100% (26/26), whereas the comparison of serum and treated urine had a sensitivity, specificity, NPV, and PPV of 100%. Kappa coefficients (k) demonstrated a fair agreement between the methodologies by using untreated urine and serum (k = 0.204). However, perfect agreement between treated urine and serum (k = 1) was observed (**Table 1**).

Discussion

Early diagnosis and treatment is an important strategy for preventing clinical disease and reducing the high lethality rate in HIV patients with detectable CrAg. Urine specimens are easier to collect than other samples, providing a convenient way to screen suspected patients, especially in remote areas with resource-limited settings without laboratory facilities.

As shown herein and in a study by Tenforde et al. (2015) [5], fresh urine samples should not be used in point-of-care settings for screening of cryptococcosis. The significant number

Table 1. Summary of the diagnostic performance of cryptococcal antigen lateral flow assay on urine samples using serum as the reference standard.

Population (*N* = 77)	Sensitivity	Specificity	PPV	NPV	Kappa
Untreated urine	100% (14/14)	**41% (26/63)**	**27% (14/51)**	100% (26/26)	0.204 ($p = 0.02$)
(95% CI)	(99%–100%)	**(30%–52%)**	**(17%–37%)**	(99%–100%)	(0.068–0.339)
Treated urine	100% (14/14)	100% (63/63)	100% (14/14)	100% (63/63)	1.0 ($p < 0.001$)
(95% CI)	(99%–100%)	(99%–100%)	(99%–100%)	(99–100%)	(0.755–1.0)

Data presented are the percentage, numerator/denominator, and 95% confidence interval.
Kappa, analysis of agreement by Kappa index; NPV, negative predictive value; PPV, positive predictive value.

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of false positives in fresh urine can induce a false diagnosis of cryptococcosis and the misuse of antifungal drugs [6]. Early studies on the CrAg LFA test showed high sensitivity and low specificity in urine. [7,8,9]. In other studies, the diluent was changed or the urine was frozen in order to improve specificity but the results didn't reach the gold standard pattern (serum, CSF) [8,9]. The present study calls attention on the unexpected high number of false positives in fresh, untreated urine samples from healthy individuals included as a negative control and also from HIV patients. Considering a possible cross-reaction with some macromolecules, which are part of the usual profile of the urine, and taking into account the presence of thermosensitive molecules, we propose a fast and reliable method of inactivation of those molecules (i.e., heating fresh urine at 100˚C for five minutes before the CrAg LFA), which has no effect on thermostable cryptococcal GXM antigen—the major component of the capsule. Heating of urine prior testing dramatically improved the test's specificity without compromising the test's sensitivity. The CrAg LFA in heated urine of HIV patients identified not only five new cases of cryptococcosis but also three cases of cryptococcal antigenemia. Further studies on CrAg test are necessary to understand which molecule or molecules could be acting as interference, producing cross reactivity in urine.

In conclusion, this crucial step has dramatically increased the specificity without compromising test sensitivity, with two additional advantages: no need of enzymatic treatment or sample dilution (box 1).

Box 1. Advantages and disadvantages of heating urine prior CrAg LFA tests

Advantages

- High specificity and sensibility as the gold standard technique (with serum).
- Easy implement without need for phlebotomy or finger stick supplies.
- Screening early cryptococcal infection at routine laboratory as well as in field work.

Disadvantages

- One more step is included in the methodology: the boiling.
- Five more minutes are added to the methodology.
- Cannot be a point-of-care test.

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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(4): 525–530. https://doi.org/10.1097/QAD.0b013e328322ffac PMID: 19182676
2. Trilles L, Lazéra MS, Wanke B, Oliveira RV, Barbosa GC, Nishikawa MM, et al. Regional pattern of the molecular types of *Cryptococcus neoformans* and *Cryptococcus gattii* in Brazil. Mem Inst Oswaldo Cruz. 2008; 103(5):455–62. PMID: 18797758
3. Huang HR, Fan LC, Rajbanshi B, Xu JF. Evaluation of a new cryptococcal antigen lateral flow immunoassay in serum, cerebrospinal fluid and urine for the diagnosis of cryptococcosis: a meta-analysis and systematic review. PLoS ONE. 2015 14; 10(5).
4. Williams DA, Kiiza T, Kwizera R, Kiggundu R, Velamakanni S, Meya DB, et al. Evaluation of fingerstick cryptococcal antigen lateral flow assay in HIV-infected persons: a diagnostic accuracy study. Clin Infect Dis. 2015; 1; 61(3):464–7. https://doi.org/10.1093/cid/civ263 PMID: 25838287
5. Tenforde MW, Longley N, Meya DB, Boulware DR, Meintjes G, Goercke I, et al. Poor specificity of urinary cryptococcal antigen testing: Reply to Drain et al. Prevalence of cryptococcal antigenuria at initial HIV diagnosis in KwaZulu-Natal. HIV Med. 2015.
6. Jarvis JN, Percival A, Bauman S, Pelfrey J, Meintjes G, Williams GN, et al. Evaluation of a novel point-of-care cryptococcal antigen test on serum, plasma, and urine from patients with HIV-associated cryptococcal meningitis. Clin Infect Dis 2011; 53: 1019–1023. https://doi.org/10.1093/cid/cir613 PMID: 21940419
7. Magambo KA, Kalluvya SE, Kapoor SW, Seni J, Chofle AA, Fitzgerald DW, et al. Utility of urine and serum lateral flow assays to determine the prevalence and predictors of cryptococcal antigenemia in HIV-positive outpatients beginning antiretroviral therapy in Mwanza, Tanzania.J Int AIDS Soc. 2014 8; 17:19040. https://doi.org/10.7448/IAS.17.1.19040 PMID: 25109284
8. Longley N, Jarvis JN, Meintjes G, Boulle A, Cross A, Kelly N, et al. Cryptococcal Antigen Screening in Patients Initiating ART in South Africa: A Prospective Cohort Study. Clin Infect Dis. 2016 1; 62(5):581–7. https://doi.org/10.1093/cid/civ936 PMID: 26565007
9. Drain PK, Kleene JM, Coleman SM, Losina E, Katz JN, Giddy J, et al. Prevalence of cryptococcal antigenuria at initial HIV diagnosis in KwaZulu-Natal. HIVMed. 2015; 16(10):640–4.

Informe Técnico

Recomendação para a vigilância epidemiológica dos agentes da criptococose na microrregião do Rio Negro, Amazonas, Brasil

Novembro 2018

1-Agentes da criptococose na Microrregião do Rio Negro, estado do Amazonas

A criptococose coloca-se entre as infecções fúngicas humanas de significativa letalidade e morbidade, principalmente sob forma de meningoencefalite, seja em imunodeprimidos ou indivíduos com imunidade normal. Infecção fúngica de disseminação sistêmica é adquirida via pulmonar por inalação de propágulos de agentes do complexo *Cryptococcus neoformans* e do complexo *Cryptococcus gattii* presentes no ambiente. Não há transmissão entre humanos ou animais. Entre as mais de três dezenas de espécies de *Cryptococcus*, somente *C. neoformans* e *C. gattii* comumente causam a doença. Há uma incidência global anual de meningite criptocócica estimada de 223.100 casos, resultando em 181.100 mortes anuais em indivíduos com HIV, onde predomina *C. neoformans* (Rajasingham et al., 2017). O surgimento de *C. gattii* tipo molecular VGII como patógeno de hospedeiros aparentemente imunocompetentes é particularmente impressionante.

Particularmente nas regiões norte e nordeste do Brasil, registros hospitalares de internação demonstram expressiva ocorrência de criptococose por *C. gattii* em crianças e adolescentes sem HIV (Trilles et al., 2008; Freire et al., 2012). Estudos regionais demonstram percentual de 18% a 33% de crianças em casuísticas hospitalares de meningite criptocócica nos estados do Amazonas, Pará, Maranhão e Piauí (Corrêa et al., 1999; Santos et al., 2000; Martins et al., 2003; Santos et al., 2008), demonstrando a importância dessa infecção fúngica nos primeiros anos de vida. Sem dúvida, o uso de dados hospitalares apresenta limitações, refletindo apenas pequena fração de casos infantis num contexto maior e pouco conhecido da criptococose na Amazônia. A criptococose infantil primária é excepcional em outras regiões do mundo. Face ao observado na Amazônia, a exposição ambiental aos agentes da criptococose ocorre provavelmente desde os primeiros anos de vida.

Com base nessas evidências e estudos de fontes ambientais realizados na microrregião do Rio Negro/Amazonas foi elaborado o presente informe técnico, uma vez que há evidências de exposição cotidiana e domiciliar de moradores desta região aos agentes da criptococose (Brito-Santos et al., 2015). Amostras de poeira doméstica foram analisadas quanto à presença dos agentes da criptococose e apresentaram uma significante frequência de positividade (11,4%), com isolamento de *C. gattii e C. neoformans,* demonstrando que as crianças, bem como os demais moradores, devem estar expostos à inalação do fungo no próprio domicílio, cotidianamente.

2-Estudos de fontes ambientais relacionadas ao domicílio na microrregião do Rio Negro.

No período de 2011 a 2017 foram encontrados agentes da criptococose na poeira domiciliar dos seguintes municípios com seus respectivos bairros:

- Município de Novo Airão: Anavilhas e Remanso
- Município de Santa Isabel do Rio Negro: Don Walter e São Judas Tadeu
- Município de Barcelos: Centro da cidade.

Foram analisadas amostras de poeira domiciliar de 79 casas distribuídas em 3 municípios (Tabela 1).

Tabela 1: Processamento da poeira domiciliar e identificação dos agentes da criptococose

Municípios	Domicílios positivos	Frequência (%)	Agentes da criptococose
Santa Isabel do Rio Negro	3/51	5,8	*C. gattii*
Barcelos	2/12	16	*C. neoformans*
Novo Airão	2/16	12,5	*C. gattii / C. neoformans*
Todos os Municípios	**7/79**	**11,4**	*C. gattii/C. neoformans*

3-Ações para vigilância e controle dos agentes da criptococose

Face aos achados positivos em diferentes municípios da microrregião do Rio Negro, estado do Amazonas, há necessidade de alertar as equipes de atenção básica e de vigilância em saúde para casos clínicos suspeitos de criptococose e atenção especial aos menores oriundos desta região que podem ser considerados um grupo vulnerável. Considerando o difícil acesso e longas distâncias para atendimento médico especializado na microrregião do Rio Negro, é importante informar e preparar os profissionais de saúde relacionados a atenção básica para triagem de casos suspeitos. A fase inicial da criptococose, seja da forma pulmonar, seja da forma de meningite, é usualmente subaguda, progressiva, e nem sempre a síndrome febril é evidente. Nesta fase, confunde com outros diagnósticos (sinusite, pneumonia, cefaleia persistente, entre outros), o estado geral é bom e pode evoluir lentamente em semanas ou meses. Na busca do diagnóstico precoce, está indicado o uso do teste rápido, que pode ser feito no local em que o paciente está ("point of care"). Trata-se de teste imunocromatográfico para detecção de antígeno polissacarídeo de *Cryptococcus* sp (CrAg-LFA), específico, com leitura em 10 minutos, de fácil realização e que

não necessita da cadeia de refrigeração no transporte. Nos casos de testes positivos, estes deverão seguir para centros com mais recursos para os demais testes, como exame direto e cultivo de espécimes clínicos para fungo, além da avaliação médica.

3.1-Objetivos da Vigilância

- Diagnosticar precocemente os casos suspeitos de criptococose pulmonar e/ou meningite, em indivíduos nativos ou moradores das mencionadas regiões.

- Realizar o "screening" diagnóstico dos casos suspeitos utilizando o teste rápido de detecção de antígeno criptocócico (CrAg-LFA) no soro.

3.1.1-Definição de caso suspeito de criptococose

• Por critério clínico-epidemiológico:

Além de manifestações clínicas sugestivas de criptococose, deve ter história de ter nascido, morado ou viajado para zona endêmica de criptococose. As manifestações clínicas não são suficientes para o diagnóstico definitivo, confundindo com quadros respiratórios, pneumonia, tumor pulmonar, meningite viral, bacteriana ou causada por tuberculose.

• Por critério clínico:

Criptococose pulmonar

A maioria dos casos se apresenta como infecção pulmonar antes de disseminar e causar meningite. Nódulo solitário subpleural, por vezes mais de um nódulo ou infiltrado pulmonar não específico são vistos ao RX.

São sintomas suspeitos de criptococose pulmonar:

1. Dor torácica ou dor torácica ao respirar, por pelo menos duas semanas
2. Perda de peso
3. Febre é incomum, mas pode ocorrer.

Meningoencefalite (meningite) criptocócica

É a principal manifestação no sistema nervoso central (SNC), com inflamação das meningites e progressiva hipertensão intracraniana, mas pode ocorrer também associada a nódulo ou nódulos cerebrais. O início é geralmente insidioso, com cefaleia progressiva, principalmente occipital, que vai ficando intolerável e não melhora com analgésico. Segue-se queixa visual, já em estado avançado com estrabismo, visão dupla, acompanhada ou não de febre e perda de peso. Este espectro clínico deve ser considerado em todas as faixas etárias, incluindo crianças.
São sintomas suspeitos de meningite criptocócica:

1. Cefaleia intensa sem causa evidente por pelo menos 10 dias
2. Queixa visual: turvação da visão, visão dupla, estrabismo (desalinhamento dos olhos)

4.2-Confirmação de caso de criptococose

Em caso suspeito de criptococose (pulmonar, cutânea, disseminada, ou outra localização), o diagnóstico baseia-se na detecção do antígeno capsular criptocócico circulante por teste rápido (CrAg-LFA).

Os casos suspeitos com teste positivo no soro devem ser encaminhados para realização de exames:

1. RX de tórax: procedimento mínimo aconselhado no rastreio de lesão pulmonar.
2. Punção lombar: deve ser feita de rotina mesmo quando não há a tríade clássica de meningite (cefaléia, rigidez de nuca e vômitos), a meningite pode ser inicialmente assintomática ou oligossintomática. O liquor deve ser submetido ao mesmo teste (CrAg-LFA) e o resultado positivo indica meningite criptocócica. Demais exames devem ser realizados como exame direto do LCR (Liquido Cefalorraquidiano) ao nanquim (tinta da china) e cultivo para fungos, porem o tratamento pode ser iniciado imediatamente após o resultado positivo do teste CrAg-LFA.

Teste CrAg positivo nos seguintes espécimes clínicos:

Sangue total, urina pré-aquecida, soro e plasma o teste positivo confirma criptococose, porém não define a forma clínica.

Confirmação de criptococose pulmonar:

Teste positivo no soro, urina pré-aquecida, sangue ou plasma, associado a evidência de lesão ao RX de tórax, presença de levedura capsulada em espécime clínico pulmonar (exame direto ao nanquim) e/ou cultivo positivo para *C. gattii* ou *C. neoformans.*

Confirmação de diagnóstico de meningite criptocócica:

Teste CrAg positivo no LCR basta para o diagnóstico. O exame direto positivo ao nanquim para levedura capsulada no LCR ou cultivo positivo para *Cryptococcus* spp no LCR têm também valor diagnóstico e devem ser feitos sempre que haja estrutura laboratorial mínima e treinamento para o diagnóstico laboratorial.

4.3-Recomendações direcionadas à atenção ao paciente com criptococose confirmada.

Feito o diagnóstico e definida a forma clínica o paciente deve ser referido a um posto ou centro de referência da região. Sendo a forma de criptococose localizada (pulmonar, cutânea isolada, entre outras) sem evidência de disseminação para SNC, bom estado geral e sem doença de base pode, ser iniciado o tratamento oral com itraconazol 200 a 400mg/dia 6 meses a 1 ano para adulto. É importante ressaltar que o tratamento antifúngico para criptococose em crianças é baseado e extrapolado pela experiência com adultos, a maioria infectados pelo HIV, uma vez que casos pediátricos são raros nas casuísticas mundiais. Portanto, considerando a concentração tecidual pulmonar do itraconazol, preconizamos o uso oral de itraconazol 10mg por kg o, tomado 1 x ao dia, após refeição (almoço).

Chamamos a atenção para a importância da punção lombar no paciente que foi positivo no soro. Caso não haja condições para realizar o procedimento, o paciente deve ser referido a outro local para realizar a punção lombar. A positividade ao teste CrAg no LCR caracteriza meningite mesmo que o LCR seja aparentemente normal, e indica tratamento combinado de

Anfotericina-B e flucitosina, conforme esquema já estabelecido (Consenso de criptococose; 2008).

4.4-Notificação dos casos de criptococose

Apesar de ser emergente e causar elevada letalidade sob forma de meningite, a criptococose não é um agravo de notificação no país, nem na região amazônica onde há evidências de endemia estabelecida. Sugerimos que uma ficha de notificação seja criada e um fluxo de vigilância seja adotado na região.

4.5-Ações de educação em saúde e cursos de formação continuada

- Folder informativo sobre a criptococose e seus agravos.
- Palestras para profissionais de saúde sobre o tema.
- Curso teórico-prático para detecção de antígeno criptocócico utilizando o teste rápido CrAg para médicos, enfermeiros, farmacêuticos, biomédicos e técnicos de saúde.

5-Referências

Trilles L, Lazera M, Wanke B, Oliveira RV, Barbosa GG, Nishikawa MM, Morales BP, Meyer W 2008. Regional pattern of the molecular types of Cryptococcus neoformans and Cryptococcus gattii in Brazil. Mem Inst Oswaldo Cruz 103: 455-462.

Freire AK, dos Santos Bentes A, de Lima Sampaio I, Matsuura AB, Ogusku MM, Salem JI, Wanke B, de Souza JV 2012. Molecular characterisation of the causative agents of Cryptococcosis in patients of a tertiary healthcare facility in the state of Amazonas-Brazil. Mycoses. May;55(3):e145-50.

Correa Mdo P, Oliveira EC, Duarte RR, Pardal PP, Oliveira Fde M, Severo LC 1999.. Cryptococcosis in children in the State of Pará, Brazil Rev Soc Bras Med Trop. 32(5):505-8.

Santos LO 2000. Criptococose no estado do Amazonas: estudo de 75 casos diagnosticados na Fundação de Medicina Tropical/FMT/IMTM, Manaus, AM (1988-1998), Msc Thesis, Instituto Oswaldo Cruz-Fiocruz, Rio de Janeiro 154 pp.

Martins LMS 2003. Epidemiologia da criptococose em crianças e adultos jovens e diversidade de Cryptococcus neoformans no Meio Norte do Brasil, MSc Thesis, Instituto Oswaldo Cruz-Fiocruz, Rio de Janeiro, 87 pp.

Santos WR, Meyer W, Wanke B, Costa SP, Trilles L, Nascimento JL, Medeiros R, Morales BP, Bezerra Cde C, Macêdo RC, Ferreira SO, Barbosa GG, Perez MA, Nishikawa MM, Lazéra M dos S. Primary endemic Cryptococcosis gattii by molecular type VGII in the state of Pará, Brazil. Mem Inst Oswaldo Cruz. 2008 Dec;103(8):813-8.

Brito-Santos F, Barbosa GG, Trilles L, Nishikawa MM, Wanke B, Meyer W, Carvalho-Costa FA, Lazéra Mdos S. 2015A. Environmental isolation of *Cryptococcus gattii* VGII from indoor dust from typical wooden houses in the deep Amazonas of the Rio Negro basin. PLoS One.Feb 17;10(2):e0115866. doi: 10.1371/journal.pone.0115866.

CONSENSO EM CRIPTOCOCOSE. Rev. Soc. Bras. Med. Trop. 41(5):524-544, set-out, 2008.

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O que é?

Criptococose é uma micose causada por um fungo que vive na natureza, sendo facilmente encontrado em fezes de aves, ocos de árvores e madeiras apodrecidas, podendo ser achado até em poeira domiciliar.

A pessoa pode adquirir a criptococose pela respiração da poeira contaminada com o fungo. No pulmão, causa doença com tosse, dor nas costas e, as vezes, pneumonia. Se a criptococose do pulmão não for tratada, o fungo pode ir para o cérebro, causando meningite.

Essa doença afeta, principalmente, crianças e adultos jovens. Quanto mais cedo for feito o diagnóstico dessa infecção, menor serão os riscos de cegueira, perda de audição e morte por meningite.

Apoio:

INI
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FIOCRUZ
Fundação Oswaldo Cruz

IOC PROGRAMA DE PÓS-GRADUAÇÃO STRICTO SENSU EM MEDICINA TROPICAL

Elaboração:

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Consultoria: Marcia Lazera
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CRIPTOCOCOSE
(Cripto)

Saiba como proteger a sua família.

Sintomas mais comuns

No início

1. Dor nas costas
2. Tosse

Depois

3. Fortes dores de cabeça (que não passam com remédios comuns)
4. Enjôo e vômito
5. Sonolência
6. Visão turva ou visão dupla

TRATAMENTO E PREVENÇÃO

Em Caso De Suspeita Procure Um Médico

Se você tiver esses sintomas procure imediatamente um médico ou seu agente de saúde no local mais próximo. Para realizar o exame da criptococose existe um teste rápido que pode ser feito no sangue. Se o teste for positivo, o médico irá tomar a conduta para o tratamento.

Prevenção

Não existe prevenção ou vacina para evitar a pneumonia ou a meningite causada pela criptococose. Mas, podemos diminuir o risco de respirar esse fungo no ar. Por exemplo, ele cresce em vários locais onde tem madeira podre, fezes de aves, que se espalham no ar e viram poeira. A umidade e escuridão ajudam o crescimento do fungo, que gosta de ocos de árvores.

Limpe Bem A Sua Casa

Então, você deve evitar madeira podre, pó de serragem ou fezes de aves, dentro da casa ou no quintal. Deve tratar ou pintar as tábuas de sua casa, manter a casa limpa, ventilada e com luz do sol sempre que possível.

7-DISCUSSÃO

Emmons, em 1951, descreve o primeiro isolado de *C. neoformans* a partir de fontes ambientais no estado da Virgínia/EUA, onde isolou o fungo de solo contaminado com excreta de aves, em particular de pombos, sedimentando o conceito de infecção adquirida a partir de ambiente contaminado (Emmons, 1951). A partir desses achados, a intensa procura pelo habitat natural de *C. neoformans* foi realizada por vários autores. Sabendo-se que a criptococose por *C. neoformans* tem um padrão cosmopolita e o pombo, ave cosmopolita, é adaptada a ambientes urbanos, Nielsen e colaboradores relatam em seus estudos *in vitro* a melhor capacidade de adaptação de *C. neoformans* na excreta de columbiformes, apresentando progênies férteis no cruzamento entre *C. neoformans* var. *grubii Mat* a e *C. neoformans* var. *neoformans Mat* alfa (Nielsen et al., 2007).

Originalmente, a investigação intensiva do possível habitat natural nas excretas de pombos em diferentes continentes levou ao isolamento exclusivo de *C. neoformans* a partir de fontes ambientais. Após a confirmação da existência de uma variedade (*C. neoformans* var. *gattii*) mais tarde reconhecida como espécie distinta (*C. gattii*) (Kwon-Chung, 1975), este agente passou a ser exaustivamente procurado no ambiente em fezes de pombo, sempre com resultados negativos e assim considerado um agente raro no ambiente. A criptococose por *C. gattii* que apresenta um padrão endêmico em climas tropicais e subtropicais, foi muito estudada na Austrália por Ellis e Pfeiffer direcionando as pesquisas ambientais para *Eucaliptus camaldulensis* árvore nativa daquela região (Ellis and Pfeiffer, 1990). Surpreendentemente os autores encontraram *C. gattii* em amostra de madeira e flores dessas árvores e estabeleceram que *C. gattii* tinha uma associação ecológica específica e exclusiva com *E. camaldulensis*. Por outro lado, estudos desenvolvidos no Brasil mostraram a positividade de *C. gattii* em árvores de diferentes espécies, ampliando o conceito de habitat natural primário relacionado à madeira em decomposição em ocos de árvores. Ficou evidente então que não havia uma especificidade de *C. gattii* por uma determinada árvore-hospedeira (Lazera et al., 1998) e estudos progressivos mostraram que tanto *C. gattii* quanto *C. neoformans* podem ser isolados de um mesmo microhabitat, isto é, oco de árvores. Neste microhabitat a madeira em decomposição apresenta substratos ricos em lignina. A produção de lacase, enzima relacionada à degradação de madeira, pelos agentes da criptococose, facilita o crescimento saprofítico em ocos de árvores, que representam habitats naturais primários de *C. gattii* e *C. neoformans* (Lazera et al., 2000). A lista

de espécies de árvores das quais *C. gattii* e/ou *C. neoformans* são isolados é crescente, em diversos países, na Argentina e Colômbia (15 gêneros); Índia (11 gêneros); Canadá, México e Estados Unidos (9 gêneros), Espanha e Holanda (4 gêneros) e Austrália (2 gêneros). No Brasil *C. neoformans* foi descrito em 14 gêneros de árvores e *C. gattii* em 7 gêneros (Barbosa et al., 2013).

A expansão geográfica das infecções por *C. gattii*, originalmente restrita a áreas tropicais e subtropicais, foi evidenciada pelo surto no Canadá. Seria impossível imaginar um surto de *C. gattii* em clima temperado, porém, na ilha de Vancouver, ocorreu infecção humana e animal caracterizando um surto de larga escala pelo tipo molecular VGII e seus subtipos VGIIa e VGIIb foram associados a mortes nessa região (Galanis et al., 2010). Além disso, em estudos ambientais, isolaram esse agente de árvores nativas, fontes naturais de água e no ar externo *"outdoor"* em florestas desta região e esse surto foi creditado ao possível aumento de temperatura nesse local (Kidd et al., 2007). O desequilíbrio ambiental gerado por fatores antrópicos, alteram os microhabitats e, consequentemente, a microbiota, contribuindo para a dispersão/proliferação/adaptação dos agentes da criptococose.

Durante décadas a região amazônica sofre as consequências da antropização, como exemplo, o desflorestamento, extinção de espécies, exploração de riquezas minerais e taxa de desmatamento em crescimento contínuo (Yanai et al., 2017). No presente trabalho utilizamos como área de estudo a microrregião do Rio Negro, localizado no Estado do Amazonas. No primeiro trabalho realizado nessa região levantamos a hipótese que *C. gattii* VGII isolado na poeira domiciliar *"indoor"* estaria associada às casas de madeira, construídas com árvores nativas, uma vez que já havia evidência de *C. gattii* em árvore nativa da região amazônica (Fortes et al., 2001). De fato, como relatado anteriormente, *C. gattii* está associado às árvores nativas em diversas regiões do mundo (Brito-Santos et al., 2015a). Com relação à microrregião, este primeiro trabalho ocorreu no município de Santa Isabel do Rio Negro. A ampliação do estudo foi necessária para verificar se os achados foram eventuais ou representavam em pequena escala um evento maior de adaptação dos agentes da criptococose a áreas antropizadas dessa vasta região de Amazônica. Foi coletada poeira domiciliar em casas mistas (alvenaria e/ou madeira). Realmente, esse segundo estudo, consolidou os achados inicias, mostrando que os agentes da criptococose ocorrem no ambiente domiciliar da microrregião do Rio Negro e possivelmente em cenários similares de assentamento de populações humanas na Amazônia. Será que essa extensa área do bioma amazônico daria suporte ao habitat **natural primário** desses agentes, dispersos na mata original e posteriormente adaptados a áreas antropizadas nesta região. Um trabalho realizado em

áreas de baixo efeito antrópico (reserva ambiental) da floresta amazônica, na ilha de Maracá, *C gattii* VGII foi isolado de árvore nativa, sugerindo a presença ancestral desse agente no bioma amazônico (Fortes et al., 2001).

Portanto, tanto *C gattii* VGII e *C. neoformans* VNI estão associados a madeira em decomposição nesta microrregião (Brito-Santos et al., 2015b). Chama atenção a ausência de pombos nos ambientes estudados, contrastando com os ambientes das grandes cidades onde pombo está associado a dispersão clonal de *C. neoformans* VNI através de excretas secos, que são verdadeiros microfocos desse agente (Baroni et al., 2006). Por outro lado, esse cenário não foi observado nas casas positivas para *C. neoformans* VNI na Amazônia.

Considerando a região de estudo, alguns aspectos devem ser considerados com relação à capacidade de adaptação tanto de *C. neoformans* VNI como *C. gattii* VGII ao ambiente de estudo, antropizado e em fase inicial de urbanização. No município de Santa Isabel do Rio Negro, observamos que ambos os agentes foram encontrados em postes feitos de árvores nativas extraídas da mata original, trazendo a biodiversidade para ambiente antropizado (Brito-Santos et al., 2015b). Quanto ao papel dos postes de madeira na formação da cidade, seria possível sugerir que atuem como corredores biológicos, ou seja, como uma extensão do ecossistema natural- que seria a mata nativa- interligado ao ambiente antropizado e possibilitando a manutenção de parte da biodiversidade e seus processos evolutivos, e incluindo os agentes da criptococose. De fato em sua maioria, os postes de madeira apresentavam carreiros de cupins, associados à decomposição de madeira. Fortes e col. relatam o isolamento dos agentes da criptococose em cupinzeiros no município de Boa Vista /Roraima (Sales et al., 2017). Seriam os cupins possíveis responsáveis pela dispersão desses agentes na microrregião? Seriam as áreas em decomposição dos postes um padrão de habitat secundário, adaptado para esses agentes no ambiente antropizado? Deve-se considerar que a madeira em decomposição corresponde a substratos favoráveis ao crescimento dos agentes da criptococose, que possuem uma enzima lacase, pertencente a grupo especializado, relacionado a decomposição de lignina (Lazera et al., 1996).

Outro aspecto a ser considerado está relacionado à ocupação antrópica histórica ao longo do tempo. O município de Barcelos tem uma população estimada de 25.589 habitantes, tendo sido a primeira capital da província do estado do Amazonas com cerca de 240 anos. Os achados da poeira domiciliar desse município revelaram um padrão exclusivo de *C. neoformans* VNI, onde esse agente foi isolado tanto na igreja local quanto na casa de uma funcionária da igreja. “Pesquisadores sugerem que *Cryptococcus* spp seria um fungo católico”, devido aos relatos de

igrejas onde já foi isolado este agente! (Baroni et al., 2006). Considerando o nosso processo de colonização, onde as igrejas eram o marco inicial das cidades e ponto de encontro de toda comunidade, esse possível ponto de dispersão deve ser considerado. Barcelos é a mais antiga das cidades estudadas e não foi possível isolar *C. gattii* das amostras coletadas, essencialmente poeira doméstica. Não foram coletadas amostras de postes e isso pode ter influenciado no resultado final. Considerando o ambiente domiciliar, *C. neoformans* predominou de maneira evidente lembrando o observado em grandes centros urbanos. Seria essa uma tendência com a progressiva urbanização de cidades da Amazônia? (Freire et al., 2012; Rocha et al., 2018).

O município de Novo Airão, corroborando os achados de Santa Isabel do Rio Negro, reforçou a ocorrência de *C. neoformans* VNI e *C. gatiii* VGII na microrregião de estudo (Brito-Santos et al., 2017). Os principais tipos moleculares responsáveis pela criptococose no Brasil, parecem estar adaptados a essa região!

O estudo da estrutura populacional por MLST é uma ferramenta aplicada a estudos epidemiológicos moleculares (Meyer et al., 2009). Com o auxílio dessa ferramenta, foi possível identificar na poeira domiciliar dos municípios da microrregião do Rio Negro os principais subtipos responsáveis pela criptococose no mundo. A primeira análise de MLST no município de Santa Isabel do Rio Negro já revelava presença dos subtipos relacionados ao surto de Vancouver (ST20/VGIIa e ST7/VGIIb) (Brito-Santos et al., 2015a). Souto et al., (2016) em análise da diversidade genética dos isolados brasileiros de *C. gattii*, chamou atenção dos isolados de Santa Isabel do Rio Negro, onde ST20 (VGIIa) mostrou padrão de dispersão na região Amazônica. Além do que, dois STs (ST266, ST267) exclusivos de Santa Isabel do Rio Negro estavam ligados ao complexo clonal do ST20 (VGIIa) (Apêndice A). Posteriormente, a presença de ST7/VGIIb foi consolidada com isolamento deste no município de Novo Arião. O município de Barcelos apresentou exclusivamente os ST5/VNI e ST93/VNI, dois subtipos já identificados em isolados clínicos e ambientais no Brasil e também comuns na Ásia e na Europa (Ferreira-Paim et al., 2017).

A evidência de 6 novos subtipos de *C gattii* STs (264, 265, 266, 267, 268 e 445), o achado de *C. gattii* VGII tipo sexuado *Mat* a e o predomínio do *Mat* alfa na poeira domiciliar da microrregião, despertaram várias possibilidades: Reprodução sexuada favorecendo o rearranjo genético com maior probabilidade de sobrevivência em ambientes mutáveis e/ou competitivos como observado em Santa Isabel do Rio Negro.

A presença de mating type **a**, em isolado de *C. gattii* reforça a possibilidade de eventos de recombinação levando a grande variabilidade e diversidade genética na poeira domiciliar da área estudo. É interessante notar que o mesmo não foi observado para *C. neoformans* que apresenta uma estrutura predominantemente clonal.

Após essa reflexão, o questionamento sobre a virulência desses agentes era de fato um desafio. Teriam esses isolados da poeira domiciliar algum potencial de virulência? Escolhemos portanto o modelo com invertebrado (Lepidoptera) *Galleria mellonella*, método alternativo ao modelo murino, para avaliar o potencial de virulência dos agentes da criptococose (García-Rodas et al., 2011; Garcia-Solache et al., 2013).

Mylonakis e Col. (2005), Trevijano e Col. (2015) e Benaducci e Col. (2016) utilizaram o modelo *G. mellonella* para avaliar a virulência de isolados clínicos (VNI, VGII e H99), além de Firacative e Col. (2014) em estudo comparando os subtipos de *C. gattii* (VGII-VGIV), incluídos sete isolados ambientais (Mylonakis et al., 2005; Firacative et al., 2014; Trevijano-Contador et al., 2015; Benaducci et al., 2016). No presente estudo comparamos a sobrevida de *G. mellonella* inoculada exclusivamente com isolados da poeira domiciliar e observamos que tanto VNI e VGII tinham potencial de virulência

Portanto, subtipos como ST7 e ST20 (VGII) comprovadamente virulentos por estarem relacionados a surto em humanos e animais, demonstraram seu potencial através do modelo *G. mellonella* utilizado no presente estudo. Demais subtipos novos encontrados na poeira domiciliar com ex: VGII (ST445) estão associados a criptococose em pessoas com HIV. Estes aspectos são comentados no artigo em fase final de redação: *"Virulent strains of the agents of cryptococcosis in indoor dust from a micro-region of Rio Negro, Amazonas, Brazil - New evidences and big challenges".*

Sabendo-se que a criptococose é uma micose com porta de entrada respiratória, pela inalação de esporos, é possível imaginar que a recombinação e diversidade genética podem contribuir para virulência desses agentes. Existia alguém doente, alguma evidência de surto, paciente assintomático ou oligossintimático na área de estudo?

O primeiro projeto voltado para essas perguntas e a primeira proposta de doutorado foi "Estudo da infecção por *Cryptococcus* spp em crianças e investigação de fontes ambientais de agentes da criptococose no município de Santa Isabel do Rio Negro, Amazonas, Brasil'. Particularmente na Amazônia, observa-se elevada ocorrência de criptococose em crianças sem

HIV negativo por *C.gattii* apontando um aspecto epidemiológico distinto e não descrito em outras regiões do mundo (Freire et al., 2012).

Segundo dados do IBGE-2017 da pirâmide de faixa etária, Novo Airão, Barcelos e Santa Isabel do Rio Negro, apresentam uma grande população na faixa de 0-14 anos de idade. O predomínio de uma população de crianças e um padrão endêmico dos agentes da criptococose nessa região incentivou a busca de casos sintomáticos, assintomáticos e oligossintomáticos de criptococose. Como buscar esses casos de criptococose no interior do estado do Amazonas e que ferramenta utilizar.

O Cryptococcus Teste Rápido CrAg-LTF (Lateral Flow Assay) é um método imunocromatográfico que utiliza o sistema sanduíche para detectar o antígeno das espécies *C. gattii* e *C. neoformans*. O teste CRAG® realizado no soro/plasma ou sangue total, pode servir como uma ferramenta de rastreio para a doença criptocócica, e a utilização do CRAG® como triagem em pacientes com CD4< 100 células/mm^3 tem sido proposto para diagnóstico precoce da criptococose principalmente em regiões com limitações laboratoriais, visto que esse exame é de fácil execução (Pongsai et al., 2010). A urina no primeiro momento era um desafio para detecção do antigeno, por apresentar um baixa especificidade. A otimização do teste, com o aumento da especificidade sem perder a sensibilidade, realizando um simples aquecimento a 100°C/5m, foi um grande avanço para o rastreio da criptococose em crianças na faixa etária de 0-14 anos de idade nessa região, devido à facilidade de obter esse material, além da capacidade de detectar rapidamente o antígeno criptocócico nesses indivíduos. Infelizmente, não foi possível realizar esse projeto, mesmo o nosso grupo sendo contemplado pelo "Auxílio Básico à pesquisa-APQ1 2014-02 FAPERJ". Mesmo não sendo possível realizar essa proposta inicial, não poderíamos desistir, visto que: O estudo da criptococose dirigido a indivíduos nativos ou moradores de regiões endêmicas em nosso país constituía um desafio considerando que a criptococose, apesar de ser emergente e causar elevada letalidade sob forma de meningite, não é notificada no Brasil.

Mediante os achados e publicações na microrregião do Rio Negro, além de relatos dos próprios moradores dessa região "Perguntando sobre os resultados das pesquisas anteriores realizadas na mesma área de estudo", nosso grupo foi estimulado a criar uma nova proposta voltada para a comunidade. Aproveitando o edital 2018 "Apresentação de Propostas para Projetos de Divulgação Científica" elaboramos o projeto "Vigilância epidemiológica dos agentes da criptococose na microrregião do Rio Negro, Amazonas, Brasil" que tinha como objetivo: Elaboração de informe técnico sobre a ocorrência dos agentes da criptococose em fontes

ambientais para cada município. Nesse documento foram expostos os achados para a secretaria de saúde de cada município, alertando sobre a ocorrência dos agentes da criptococose isolados a partir de fontes ambientais, e como proposta de vigilância foram informadas as possíveis consequências sendo fornecidos subsídios técnicos para tomada de decisão. Foi confeccionado também um informativo popular (Folder informativo) sobre a criptococose e seus agravos. Esse folder informativo voltado para comunidade com uma linguagem popular, tinha como principal objetivo apresentar a doença à população. As pesquisas que envolvem seres humanos necessitam atender a alguns fundamentos éticos e científicos pertinentes, como o de assegurar aos participantes da pesquisa os benefícios resultantes do projeto, ou seja, o retorno social (BRASIL, 2012). Não existem, até o momento, relatos de proposta de um modelo de vigilância da criptococose, voltada para áreas endêmicas no Brasil. O Canadá, após o surto de Vancouver, colocou a criptococose como doença de notificação e até hoje tem um modelo de vigilância preventiva para evitar novos surtos (BC Centre for Disease Control).

Nos últimos anos, o termo ***One Health*** (Saúde Única) vem ganhando espaço cada vez maior dentro das discussões científicas que tratam de questões ligadas à saúde e epidemiologia. Ressalta-se a integração entre a saúde humana, a saúde animal, o ambiente e a adoção de políticas públicas efetivas para prevenção e controle de enfermidades trabalhando nos níveis local, regional, nacional e global (Davis et al., 2017). Com o estudo na microrregião do Rio Negro, foi possível integrar a saúde humana com a proposta da implementação do CrAg na urina para diagnosticar casos precoces de criptococose. Quanto ao ambiente "*indoor environment*" identificamos e confirmamos o potencial de virulência dos principais agentes da criptococose na poeira domiciliar. A adoção de políticas públicas foi sugerida através da proposta de vigilância desses agentes na área de estudo. De fato não foi possível estudar a saúde animal nesta região, porém identificamos em outro estudo na região sudeste 2 casos autóctones de felinos com criptococose por *C. gattii* VGII até então somente endêmico na região norte e nordeste **(Apêndice B)**. Seriam esses felinos sentinelas dos agentes da criptococose no ambiente domiciliar? Portanto, o conceito de saúde única para os agentes da criptococose pode ser uma estratégia promissora na região rural e nos grandes centros urbanos.

8-CONCLUSÕES

- *C. gattii* VGII e *C. neoformans* VNI estão presentes no ambiente da microrregião do Rio Negro no estado do Amazonas, especificamente em poeira de domicílios e dos postes de madeira estudados nesta região.
- Mesmo sem a evidência de casos de criptococose em humanos, *C. gattii* VGII e *C. neoformans* VNI foram isolados do ambiente e apresentaram potencial de virulência em modelo alternativo, *in vivo*, *Galleria mellonella*.
- Os principais subtipos moleculares globalmente conhecidos de *C. gattii* VGII (ST7 e ST20) e *C. neoformans* (ST5 e ST93) estão presentes na poeira dos domicílios da microrregião do Rio Negro.
- A população de *C. gattii* da microrregião do Rio Negro apresenta elevada diversidade genética, enquanto que *C. neoformans* possui significativa clonalidade.

9-PERSPECTIVAS

- O retorno aos municípios da microrregião do Rio Negro se faz necessário, para divulgação dos resultados da pesquisa.

- A investigação da qualidade do ar domiciliar das casas positivas e negativas, utilizando amostradores de partículas vivas para coletar e analisar os agentes da criptococose nessa região é necessária para investigar a carga fúngica à qual as pessoas estão expostas.

- A realização de pesquisa de antígeno e anticorpo dos agentes (ELISA) da criptococose nos moradores da microrregião é necessária para detecção precoce de casos assintomáticos ou oligossintomáticos.

- Ampliar o estudo para São Gabriel da Cachoeira.

- Aplicar o conceito *"One Health"* na região, avaliando possíveis casos de criptococose animal.

- Ampliar os estudos da virulência dos isolados de *C. gattii* e *C. neoformans* da microrregião do Rio Negro, para entender o porquê de isolados virulentos não estarem causando doença como ocorreu na ilha de Vancouver no Canadá.

REFERÊNCIAS BIBLIOGRÁFICAS

Acheson, E.S., Galanis, E., Bartlett, K., Mak, S., Klinkenberg, B., 2017. Searching for clues for eighteen years: Deciphering the ecological determinants of Cryptococcus gattii on Vancouver Island, British Columbia. Medical Mycology. https://doi.org/10.1093/mmy/myx037

Alspaugh, J.A., 2015. Virulence mechanisms and *Cryptococcus neoformans* pathogenesis. Fungal Genet. Biol. 78, 55–58. https://doi.org/10.1016/j.fgb.2014.09.004

Alves, G. S., Freire, A. K., Bentes, A. d., Pinheiro, J. F., Souza, J. V., Wanke, B., Matsuura, T.and Jackisch-Matsuura, A. B. (2016), Molecular typing of environmental *Cryptococcus neoformans/C. gattii* species complex isolates from Manaus, Amazonas, Brazil.Mycoses, 59: 509-515. doi:10.1111/myc.12499

Andrade-Silva, L.E., Ferreira-Paim, K., Ferreira, T.B., Vilas-Boas, A., Mora, D.J., Manzato, V.M., Fonseca, F.M., Buosi, K., Andrade-Silva, J., Prudente, B. da S., Araujo, N.E., Sales-Campos, H., da Silva, M.V., Júnior, V.R., Meyer, W., Silva-Vergara, M.L., 2018. Genotypic analysis of clinical and environmental *Cryptococcus neoformans* isolates from Brazil reveals the presence of VNB isolates and a correlation with biological factors. PLoS ONE 13, e0193237. https://doi.org/10.1371/journal.pone.0193237

Barbosa, G., 2013. *Cryptococcus gattii* VGI and *Cryptococcus neoformans* VNI Associated with Wood Decay in Ficus Hollow Trees in Rio de Janeiro, Brazil. British Microbiology Research Journal 3, 106–115. https://doi.org/10.9734/BMRJ/2013/2682

Baró, T., Torres-Rodríguez, J.M., De Mendoza, M.H., Morera, Y., Alía, C., 1998. First identification of autochthonous *Cryptococcus neoformans* var. *gattii* isloated from goats with predominantly severe pulmonary disease in Spain. J. Clin. Microbiol. 36, 458–461.

Baroni, F. de A., Paula, C.R., Silva, E.G. da, Viani, F.C., Rivera, I.N.G., Oliveira, M.T.B. de, Gambale, W., 2006. *Cryptococcus neoformans* strains isolated from church towers in Rio de Janeiro City, RJ, Brazil. Rev. Inst. Med. Trop. Sao Paulo 48, 71–75. https://doi.org//S0036-46652006000200003

Benaducci, T., Sardi, J. de C.O., Lourencetti, N.M.S., Scorzoni, L., Gullo, F.P., Rossi, S.A., Derissi, J.B., de Azevedo Prata, M.C., Fusco-Almeida, A.M., Mendes-Giannini, M.J.S., 2016. Virulence of *Cryptococcus* sp. Biofilms In Vitro and In Vivo using Galleria

mellonella as an Alternative Model. Front Microbiol 7, 290. https://doi.org/10.3389/fmicb.2016.00290

Boekhout, T., Theelen, B., Diaz, M., Fell, J.W., Hop, W.C., Abeln, E.C., Dromer, F., Meyer, W., 2001. Hybrid genotypes in the pathogenic yeast Cryptococcus neoformans. Microbiology (Reading, Engl.) 147, 891–907. https://doi.org/10.1099/00221287-147-4-891

BC Centre for Disease Control [homepage na internet]. Information for Health Professionals: Case definitions of *Cryptococcus gattii* infection and Laboratory Testing [acesso em 12 Dez 2018]. Disponível em: http://www.bccdc.ca/health-info/diseases-conditions/cryptococcus-gattii

BRASIL. Ministério da Saúde. Conselho Nacional de Saúde. Resolução nº 466, de 12 de dezembro de 2012.

Brito-Santos F, Trilles L, Meyer W, Nishikawa MM, Junqueira ACV, Souza AC, Carvalho-Costa FA, Wanke B and Lazera M., 2017. Agents of cryptococcosis indoor dust- New evidences and challenges in Novo Airão municipality, Rio Negro basin, the Brazilian Amazon, in: abstract Book of the 10th International Conference on *Cryptococcus* and Cryptococcosis, Paraná, Foz do Iguaçu, Brasil, p81.

Brito-Santos, F., Barbosa, G.G., Trilles, L., Nishikawa, M.M., Wanke, B., Meyer, W., Carvalho-Costa, F.A., Lazéra, M. dos S., 2015a. Environmental Isolation of Cryptococcus gattii VGII from Indoor Dust from Typical Wooden Houses in the Deep Amazonas of the Rio Negro Basin. PLOS ONE 10, e0115866. https://doi.org/10.1371/journal.pone.0115866

Brito-Santos F, Barbosa GG, Trilles L, Nishikawa MM, Wanke B, Meyer W, Carvalho-Costa FA, Lazéra Mdos S. 2015b. Environmental isolation of *Cryptococcus gattii* VGII from indoor dust from typical wooden houses in the deep Amazonas of the Rio Negro basin. Supplement on line. Infection and Immunity. http://biomedfrontiers.org/inf-2015-11-6/

Brito-Santos F.2013.Isolamento e caracterização molecular dos agentes da criptococose em poeira domiciliar em bairros de Santa Isabel do Rio Negro no estado do Amazonas.Rio de Janeiro. Dissertação. [Mestrado em Medicina Tropical] -Instituto Oswaldo Cruz-FIOCRUZ;2013

Casadevall, A., Freij, J.B., Hann-Soden, C., Taylor, J., 2017. Continental Drift and Speciation of the *Cryptococcus neoformans* and *Cryptococcus gattii* Species Complexes. mSphere 2. https://doi.org/10.1128/mSphere.00103-17

Casadevall, A., Pirofski, L., 2007. Accidental virulence, cryptic pathogenesis, Martians, lost hosts, and the pathogenicity of environmental microbes. Eukaryotic Cell 6, 2169–2174. https://doi.org/10.1128/EC.00308-07

Cavalcanti MAS. 1995. Criptococose e seu agente no Meio Norte, estados do Piauí e Maranhão, Brasil. Tese de Doutorado, Fundação Oswaldo Cruz (Rio de Janeiro) e Universidade Federal do Piauí (Teresina).

Cogliati, M., Puccianti, E., Montagna, M.T., De Donno, A., Susever, S., Ergin, C., Velegraki, A., Ellabib, M.S., Nardoni, S., Macci, C., Trovato, L., Dipineto, L., Rickerts, V., Akcaglar, S., Mlinaric-Missoni, E., Bertout, S., Vencà, A.C.F., Sampaio, A.C., Criseo, G., Ranque, S., Çerikçioğlu, N., Marchese, A., Vezzulli, L., Ilkit, M., Desnos-Ollivier, M., Pasquale, V., Polacheck, I., Scopa, A., Meyer, W., Ferreira-Paim, K., Hagen, F., Boekhout, T., Dromer, F., Varma, A., Kwon-Chung, K.J., Inácio, J., Colom, M.F., 2017. Fundamental niche prediction of the pathogenic yeasts *Cryptococcus neoformans* and *Cryptococcus gattii* in Europe. Environ. Microbiol. 19, 4318–4325. https://doi.org/10.1111/1462-2920.13915

Correa Mdo P, Oliveira EC, Duarte RR, Pardal PP, Oliveira Fde M, Severo LC 1999.. Cryptococcosis in children in the State of Pará, Brazil *Rev Soc Bras Med Trop.* 32(5):505-8,

Costa, S. do P.S.E., Lazéra, M. dos S., Santos, W.R.A., Morales, B.P., Bezerra, C.C.F., Nishikawa, M.M., Barbosa, G.G., Trilles, L., Nascimento, J.L.M. do, Wanke, B., 2009. First isolation of *Cryptococcus gattii* molecular type VGII and *Cryptococcus neoformans* molecular type VNI from environmental sources in the city of Belém, Pará, Brazil. Mem. Inst. Oswaldo Cruz 104, 662–664.

Davis, M.F., Rankin, S.C., Schurer, J.M., Cole, S., Conti, L., Rabinowitz, P., COHERE Expert Review Group, 2017. Checklist for One Health Epidemiological Reporting of Evidence (COHERE). One Health 4, 14–21. https://doi.org/10.1016/j.onehlt.2017.07.001

Diaz, M.R., Boekhout, T., Kiesling, T., Fell, J.W., 2005. Comparative analysis of the intergenic spacer regions and population structure of the species complex of the pathogenic yeast *Cryptococcus neoformans*. FEMS Yeast Res. 5, 1129–1140. https://doi.org/10.1016/j.femsyr.2005.05.005

Ellis, D.H., Pfeiffer, T.J., 1990. Natural habitat of *Cryptococcus neoformans* var. *gattii*. J. Clin. Microbiol. 28, 1642–1644.

Emmons, C.W., 1951. Isolation of *Cryptococcus neoformans* from soil. J. Bacteriol. 62, 685–690.

Engelthaler, D.M., Hicks, N.D., Gillece, J.D., Roe, C.C., Schupp, J.M., Driebe, E.M., Gilgado, F., Carriconde, F., Trilles, L., Firacative, C., Ngamskulrungroj, P., Castaneda, E., Lazera, M. d. S., Melhem, M.S.C., Perez-Bercoff, A., Huttley, G., Sorrell, T.C., Voelz, K., May, R.C., Fisher, M.C., Thompson, G.R., Lockhart, S.R., Keim, P., Meyer, W., 2014. Cryptococcus gattii in North American Pacific Northwest: Whole-Population Genome Analysis Provides Insights into Species Evolution and Dispersal. mBio 5, e01464-14-e01464-14. https://doi.org/10.1128/mBio.01464-14

Ferreira MF.2016.Prevalência de Antigenemia Criptocócica em Pacientes HIV Positivos com Imunossupressão Avançada Acompanhados no Instituto Nacional de Infectologia Evandro Chagas. Rio de Janeiro. Dissertação [Mestrado em Pesquisa Clínica em Doenças Infecciosas] - Instituto de Pesquisa Clínica Evandro Chagas;

Ferreira-Paim, K., Andrade-Silva, L., Fonseca, F.M., Ferreira, T.B., Mora, D.J., Andrade-Silva, J., Khan, A., Dao, A., Reis, E.C., Almeida, M.T.G., Maltos, A., Junior, V.R., Trilles, L., Rickerts, V., Chindamporn, A., Sykes, J.E., Cogliati, M., Nielsen, K., Boekhout, T., Fisher, M., Kwon-Chung, J., Engelthaler, D.M., Lazéra, M., Meyer, W., Silva-Vergara, M.L., 2017. MLST-Based Population Genetic Analysis in a Global Context Reveals Clonality amongst *Cryptococcus neoformans* var. grubii VNI Isolates from HIV Patients in Southeastern Brazil. PLoS Negl Trop Dis 11, e0005223. https://doi.org/10.1371/journal.pntd.0005223

Firacative, C., Duan, S., Meyer, W., 2014. Galleria mellonella Model Identifies Highly Virulent Strains among All Major Molecular Types of *Cryptococcus gattii*. PLoS ONE 9, e105076. https://doi.org/10.1371/journal.pone.0105076

Firacative, C., Lizarazo, J., Illnait-Zaragozí, M.T., Castañeda, E., 2018. The status of cryptococcosis in Latin America. Memórias do Instituto Oswaldo Cruz 113. https://doi.org/10.1590/0074-02760170554

Firacative, C., Trilles, L., Meyer, W., 2012. MALDI-TOF MS enables the rapid identification of the major molecular types within the *Cryptococcus neoformans*/*C. gattii* species complex. PLoS ONE 7, e37566. https://doi.org/10.1371/journal.pone.0037566

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

Franzot, S.P., Salkin, I.F., Casadevall, A., 1999. *Cryptococcus neoformans* var. *grubii*: separate varietal status for *Cryptococcus neoformans* serotype A isolates. J. Clin. Microbiol. 37, 838–840.

Freire, A.K.L., dos Santos Bentes, A., de Lima Sampaio, I., Matsuura, A.B.J., Ogusku, M.M., Salem, J.I., Wanke, B., de Souza, J.V.B., 2012. Molecular characterisation of the causative agents of Cryptococcosis in patients of a tertiary healthcare facility in the state of Amazonas-Brazil: Cryptococcosis in the state of Amazonas-Brazil. Mycoses 55, e145–e150. https://doi.org/10.1111/j.1439-0507.2012.02173.x

Galanis, E., 2010. Epidemiology of *Cryptococcus gattii*, British Columbia, Canada, 1999–2007. Emerging Infectious Diseases. https://doi.org/10.3201/eid1601.090900

García-Rodas, R., Casadevall, A., Rodríguez-Tudela, J.L., Cuenca-Estrella, M., Zaragoza, O., 2011. Cryptococcus neoformans capsular enlargement and cellular gigantism during Galleria mellonella infection. PLoS ONE 6, e24485. https://doi.org/10.1371/journal.pone.0024485

Garcia-Solache, M.A., Izquierdo-Garcia, D., Smith, C., Bergman, A., Casadevall, A., 2013. Fungal virulence in a lepidopteran model is an emergent property with deterministic features. MBio 4, e00100-00113. https://doi.org/10.1128/mBio.00100-13

Guidelines for The Diagnosis, Prevention and Management of Cryptococcal Disease in HIV-Infected Adults, Adolescents and Children: Supplement to the 2016 Consolidated Guidelines on the Use of Antiretroviral Drugs for Treating and Preventing HIV Infection, 2018. , WHO Guidelines Approved by the Guidelines Review Committee. World Health Organization, Geneva.

Hagen, F., Gilgado, F., Boekhout, T., Trilles, L., Castañeda, E., Meyer, W., Ngamskulrungroj, P., 2011. Molecular Typing of the *Cryptococcus neoformans*/*Cryptococcus gattii* Species Complex, in: Kwon-Chung, K.J., Kozel, T.R., Perfect, J.R., Heitman, J., Casadevall, A. (Eds.), Cryptococcus. American Society of Microbiology, pp. 327–357. https://doi.org/10.1128/9781555816858.ch24

Hagen, F., Khayhan, K., Theelen, B., Kolecka, A., Polacheck, I., Sionov, E., Falk, R., Parnmen, S., Lumbsch, H.T., Boekhout, T., 2015. Recognition of seven species in the *Cryptococcus*

gattii/Cryptococcus neoformans species complex. Fungal Genetics and Biology 78, 16–48. https://doi.org/10.1016/j.fgb.2015.02.009

Heitman J, KozelTR, Kwon-Chung J, Perfect JR , Casadeval A 2010.*Cryptococcus*:*From Human Pathogen To Model Yeast.* l (Eds.). ASM Press, Washington, DC.

Igreja, R.P., Santos Lazéra, M.D., Wanke, B., Gutierrez Galhardo, M.C., Kidd, S.E., Meyer, W., 2004. Molecular epidemiology of *Cryptococcus neoformans* isolates from AIDS patients of the Brazilian city, Rio de Janeiro. Medical Mycology 42, 229–238. https://doi.org/10.1080/13693780310001644743

Jarvis, J.N., Lawn, S.D., Vogt, M., Bangani, N., Wood, R., Harrison, T.S., 2009. Screening for cryptococcal antigenemia in patients accessing an antiretroviral treatment program in South Africa. Clin. Infect. Dis. 48, 856–862. https://doi.org/10.1086/597262

Kambugu, A., Meya, D.B., Rhein, J., O'Brien, M., Janoff, E.N., Ronald, A.R., Kamya, M.R., Mayanja-Kizza, H., Sande, M.A., Bohjanen, P.R., Boulware, D.R., 2008. Outcomes of cryptococcal meningitis in Uganda before and after the availability of highly active antiretroviral therapy. Clin. Infect. Dis. 46, 1694–1701. https://doi.org/10.1086/587667

Kidd, S.E., Chow, Y., Mak, S., Bach, P.J., Chen, H., Hingston, A.O., Kronstad, J.W., Bartlett, K.H., 2007. Characterization of Environmental Sources of the Human and Animal Pathogen *Cryptococcus gattii* in British Columbia, Canada, and the Pacific Northwest of the United States. Applied and Environmental Microbiology 73, 1433–1443. https://doi.org/10.1128/AEM.01330-06

Kidd, S.E., Hagen, F., Tscharke, R.L., Huynh, M., Bartlett, K.H., Fyfe, M., Macdougall, L., Boekhout, T., Kwon-Chung, K.J., Meyer, W., 2004. 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 101, 17258–17263.

Kishi, K., Homma, S., Kurosaki, A., Kohno, T., Motoi, N., Yoshimura, K., 2006. Clinical features and high-resolution CT findings of pulmonary cryptococcosis in non-AIDS patients. Respir Med 100, 807–812. https://doi.org/10.1016/j.rmed.2005.09.017

Klepeis, N.E., Nelson, W.C., Ott, W.R., Robinson, J.P., Tsang, A.M., Switzer, P., Behar, J.V., Hern, S.C., Engelmann, W.H., 2001. The National Human Activity Pattern Survey

(NHAPS): a resource for assessing exposure to environmental pollutants. J Expo Anal Environ Epidemiol 11, 231–252. https://doi.org/10.1038/sj.jea.7500165

Kwon-Chung, K., Bennett, J.E., 1992. Medical mycology. Lea & Febiger, Philadelphia.

Kwon-Chung, K.J., 1976. A new species of Filobasidiella, the sexual state of *Cryptococcus neoformans* B and C serotypes. Mycologia 68, 943–946.

Kwon-Chung, K.J., 1975. A new genus, *Filobasidiella*, the perfect state of *Cryptococcus neoformans*. Mycologia 67, 1197–1200.

Kwon-Chung, K.J., Bennett, J.E., Rhodes, J.C., 1982. Taxonomic studies on *Filobasidiella* species and their anamorphs. Antonie Van Leeuwenhoek 48, 25–38.

Kwon-Chung, K.J., Bennett, J.E., Wickes, B.L., Meyer, W., Cuomo, C.A., Wollenburg, K.R., Bicanic, T.A., Castañeda, E., Chang, Y.C., Chen, J., Cogliati, M., Dromer, F., Ellis, D., Filler, S.G., Fisher, M.C., Harrison, T.S., Holland, S.M., Kohno, S., Kronstad, J.W., Lazera, M., Levitz, S.M., Lionakis, M.S., May, R.C., Ngamskulrongroj, P., Pappas, P.G., Perfect, J.R., Rickerts, V., Sorrell, T.C., Walsh, T.J., Williamson, P.R., Xu, J., Zelazny, A.M., Casadevall, A., 2017. The Case for Adopting the "Species Complex" Nomenclature for the Etiologic Agents of Cryptococcosis. mSphere 2. https://doi.org/10.1128/mSphere.00357-16

Kwon-Chung, K.J., Boekhout, T., Fell, J.W., Diaz, M., 2002. (1557) Proposal to Conserve the Name *Cryptococcus gattii* against C. hondurianus and C. bacillisporus (Basidiomycota, Hymenomycetes, Tremellomycetidae). Taxon 51, 804. https://doi.org/10.2307/1555045

Kwon-Chung, K.J., Varma, A., 2006. Do major species concepts support one, two or more species within *Cryptococcus neoformans*? FEMS Yeast Research 6, 574–587. https://doi.org/10.1111/j.1567-1364.2006.00088.x

Kurtzman CP, The yeasts (5th Ed.): A taxonomic study: 04-2011 ;Langue : Anglais ;Ouvrage 2354 p ; Vol 3 ; Cap138 *Cryptococcus.*

Lacaz, C.S., Porto, E., Martins, J.E.C., Heins-Vaccari, E.M., Takahashi De Melo, N., 2002. [NO TITLE AVAILABLE]. Revista do Instituto de Medicina Tropical de São Paulo 44, 297–298. https://doi.org/10.1590/S0036-46652002000500013

Lazera, M.S., Cavalcanti, M.A.S., Trilles, L., Nishikawa, M.M., Wanke, B., 1998. *Cryptococcus neoformans* var. *gattii*—evidence for a natural habitat related to decaying wood in a pottery tree hollow. Medical Mycology 36, 119–122.

Lazera, M.S., Cavalcanti, M.S., Londero, A.T., Trilles, L., Nishikawa, M.M., Wanke, B., 2000. Possible primary ecological niche of *Cryptococcus neoformans*. Medical Mycology 38, 379–383.

Lazera, M.S., Pires, F.D.A., Camillo-Coura, L., Nishikawa, M.M., Bezerra, C.C.F., Trilles, L., Wanke, B., 1996. Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees. Journal of medical and veterinary mycology 34, 127–131.

Liechty, C.A., Solberg, P., Were, W., Ekwaru, J.P., Ransom, R.L., Weidle, P.J., Downing, R., Coutinho, A., Mermin, J., 2007. Asymptomatic serum cryptococcal antigenemia and early mortality during antiretroviral therapy in rural Uganda. Trop. Med. Int. Health 12, 929–935. https://doi.org/10.1111/j.1365-3156.2007.01874.x

Lin, X., Heitman, J., 2006. The Biology of the *Cryptococcus neoformans* Species Complex. Annual Review of Microbiology 60, 69–105. https://doi.org/10.1146/annurev.micro.60.080805.142102

Litvintseva, A.P., Thakur, R., Vilgalys, R., Mitchell, T.G., 2006. Multilocus sequence typing reveals three genetic subpopulations of Cryptococcus neoformans var. grubii (serotype A), including a unique population in Botswana. Genetics 172, 2223–2238. https://doi.org/10.1534/genetics.105.046672

Longley, N., Jarvis, J.N., Meintjes, G., Boulle, A., Cross, A., Kelly, N., Govender, N.P., Bekker, L.-G., Wood, R., Harrison, T.S., 2016. Cryptococcal Antigen Screening in Patients Initiating ART in South Africa: A Prospective Cohort Study. Clin. Infect. Dis. 62, 581–587. https://doi.org/10.1093/cid/civ936

MacDougall, L., Kidd, S.E., Galanis, E., Mak, S., Leslie, M.J., Cieslak, P.R., Kronstad, J.W., Morshed, M.G., Bartlett, K.H., 2007. Spread of *Cryptococcus gattii* in British Columbia, Canada, and detection in the Pacific Northwest, USA. Emerging infectious diseases 13, 42.

Machado Siqueira, L.P., Favero Gimenes, V.M., de Freitas, R.S., de Souza Carvalho Melhem, M., Bonfietti, L.X., da Silva, A.R., Souza Santos, L.B., Motta, A.L., Rossi, F., Benard, G., de Almeida, J.N., 2018. Evaluation of Vitek MS^{TM} for the differentiation of Cryptococcus neoformans and *Cryptococcus gattii* genotypes. J. Clin. Microbiol. https://doi.org/10.1128/JCM.01282-18

Martins, L.M.S., Wanke, B., Lazéra, M. dos S., Trilles, L., Barbosa, G.G., Macedo, R.C.L. de, Cavalcanti, M. do A.S., Eulálio, K.D., Castro, J.A.F. de, Silva, A.S. da, others, 2011. Genotypes of *Cryptococcus neoformans* and *Cryptococcus gattii* as agents of endemic cryptococcosis in Teresina, Piauí (northeastern Brazil). Memorias do Instituto Oswaldo Cruz 106, 725–730.

Martins LMS, Lazéra MS, Leal MJS, Cavalcanti MAS, Eulálio KD, Wanke B 2003. Infecção mista por sorotipo A e B de *Cryptococcus neoformans* como causa de meningoencefalite em criança de Itaituba-PA: relato de caso. In: XXXIX Congresso Sociedade Brasileira de Medicinal Tropical, 2003, Belém. Revista da Sociedade Brasileira de Medicinal Tropical.. v.36. p.216 – 216

Matsumoto, M.T., Fusco-Almeida, A.M., Baeza, L.C., Melhem, M. de S.C., Medes-Giannini, M.J.S., 2007. Genotyping, serotyping and determination of mating-type of *Cryptococcus neoformans* clinical isolates from São Paulo State, Brazil. Revista do Instituto de Medicina Tropical de São Paulo 49, 41–47.

McTaggart, L.R., Lei, E., Richardson, S.E., Hoang, L., Fothergill, A., Zhang, S.X., 2011. Rapid Identification of *Cryptococcus neoformans* and *Cryptococcus gattii* by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry. Journal of Clinical Microbiology 49, 3050–3053. https://doi.org/10.1128/JCM.00651-11

Meyer W, Gilcagdo F, Ngamskulrungroj P, Trilles L, Hange F, Castañeda, Boekhout T 2011. Molecular Typing of the *Cryptococcus neoformans*/*Cryptococcus gattii* Species complex; in: Cryptococcus: From Human Pathogen to Model Yeast (eds) J.Heitman *et al.*. ed ASM Press,Washington, DC.

Meyer, W., Aanensen, D.M., Boekhout, T., Cogliati, M., Diaz, M.R., Esposto, M.C., Fisher, M., Gilgado, F., Hagen, F., Kaocharoen, S., Litvintseva, A.P., Mitchell, T.G., Simwami, S.P., Trilles, L., Viviani, M.A., Kwon-Chung, J., 2009. Consensus multi-locus sequence typing scheme for *Cryptococcus neoformans* and *Cryptococcus gattii*. Medical Mycology 47, 561–570. https://doi.org/10.1080/13693780902953886

Meyer, W., Castañeda, A., Jackson, S., Huynh, M., Castañeda, E., Group, I.C.S., others, 2003. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. Emerging infectious diseases 9, 189.

Micol, R., Lortholary, O., Sar, B., Laureillard, D., Ngeth, C., Dousset, J.-P., Chanroeun, H., Ferradini, L., Guerin, P.J., Dromer, F., Fontanet, A., 2007. Prevalence, determinants of positivity, and clinical utility of cryptococcal antigenemia in Cambodian HIV-infected patients. J. Acquir. Immune Defic. Syndr. 45, 555–559. https://doi.org/10.1097/QAI.0b013e31811ed32c

Mylonakis, E., Moreno, R., El Khoury, J.B., Idnurm, A., Heitman, J., Calderwood, S.B., Ausubel, F.M., Diener, A., 2005. Galleria mellonella as a model system to study *Cryptococcus neoformans* pathogenesis. Infect. Immun. 73, 3842–3850. https://doi.org/10.1128/IAI.73.7.3842-3850.2005

Ngamskulrungroj, P., Chang, Y., Roh, J., Kwon-Chung, K.J., 2012. Differences in Nitrogen Metabolism between *Cryptococcus neoformans* and *C. gattii*, the Two Etiologic Agents of Cryptococcosis. PLoS ONE 7, e34258. https://doi.org/10.1371/journal.pone.0034258

Nielsen, K., De Obaldia, A.L., Heitman, J., 2007. *Cryptococcus neoformans* Mates on Pigeon Guano: Implications for the Realized Ecological Niche and Globalization. Eukaryotic Cell 6, 949–959. https://doi.org/10.1128/EC.00097-07

Nishikawa, M.M., Lazera, M.S., Barbosa, G.G., Trilles, L., Balassiano, B.R., Macedo, R.C.L., Bezerra, C.C.F., Perez, M.A., Cardarelli, P., Wanke, B., 2003. Serotyping of 467 *Cryptococcus neoformans* Isolates from Clinical and Environmental Sources in Brazil: Analysis of Host and Regional Patterns. Journal of Clinical Microbiology 41, 73–77. https://doi.org/10.1128/JCM.41.1.73-77.2003

Nishikawa, M.M., Sant'Anna, O.D., Lazera, M.S., Wanke, B., 1996. Use of D-proline assimilation and CGB medium for screening Brazilian *Cryptococcus neoformans* isolates. J. Med. Vet. Mycol. 34, 365–366.

Osazuwa, F., Dirisu, J.O., Okuonghae, P.E., Ugbebor, O., 2012. Screening for cryptococcal antigenemia in anti-retroviral naïve AIDS patients in Benin city, Nigeria. Oman Med J 27, 228–231. https://doi.org/10.5001/omj.2012.51

Passoni, L.F.C., Wanke, B., Nishikawa, M.M., Lazéra, M.S., 1998. *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 36, 305–311.

Pongsai, P., Atamasirikul, K., Sungkanuparph, S., 2010. The role of serum cryptococcal antigen screening for the early diagnosis of cryptococcosis in HIV-infected patients with different

ranges of CD4 cell counts. J. Infect. 60, 474–477. https://doi.org/10.1016/j.jinf.2010.03.015

Posteraro, B., Vella, A., Cogliati, M., De Carolis, E., Florio, A.R., Posteraro, P., Sanguinetti, M., Tortorano, A.M., 2012. Matrix-assisted laser desorption ionization-time of flight mass spectrometry-based method for discrimination between molecular types of *Cryptococcus neoformans* and *Cryptococcus gattii*. J. Clin. Microbiol. 50, 2472–2476. https://doi.org/10.1128/JCM.00737-12

Prado, M., Silva, M.B. da, Laurenti, R., Travassos, L.R., Taborda, C.P., 2009. Mortality due to systemic mycoses as a primary cause of death or in association with AIDS in Brazil: a review from 1996 to 2006. Mem. Inst. Oswaldo Cruz 104, 513–521.

Rajasingham, R., Smith, R.M., Park, B.J., Jarvis, J.N., Govender, N.P., Chiller, T.M., Denning, D.W., Loyse, A., Boulware, D.R., 2017. Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis. The Lancet Infectious Diseases 17, 873–881. https://doi.org/10.1016/S1473-3099(17)30243-8

Ramadan, M.F., Kroh, L.W., Mörsel, J.-T., 2003. Radical scavenging activity of black cumin (Nigella sativa L.), coriander (Coriandrum sativum L.), and niger (Guizotia abyssinica Cass.) crude seed oils and oil fractions. J. Agric. Food Chem. 51, 6961–6969. https://doi.org/10.1021/jf0346713

Raso, T.F., Werther, K., Miranda, E.T., Mendes-Giannini, M.J.S., 2004. Cryptococcosis outbreak in psittacine birds in Brazil. Medical Mycology 42, 355–362. https://doi.org/10.1080/13693780410001712061

Rocha, D.F.S., Cruz, K.S., Santos, C.S. da S., Menescal, L.S.F., Neto, J.R. da S., Pinheiro, S.B., Silva, L.M., Trilles, L., Braga de Souza, J.V., 2018. MLST reveals a clonal population structure for *Cryptococcus neoformans* molecular type VNI isolates from clinical sources in Amazonas, Northern-Brazil. PLoS ONE 13, e0197841. https://doi.org/10.1371/journal.pone.0197841

Sales KK.B, Neve MA, Brito-Santos F, Trilles L, Lazera M, Fortes ST.2017. Cryptococcus gattii VGII associated with termites trial and nest in Roraima–Brazil.In:abstract Book of the 10th International Conference on *Cryptococcus* and Cryptococcosis, Paraná, Foz do Iguaçu, Brasil.

Santos, W.R.A. dos, Meyer, W., Wanke, B., Costa, S.P.S.E., Trilles, L., Nascimento, J.L.M. do, Medeiros, R., Morales, B.P., Bezerra, C. de C.F., Macêdo, R.C.L. de, Ferreira, S.O.,

Barbosa, G.G., Perez, M.A., Nishikawa, M.M., Lazéra, M. dos S., 2008. Primary endemic *Cryptococcosis gattii* by molecular type VGII in the state of Pará, Brazil. Mem. Inst. Oswaldo Cruz 103, 813–818.

Santos L.O. 2000. Criptococose no estado do Amazonas: estudo de 75 casos diagnosticados na Fundação de Medicina Tropical/FMT/IMTM, Manáus, AM (1988-1998). Tese de Mestrado, Instituto Oswaldo Cruz, FIOCRUZ.

Soares EA. 2015. Mortalidade por criptococose no Brasil (2000 a 2012). Rio de Janeiro. Dissertação [Mestrado Profissional em Epidemiologia em Saúde Pública] -Escola Nacional de Saúde Pública.

Souto, A.C.P., Bonfietti, L.X., Ferreira-Paim, K., Trilles, L., Martins, M., Ribeiro-Alves, M., Pham, C.D., Martins, L., dos Santos, W., Chang, M., Brito-Santos, F., Santos, D.C.S., Fortes, S., Lockhart, S.R., Wanke, B., Melhem, M.S.C., Lazéra, M.S., Meyer, W., 2016. Population Genetic Analysis Reveals a High Genetic Diversity in the Brazilian *Cryptococcus gattii* VGII Population and Shifts the Global Origin from the Amazon Rainforest to the Semi-arid Desert in the Northeast of Brazil. PLOS Neglected Tropical Diseases 10, e0004885. https://doi.org/10.1371/journal.pntd.0004885

Temfack, E., Bigna, J.J., Luma, H.N., Spijker, R., Meintjes, G., Jarvis, J.N., Dromer, F., Harrison, T., Cohen, J.F., Lortholary, O., 2018. Impact of routine cryptococcal antigen screening and targeted pre-emptive fluconazole therapy in antiretroviral naive HIV-infected adults with less than 100 CD4 cells/μL: a systematic review and meta-analysis. Clin. Infect. Dis. https://doi.org/10.1093/cid/ciy567

Thomaz, D.Y., Grenfell, R.C., Vidal, M.S.M., Giudice, M.C., Del Negro, G.M.B., Juliano, L., Benard, G., de Almeida Júnior, J.N., 2016. Does the Capsule Interfere with Performance of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for Identification of *Cryptococcus neoformans* and *Cryptococcus gattii*? J. Clin. Microbiol. 54, 474–477. https://doi.org/10.1128/JCM.02635-15

Trevijano-Contador, N., Herrero-Fernández, I., García-Barbazán, I., Scorzoni, L., Rueda, C., Rossi, S.A., García-Rodas, R., Zaragoza, O., 2015. *Cryptococcus neoformans* induces antimicrobial responses and behaves as a facultative intracellular pathogen in the non mammalian model Galleria mellonella. Virulence 6, 66–74. https://doi.org/10.4161/21505594.2014.986412

Trilles, L., Lazéra, M. dos S., Wanke, B., Oliveira, R.V., Barbosa, G.G., Nishikawa, M.M., Morales, B.P., Meyer, W., 2008. Regional pattern of the molecular types of *Cryptococcus neoformans* and *Cryptococcus gattii* in Brazil. Memórias do Instituto Oswaldo Cruz 103, 455–462.

Wajanga, B.M., Kalluvya, S., Downs, J.A., Johnson, W.D., Fitzgerald, D.W., Peck, R.N., 2011. Universal screening of Tanzanian HIV-infected adult inpatients with the serum cryptococcal antigen to improve diagnosis and reduce mortality: an operational study. J Int AIDS Soc 14, 48. https://doi.org/10.1186/1758-2652-14-48

Yanai, A.M., Nogueira, E.M., de Alencastro Graça, P.M.L., Fearnside, P.M., 2017. Deforestation and Carbon Stock Loss in Brazil's Amazonian Settlements. Environ Manage 59, 393–409. https://doi.org/10.1007/s00267-016-0783-2

APÊNDICE

APÊNDICE A – ARTIGO 1 "Population Genetic Analysis Reveals a High Genetic Diversity in the Brazilian *Cryptococcus gattii* VGII Population and Shifts the Global Origin from the Amazon Rainforest to the Semi-arid Desert in the Northeast of Brazil."

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Population Genetic Analysis Reveals a High Genetic Diversity in the Brazilian *Cryptococcus gattii* VGII Population and Shifts the Global Origin from the Amazon Rainforest to the Semi-arid Desert in the Northeast of Brazil

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Abstract

Cryptococcus neoformans and *Cryptococcus gattii* are responsible globally for almost one million cryptococcosis cases yearly, mostly in immunocompromised patients, such as those living with HIV. Infections due to *C. gattii* have mainly been described in tropical and subtropical regions, but its adaptation to temperate regions was crucial in the species evolution and highlighted the importance of this pathogenic yeast in the context of disease. *Cryptococcus gattii* molecular type VGII has come to the forefront in connection with an on-going emergence in the Pacific North West of North America. Taking into account that previous work pointed towards South America as an origin of this species, the present work aimed to assess the genetic diversity within the Brazilian *C. gattii* VGII population in order to gain new insights into its origin and global dispersal from the South American continent using the ISHAM consensus MLST typing scheme. Our results corroborate the finding that the Brazilian *C. gattii* VGII population is highly diverse. The diversity is likely due to recombination generated from sexual reproduction, as evidenced by the presence of both mating types in clinical and environmental samples. The data presented herein strongly supports the emergence of highly virulent strains from ancestors in the Northern regions of Brazil, Amazonia

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and the Northeast. Numerous genotypes represent a link between Brazil and other parts of the world reinforcing South America as the most likely origin of the *C. gattii* VGII subtypes and their subsequent global spread, including their dispersal into North America, where they caused a major emergence.

Author Summary

Cryptococcus neoformans and *Cryptococcus gattii* are fungal agents responsible globally for almost one million cryptococcosis cases yearly, mostly in immunocompromised patients, such as those living with HIV. Cryptococcosis is a life-threatening mycosis, frequently causing meningoencephalitis. Infections due to *C. gattii* were originally described in tropical and subtropical regions, but its adaptation to temperate regions was highlighted by the emergence in the Pacific North West of North America by *C. gattii* molecular type VGII. The present work aimed to assess the genetic diversity within the Brazilian *C. gattii* VGII population to gain new insights into its origin and global dispersal from the South American continent using the ISHAM MLST consensus typing scheme. Our results corroborate that the Brazilian *C. gattii* VGII population is highly diverse, and strongly supports the emergence of highly virulent strains from ancestors in the Northern regions of Brazil. Numerous genotypes represent a link between Brazil and other parts of the world, and the isolates from the transitional ecological area in Northeast Brazil are the most likely ancestor lineages, translocating from caatinga/cerrado by adapting progressively throughout Amazonia in South America, and spread to the North American Pacific Northwest region and other parts of the world on multiple occasions.

Introduction

Cryptococcosis is a life-threatening mycosis with high lethality rates, especially in underdeveloped countries [1]. Infection occurs via the respiratory route by inhalation of infectious propagules (desiccated yeast cells or basidiospores) of *Cryptococcus neoformans* and *C. gattii*, frequently spreading to the central nervous system causing meningoencephalitis, with a lethality rate of up to 70% within three months after diagnosis [1, 2]. *C. neoformans* is a cosmopolitan and primarily opportunistic agent, comprising the major molecular types VNI, VNII (VNB), VNIII and VNIV. By contrast, *C. gattii* infects mainly otherwise immunocompetent hosts, although a previous study suggests that some immune profile deficiency not detected by routine tests may predispose immunocompetent individuals to meningoencephalitis by *C. gattii* [3]. Besides the well-known outbreak in North America, *C. gattii* infections occur in large areas of the Amazon region and in the semi-arid Northeast region of Brazil [4, 5, 6, 7], being the major molecular types VGI, VGII, VGIII and VGIV. The molecular types of both species have been recently described as new species [8]. To enable a clear connection to previous published work this report maintains the two species concept with its molecular type-based nomenclature.

C. gattii VGI and VGIII had been the primary cause of human and animal infections until 1999 in North America, when isolates of the molecular type VGII were reported as the cause of an outbreak affecting hundreds of healthy humans and animals in British Columbia, Canada. This outbreak lineage subsequently spread to the Pacific Northwest (PNW) of the USA in the following years [9]. Alternatively, based on one clinical case reported from the 1970s, which

described a VGII isolate NIH444 from Seattle (USA), it could be suggested that the VGII outbreak lineage was already present in the temperate region several decades before its emergence on Vancouver Island [10]. However, the genotype of this isolate is very different from the Vancouver Island outbreak lineages [11] making in unlikely to be the source of the Vancouver Island outbreak.

Later on, PCR-fingerprinting, Amplification Fragment Length Polymorphism (AFLP) analysis and Multilocus Sequence Typing (MLST) identified three distinct clonal lineages (subtypes) responsible for the majority of cases in the PNW [9, 12]: VGIIa, the most common genotype, VGIIb, the less common [13], and VGIIc, a subsequently identified genotype with a confined geographic distribution [12]. Following this, the ISHAM working group of the International Society for Human and Animal Mycology (ISHAM) on genotyping of *C. neoformans* and *C. gattii* proposed a standardized MLST scheme, using six housekeeping genes and the IGS1 region as method of choice for strain subtyping to obtain comparable subtyping results worldwide [14]. MLST confirmed the same three major genotypes within North America [11, 15].

The emergence of infections by *C. gattii* VGII in temperate regions initiated a pursuit of the origin of the Vancouver Island outbreak strains. One hypothesis is the occurrence of same-sex mating from an Australasian population, giving rise to a virulent genotype, which was subsequently dispersed [16, 17]. However, a study using coalescence gene genealogy, phylogenetic and recombination analysis suggested that it may alternatively have emerged from a highly-recombining *C. gattii* population in the native rainforest of Northern Brazil, subsequently dispersed out of the original tropical area, reaching North America [18]. Similarly, two recent population genetic analyses using Single Nucleotide Polymorphism (SNP) analysis based on whole genome sequence data provided additional evidence that the PNW strains originated from South America [11, 16].

Based on the above mentioned findings the present work aimed to assess the genetic variability within the Brazilian VGII population and to gain new insights related to the population structure, its origin and global dispersal from the South American continent.

Methods

Isolates

One hundred and forty five Brazilian clinical and environmental isolates of the major *C. gattii* molecular type VGII identified by *URA5*-RFLP analysis [19] stored in the Culture Collection of Pathogenic Fungi, at the Oswaldo Cruz Foundation, Rio de Janeiro, and in the Research Collection of the Adolf Lutz Institute, São Paulo, Brazil were studied retrospectively. In addition to the Brazilian isolates, 42 published sequence types (STs) from Brazil and other countries, representing all previously published VGII sequence types, maintained in the MLST database (mlst.mycologylab.org), were used for comparison, in order to place the Brazilian population in an international context. For isolate information, see S1 Table.

MultiLocus Sequence Typing (MLST)

The molecular subtypes and the genetic diversity of the Brazilian *C. gattii* VGII isolates were investigated using the ISHAM MLST consensus scheme for *C. neoformans* and *C. gattii* [14]. Seven unlinked genetic loci were amplified, including the genes *CAP59*, *GPD1*, *LAC1*, *PLB1*, *SOD1* and *URA5* and the IGS1 region, using the published PCR conditions for all seven loci [14]. The sequences were manually edited using the software Sequencher 5.3 (Gene Codes Corporation, MI, USA) and aligned using MEGA 6.06 [20]. The allele types and the sequence types (ST) were identified via sequence alignments against the *C. gattii* MLST database

available at http://mlst.mycologylab.org/. The sequences of all newly identified allele types have been submitted to the *C. gattii* MLST database and GenBank.

Phylogenetic analyses

In order to infer the phylogenetic relationships of the isolates, the best evolutionary model for concatenated sequences of the seven loci was selected using the software jModelTest 2.1.7 [21, 22] applying the corrected Akaike Information Criterion (AIC) and/or Bayesian information criteria (BIC). The model K80 + I + G with Ti/Tv: 3.4548 and gamma shape 0.4430 [23] was the best model for the concatenated dataset, which was then used in the software MEGA 6.06 [24] to construct an unrooted Maximum Likelihood (ML) phylogenetic tree. In addition, the dataset was submitted to Neighbour Joining (NJ) analysis based on the K80 [23] model and Maximum Parsimony (MP) based on the nucleotide substitution model and using the Subtree-Pruning-Regrafting (SPR) algorithm [11]. For the ML and MP methods, all sites were included in the analysis while for NJ, all positions containing alignment gaps were eliminated. Bootstrap analysis using 1,000 replicates was used to estimate support for the identified clades of the concatenate dataset in all analysis.

The minimum spanning tree using the goeBURST algorithm in the PHILOVIZ software (http://www.phyloviz.net/wiki/) [25] was generated from concatenated sequence regions to visualize the relatedness of the *C. gattii* isolates with their region of origin. The diagrams show where the ST differs in the single locus variant (SLV), double locus variant (DLV), and triple locus variant (TLV), respectively. A clonal complex (CC) concept was adopted when a SLV linkage with the founder ST was found [24, 25].

Population structure

In order to better understand the correct number of *C. gattii* VGII populations (*K*) that were geographically homogeneous and maximally differentiated from each other, and to evaluate the presence of immigrant individuals with respect to their geographical population, we used a Bayesian statistical model [26], which calculates the membership coefficient to each of the population using the software STRUCTURE 2.3.4, available at http://pritchardlab.stanford.edu/structure.html [27]. Twenty runs were performed for each value of the number of populations (*K*) ranging from 1 to 10. Each run consisted of Markov-chain Monte Carlo (MCMC) simulations of 1,000,000 interactions with a burn-in period of 100,000 generations. The model selected was Admixture model that takes into account the presence of migrants in the population. The actual number of *K* was calculated using the average and standard deviation of each *K* using the *ad hoc* statistic of the software Structure Harvester available at http://taylor0.biology.ucla.edu/structureHarvester/ [28]. The results of the coefficients of the optimal *K* were graphed using the software Clumpp version 1.1.2 available at https://web.stanford.edu/group/rosenberglab/clumpp.html [29] and Structure plot [30].

Recombination, clonality and nucleotide diversity analysis

The software DnaSP 5.10 (http://www.ub.edu/dnasp/) [31] was used to analyse the haplotype diversity (Hd) and nucleotide diversities. The presence of recombination in the dataset was checked by phylogenetic compatibilities of nearby polymorphic sites along single and concatenated sequences in the software SplitsTree v. 4.13.1 (http://www.splitstree.org/) [32]. Recombination events can be visualized by the formation of parallelograms between the neighbours using the reticulated algorithm NeighborNet. The Pairwise Homoplasy Index (PHY) test implemented in SplitsTree v. 4.13.1 and the pairwise linkage disequilibrium (D) available in the software DnaSP v. 5.10 were also used to detect the presence of recombination. To perform

the recombination analysis, the optimal molecular evolutionary model per gene was selected in the software jModelTest 2.1.7 as described above for the phylogenetic analysis and applied in the software SplitsTree v. 4.13.1. Thus, the parameters were used as follows: *CAP59*: K80 + I, Ti/Tv: 31.3584, and pinv: 0.9740; *GPD1*: K80, Ti/Tv: 2.7548; IGS1: F81; FA (0.2655), FC (0.1533), FG (0.2906), FT (0.2903); *LAC1*: K80, Ti/Tv: 3.0044; *SOD1*: K80 + I + G, Ti/Tv: 1.8129, pinv: 0.9360, and alpha: 0.7400; *URA5*: JC; *PLB1*: F81, FA (0.2336), FC (0.2036), FG (0.2908), and FT (0.2725).

The standard index of association (I_A) is a measure of linkage disequilibrium of genotypes and/or population [33]. This test checks the null hypothesis of linkage equilibrium and $p < 0.05$ indicates that the null hypotheses of linkage equilibrium should be rejected, which means that the population is under clonal reproduction. In this study we applied also the standardized I_A (I_A^S) with 10,000 randomizations available in the program LIAN 3.5 (http://guanine.evolbio.mpg.de/cgi-bin/lian/lian.cgi.pl) using both the parametric method and the Monte Carlo simulation for the concatenated dataset to infer the presence of linkage disequilibrium.

Mating typing

The mating type was characterized by PCR of the pheromone genes using primers specific for MATalpha, MFalfaU (5'TTCACTGCCATCTTCACCACC 3') in combination with MFalfaL (5'TCTAGGCGATGACACAAAGGG 3'); and for MAT**a** JOHE9787 (5' ACACCGCCTGT TACAATGGAC 3') in combination with JOHE9788 (5' CAGCGTTTGAAGATGGACTTT 3') [34]. Amplifications of the pheromone genes MATalpha and MAT**a** were performed independently, in a final volume of 50μL containing 50 ng of DNA, 1X PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl—Invitrogen], 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Invitrogen), 2 mM magnesium cloride, 2.5 U Taq DNA polymerase (Invitrogen), and 50 ng of each primer. The amplification was carried out in a thermocycler (Eppendorf mastercycler gradient, California, USA) at 95°C for 3-min initial denaturation, 30 cycles at 94°C for 1 min, annealing at 57.5°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The unique fragment corresponding to each mating type was visualized after 3% agarose gel electrophoresis at 100 V.

Results

Genetic variability and regional distribution in Brazil

A total of 145 *C. gattii* VGII isolates, including 127 clinical and 18 environmental isolates, collected between 1989 and 2010 in 4 out of the 5 Brazilian regions: 1. Northeast (n = 39), including isolates from Piauí (PI) and Bahia (BA); 2. North (n = 38), including isolates from Pará (PA), Amazonas (AM) and Roraima (RR); 3. Southeast (n = 59), including isolates from Rio de Janeiro (RJ) and São Paulo (SP); and 4. Central-West (n = 9), including isolates from Mato Grosso do Sul (MS). No VGII isolates were collected in the South region (Rio Grande do Sul, Santa Catarina and Paraná) of the country. The individual isolate data are in S1 Table.

MLST analysis identified 24 allele types for the *CAP59* locus, 13 for *GPD1*, nine for *LAC1*, 11 for *PLB1*, 38 for *SOD1*, eight for *URA5* and 34 for the IGS1 region. Based on the combined analysis of the seven loci, a total of 81 sequence types were observed (Table 1, S1 Table), with 100 polymorphic sites detected in 4,186 sites analysed. The haplotype diversity (Hd) of all strains was equal to 0.978, revealing a high genetic variability among the Brazilian *C. gattii* VGII strains. All Brazilian regions showed high haplotype diversity, with the highest one found in the Northeast (NE) region (Hd = 0.981) with 31 STs, and the lowest one in the Central-West (CW) region (Hd = 0.889) with five STs (Table 1).

Table 1. Characteristics of the studied Brazilian regions.

Region (studied States*)	# of Isolates	# of Sequence Types	# of Polymorphic Sites	Haplotype Diversity (Hd)	Nucleotide Diversity (π)
Central-West (MS)	9	5	34	0.889	0.00353
Northeast (PI, BA)	39	31	68	0.981	0.00328
North (AM, PA, RR)	38	20	55	0.905	0.00296
Southeast (RJ, SP)	59	33	68	0.944	0.00334
Total	145	81¶	100¶	0.978	0.00349

* MS, Mato Grosso do Sul; PI, Piauí; BA, Bahia; AM, Amazonas; PA, Pará; RR, Roraima; RJ, Rio de Janeiro; SP, São Paulo.

¶ The repeated sequence types from different regions are not included in the total number.

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The genetic relationships of the obtained MLST genotypes may be separated into two main groups, the first one with 35 STs, including the VGIIa sub-genotype (ST20, major outbreak genotype on Vancouver Island), and the second one with 46 STs, including the VGIIb sub-genotype (ST7, globally present and minor outbreak genotype on Vancouver Island). No isolates of the third North American sub-genotype VGIIc (ST6) were identified in Brazil, but a closely related sequence type ST272 (strain 438BP) from MS, the CW region of Brazil was identified (Fig 1).

Among the 81 sequence types identified in Brazil, 54 are represented by a single isolate. The most frequent subtype, ST40, accounts for 13 isolates found in the Central-West (CW) and Southeast (SE) regions, followed by ST20 (VGIIa) and ST5, which contained nine and seven isolates, respectively (S1 Table). In general, the different regions harboured different genotypes. The majority of the sequence types (n = 73) are unique for each of the Brazilian regions analysed. Only six sequence types were identified in more than one region (ST20, ST28, ST40, ST133, ST185 and ST287) (S1 Table, Fig 2A).

The regional distribution of the STs within the regions was also evaluated with the goeBURST analysis including all 145 *C. gattii* isolates from this study, and another nine different Brazilian STs obtained from previously published data (S1 Table, Fig 2A). In this analysis, 9 clonal complexes (CC) were identified (e.g. 9 groups presenting SLV). Clonal complex CC 278 is composed of the clinical sequence type ST278, isolated from a patient in Piauí, ST7 isolated from clinical and environmental samples from Amazonia, and ST124, isolated from clinical and environmental samples from Piauí state. ST278 seems to play an important role in the epidemiological distribution of *C. gattii* due to its link with the less virulent ST7 (VGIIb). In addition, three main groups are linked to CC 278: 1) ST301 and all its descendants, mainly present in the SE region of Brazil, which is a triple-locus variant (TLV) (IGS1, *PLB1*, *URA5* allele) of ST124; 2) ST277 and all its descendants, mainly present in the North (N) region of Brazil, which is a double-locus variant (DLV) (*GPD1*, *PLB1*) of ST7 (VGIIb); and 3) ST281 and all its descendants, a mixed group of strains from all regions of the country, which is a TLV (IGS1, *PLB1*, *SOD1* allele) of ST278. The important role played by ST278 isolated from the semi-arid NE region was confirmed after addition of 34 STs from other countries (Fig 2B).

Within the above mentioned three main groups, some representative clonal complexes can be identified: Clonal complex 40, composed of the ancestor ST40, which is the dominant ST in the SE and CW regions of Brazil, isolated from 13 clinical samples from São Paulo, Rio de Janeiro, and Mato Grosso do Sul, and its two single-locus variants (SLV) (ST325 and ST313), both isolated from clinical samples. Clonal complex 5 is represented by the ancestor ST5 and constituted of eight clinical and environmental isolates from the North of Brazil. The other three SLVs of ST5 are ST265, ST288, and ST296, all isolated from clinical and environmental samples. Clonal complex 20 is represented by five STs (ST20, ST122, ST252, ST266 and

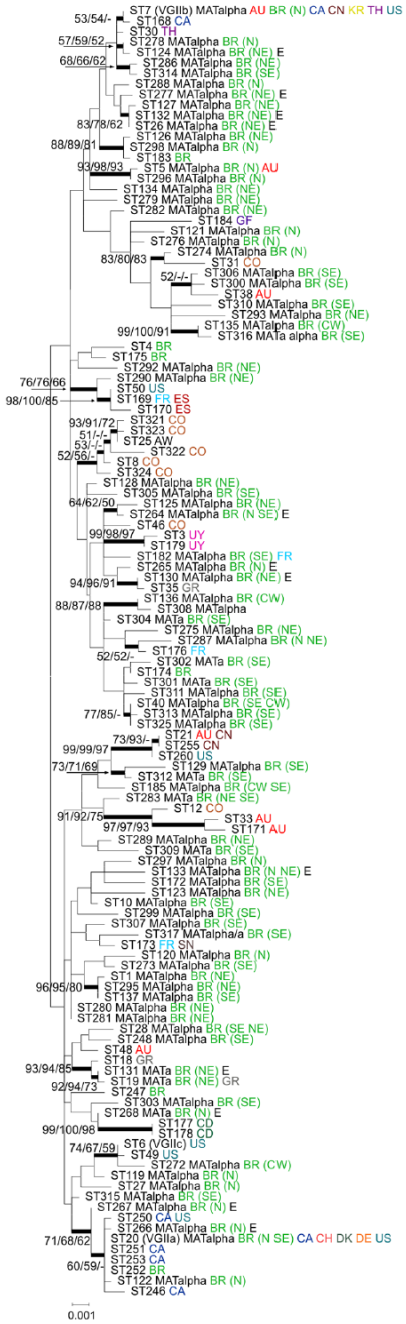

Fig 1. Phylogenetic analysis of Brazilian *Cryptococcus gattii* VGII isolates inferred by maximum likelihood (ML), neighbour-joining (NJ), and maximum parsimony (MP) methods using the concatenated data set of the seven MLST genes. All Brazilian Sequence types (81) from this study and 42 additional Sequence types representing all previously published VGII sequence types maintained in the MLST database (mlst.mycologylab.org) were included in the analysis. The tree with the highest log likelihood

(-9194.7663) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories [+G, parameter = 0.0500]). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 6.6983% sites). The tree is drawn to scale, with branch lengths measuring the number of substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 4,172 positions in the final dataset. Numbers at each branch indicate bootstrap values >50% based on 1,000 replicates by each of the three (ML/NJ/MP) algorithms which presented similar topologies. The taxa nomenclature includes the sequence type number (ST), Mating Type (**a** or alpha), country of isolation and for those isolates from Brazil the region of isolation [N = North, NE = Northeast, CW = Central-West, and SE = Southeast], and source (E = environmental; all others are clinical). All country abbreviations are designated according to the alpha-2 code of ISO 3166–1. AU: Australia, BR: Brazil, CA: Canada, CN: China, CO: Colombia, CD: Democratic Republic of the Congo, CH: Switzerland, DK: Denmark, DE: Germany, ES: Spain, FR: France, GF: French Guiana, GR: Greece, KR: Republic of Korea, SN: Senegal, TH: Thailand, US: United States of America, UY: Uruguay.

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ST267), being the ST20 (VGIIa) the founder ST of this complex and composed by isolates from the SE and N (Fig 2A).

Population structure

In order to better understand the number of populations and their distribution throughout the country, we applied the admixture model of Structure in our dataset and identified $K = 3$ populations (Fig 3A). A high proportion of admixture was observed in our sample (Fig 4A). One of the populations, here presented in green, was mainly found in those States from the N/NE part of the country while the population described in blue was mainly presented in the States of the SE/CW part of the country, such as São Paulo and Mato Grosso do Sul. The third population, presented in red was found to be distributed all over the country and seems to act as an important contributor of genetic material to the remaining populations. We then compared the 87 Brazilian isolates included in the 97 South American isolates, representing all STs obtained in Brazil, with isolates recovered from different regions of the world in order to see how the Brazilian population contributed to the global *C. gattii* VGII distribution, detecting K = 4 number of populations (Fig 3B). In this analysis, a high proportion of admixture was also detected within the whole population of *C. gattii* VGII and among the South American isolates one more population was detected (here identified in yellow), which mainly derived from North America, Asia and Australia (Fig 4B).

Mating type and multilocus linkage disequilibrium

The majority of the isolates (129/145 = 89%) were identified as mating type alpha, 10% (15/145) were mating type **a**, including 13 clinical and two environmental isolates, and one isolate of clinical origin was mating type alpha/**a** (S1 Table).

Random mating can be evidenced by the linkage disequilibrium (D), converging to zero. The SNPs present in the seven loci were used to detect the evidence of recombination separately. Pairwise Linkage Disequilibrium (D) between SNPs suggested at least six recombination events responsible for the polymorphism at the *SOD1* locus and four at the *CAP59* locus ($D' < 0.2$) (Fig 5). The other five loci showed alleles in total disequilibrium ($D' = 1$). The Brazilian VGII isolates also showed evidence of recombination with a high degree of homoplasy demonstrated by a Consistency Index (CI) of 0.27 ($p<0.05$) (Table 2).

In order to confirm these results we applied two other recombination tests to our dataset. The strong reticulation in the networks and phi test implemented in the SplitsTree software for the single sequences also indicate recombination within the Brazilian VGII isolates for *CAP59* and *SOD1* ($p<0.05$) (Fig 6). These results were confirmed in the concatenated data set with an

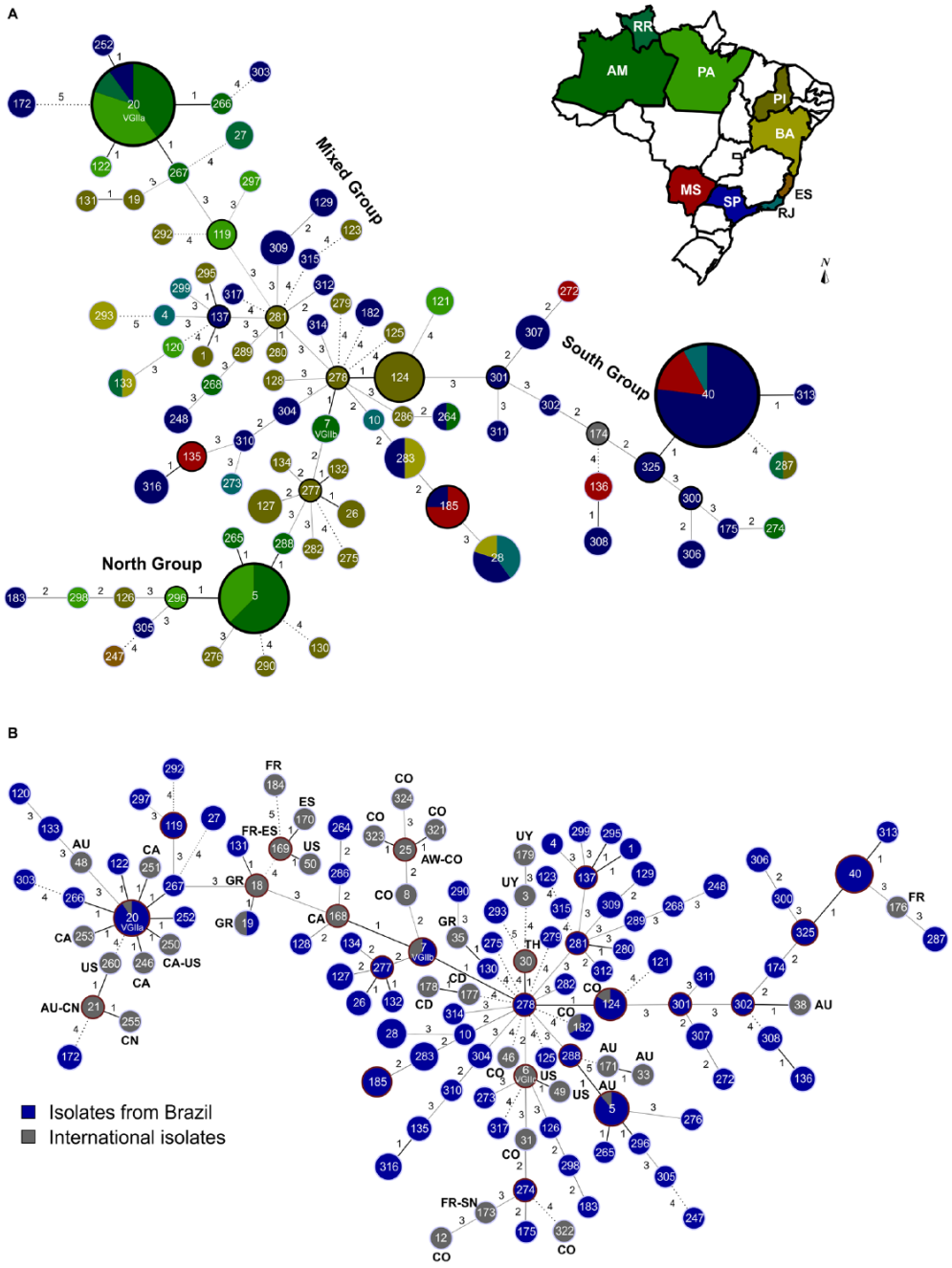

Fig 2. Minimum spanning tree using the goeBURST algorithm. (A) Minimum spanning tree using the goeBURST algorithm showing the high diversity identified among the 145 Brazilian *Cryptococcus gattii* VGII isolates and nine Brazilian sequence types (ST) according to the state where they were recovered. Dividing the country in two macro regions such as North (composing of the States of Amazonas, Roraima, Pará, Piauí, and Bahia) and South (States of Mato Grosso do Sul, São Paulo, and Rio de Janeiro) three main groups can be identified: 1) isolates mainly recovered from the South, representing

all isolates derived from the ST301; 2) isolates mainly recovered from the North, representing those originated from the ST7 (VGIIb), and 3) the mixed group, which contains isolates derived from ST281. The main clonal complexes (CC) in each of these groups are: CC40, CC5, and CC20. The ancestors of the CC is highlighted by a black line. **(B)** Minimum spanning tree using the goeBURST algorithm of the isolates presented in (**A**), and their comparison with 42 STs identified in different countries previously published. All country abbreviations are designated according to the alpha-2 code of ISO 3166–1. In both figures each circle represents a unique ST, and the circumference is proportional to the number of isolates within each ST. Solid, grey and dashed branches represent at least one, two to three, and more than four or five differences, respectively. All STs are different VGII lineages, only the three PNW outbreak genotypes are labelled specifically as VGIIa (ST20), VGIIb (ST7), and VGIIc (ST6).

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I_A^S value of 0.0407 and statistically significant for recombination ($p<0.0001$) in the Brazilian population.

Discussion

Since the unexpected emergence of cryptococcosis caused by the VGII subtype of *C. gattii* in temperate North America in 1999, it has been recognized as a major agent of severe pulmonary and neurological infections in this region [reviewed in 36]. The North American cases of human and animal cryptococcosis caused by distinct highly clonal populations (VGIIa, VGIIb and VGIIc) [11, 16] point to their capacity to emerge from original habitats to adapt and colonize new environments and hosts, rapidly multiplying the new adapted populations.

The current study shows a high genetic variability amongst Brazilian *C. gattii* VGII isolates, presenting 81 MLST STs in 145 clinical and environmental isolates. In addition, a high level of haplotype diversity was observed, while also demonstrating a high degree of homoplasy, with the Consistency Index suggesting the absence of a selective genetic pressure. The patterns of the polymorphisms identified among the Brazilian strains surveyed in this study indicated a history of recombination for the genetic loci *CAP59* and *SOD1* (Figs 5 and 6, Table 2), which contributed to the haplotype diversity observed. The fact that both mating types were present among the clinical and environmental Brazilian VGII isolates, with 10% of them being mating type **a**, emphasizes that recombination events are likely to occur in Brazil, leading to the great

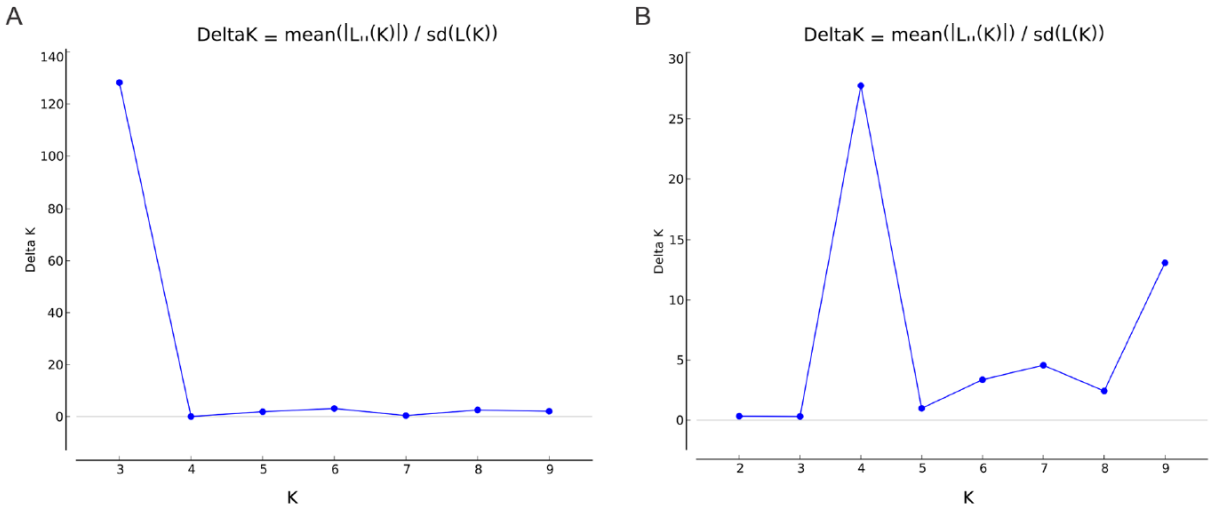

Fig 3. Number of populations using in the STRUCTURE analysis calculated according to [35]. The results presenting in (A) show three populations in the Brazilian *Cryptococcus gattii* VGII and in (B) show four populations in the STs identified in different countries previously published and in Brazil.

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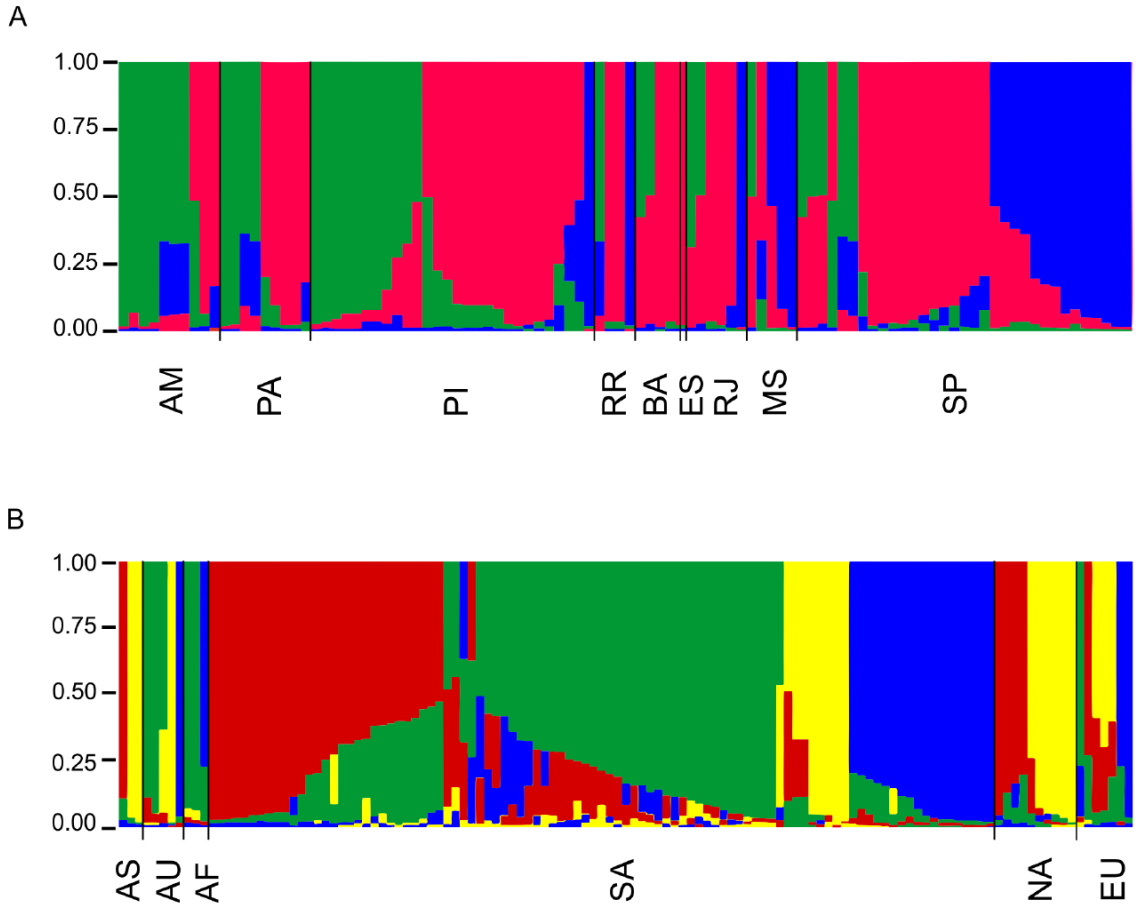

Fig 4. Population structure analysis inferred using multilocus sequence genotypes of *Cryptococcus gattii* isolates recovered from Brazil and using *K* = 3 (A), and comparison of these isolates with isolates recovered from different continents using *K* = 4 (B). Clusters of individuals based on prior-defined populations are referred according to the Brazilian States and/or Continent. Each vertical line represents one of the isolates included and each color (red, dark blue, dark green, and yellow) represents the most likely ancestry of each isolate from one of the three populations (A) or four populations (B). Individuals with multiple colours have admixed genotypes from the prior-defined populations. One clone per region was included, thus Figure A contains 100 isolates while Figure B contain 125 isolates. The taxa nomenclature includes AM: Amazonas, PA: Pará, PI: Piauí, RR: Roraima, BA: Bahia, ES: Espírito Santo, RJ: Rio de Janeiro, MS: Mato Grosso do Sul, AS: Asia, AU: Australia, AF: Africa, SA: South America, NA: North America, EU: Europe.

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variability/high genetic diversity observed. These findings are also reinforced by the mosaic of multiple small chromosomal chunks presented in most of the isolates studied (Fig 4). Recombination amongst VGII genotypes has also been detected previously at global [15, 37] and local [38, 39] scales. Although limited number of isolates have been analysed and a very limited number of sequence types have been identified [13, 39, 40], they already indicated the occurrence of high molecular polymorphisms in South American *Cryptococcus* strains.

Despite the high genetic diversity in the Brazilian *C. gattii* VGII population, nine clonal complexes were found. Some are represented by very common and frequently recovered STs in clinical and environmental samples (e.g. ST20-VGIIa, ST40, and ST5). The persistence of successful STs, which are stable in space and time and most significant in cases of widespread

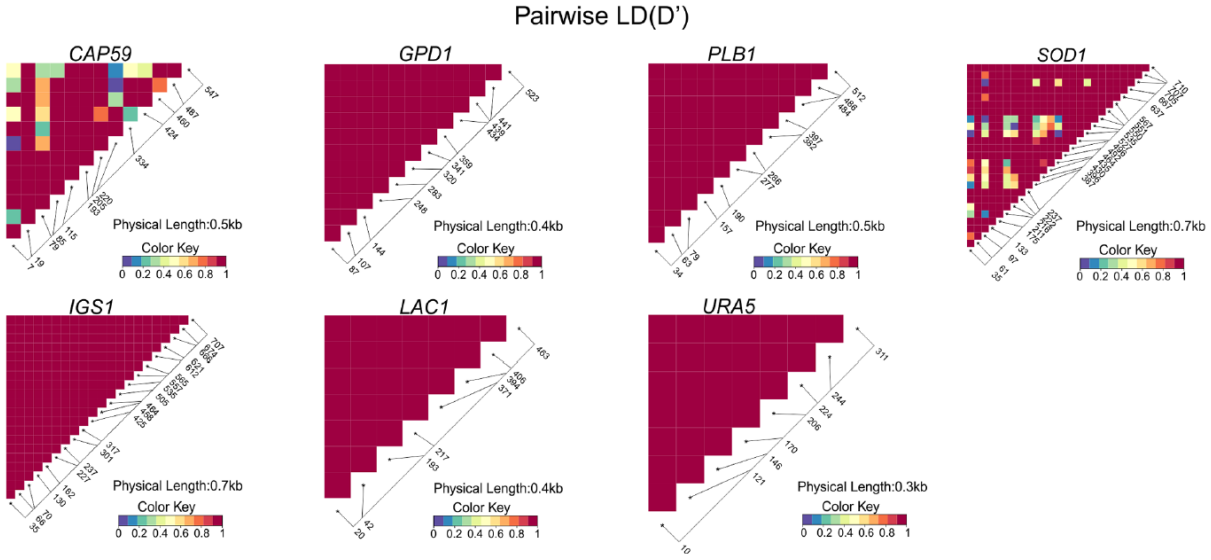

Fig 5. Linkage disequilibrium heat maps between polymorphic sites for all studied MLST loci (*CAP59*, *GPD1*, *PLB1*, *SOD1*, IGS1, *LAC1* and *URA5*). Pairwise D' metricises are represented by heat colours (Colour Key). Recombination amongst Brazilian VGII strains has been shown, as evidenced amongst four sites (0.7%) in the locus *CAP59* and six sites (0.8%) in the locus *SOD1* (Fisher's Exact Test P-value $\leq$ 0.05).

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adapted clones, may follow the features of clonal evolution which is defined as strongly restrained recombination [41]. This has been described for several microorganisms, in bacteria [42], protozoa [43], and fungi [44]. Linked populations have been identified as most likely being stepping stones in the global spread of VGII. Analysis of VGII in Australia [39] identified six sequence types (ST7 (VGIIb), ST38, ST5, ST21, ST33 and ST48), suggesting an introduction into Australia, which created a possible founder effect followed by a clonal expansion of the subtypes. In Thailand, the majority of the *C. gattii* isolates belonged to the sub-genotype VGIIb (11 out of 12) [45], suggesting again a clonal expansion of this subtype.

Despite some well-adapted clonal isolates, the herein described population is recombining. The evolutionary processes, sex crossing and consequently recombination, generates new combinations of genes, some of which may increase adaptation of the population to harsh environments to increase the chance of their survival [41]. On the other hand, DNA repair is a reasonable explanation for the high rate of recombination in diploid and haploid organisms, and could be an ancestral mechanism of general sexuality [46]. As recombination acts as ancient

Table 2. Characteristics of the MLST Loci studied in 145 Brazilian *C. gattii* VGII isolates.

Locus	Length	Polymorphic sites	Recombining position	phi test
CAP59	557	13	(7,79), (79,220), (334,424), (460,487)	0.005
GPD1	550	12	-	1.0
IGS1	717	21	-	1.0
LAC1	475	8	-	1.0
PLB1	535	12	-	0.236
SOD1	713	27	(35,97), (97,211), (216,387), (396,435), (435,527), (527,705)	0.049
URA5	638	8	-	1.0

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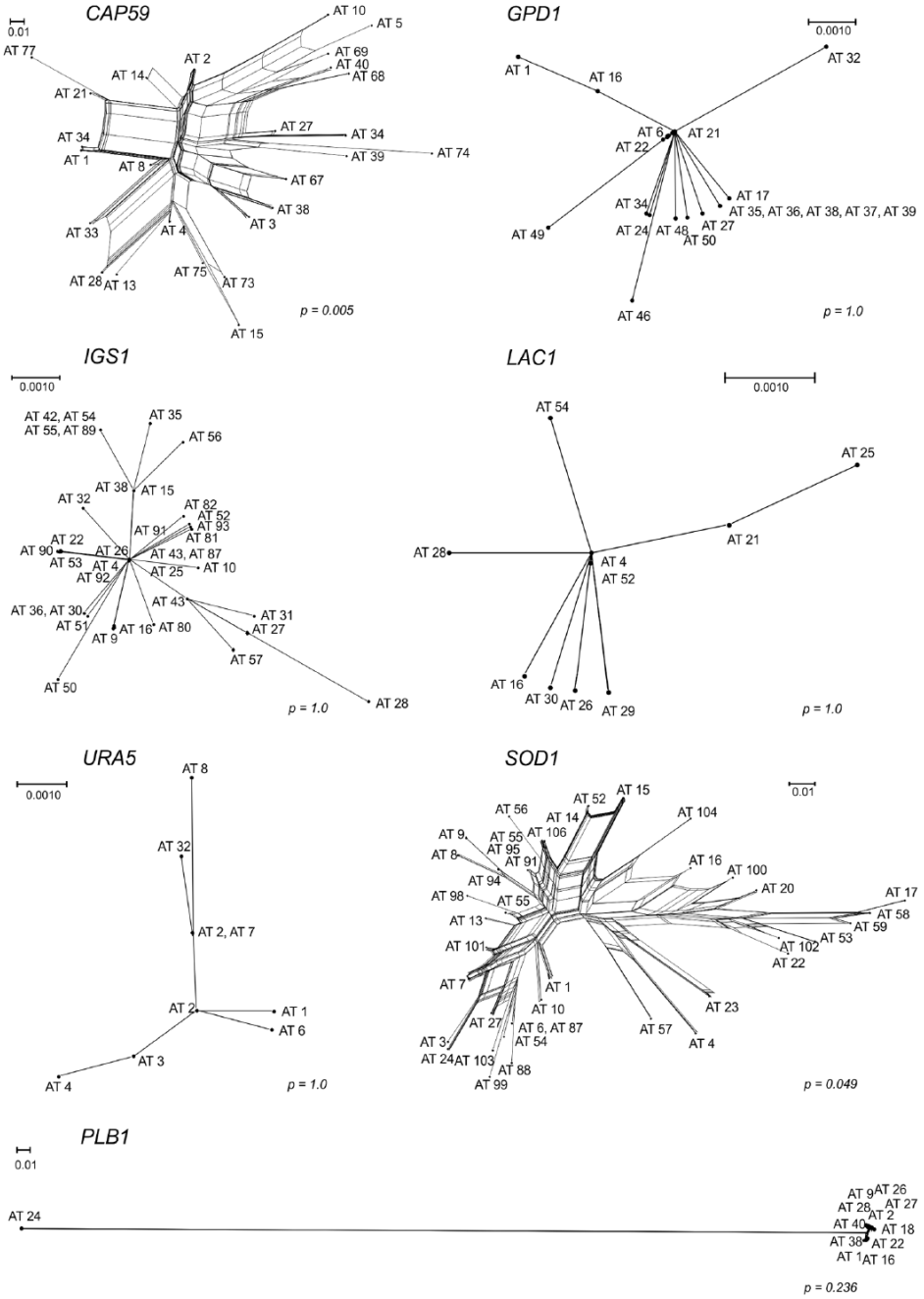

Fig 6. Split decomposition analysis using the Neighbor-net algorithm of the each of seven MLST genes evidencing the diversity and branching ambiguities attributable to recombination events in the *CAP59* and *SOD1*. The phi test result implemented in the software SplitsTree is presented next to each allele.

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machinery of DNA repair, which is not only related to sexual reproduction, but also associated with a fast and simple way of propagation observed in the clonal reproduction, it is an advantage in overcoming the challenges of the environment [46, 47], with some of the well-adapted cells could become more virulent pathogens to humans (*e.g.* outbreak strains), as the killing of the host, in the case of an opportunistic *Cryptococcus* infection, will not interfere with fungal cell propagation.

Although the Brazilian isolates do not show a very well established population structure according to the geographic origin (Fig 4), we showed that the different Brazilian regions are dominated by different genotypes (Fig 1). The six sequence types identified (Fig 2) in more than one region may reflect the Brazilian human population migration patterns, *e.g.* as São Paulo and Rio de Janeiro (SE) are the biggest cities in the country, many people from other regions migrate to the these regions to find greater and better work possibilities. In order to check the influence of migration of *C. gattii* throughout the country, which could also be due to human migration, the clone corrected dataset was submitted to the admixture model in Structure and showed one basal population distributed all over the country (presented in red in Fig 4), one mainly found in the N and NE part of the country (presented in green in Fig 4), one more frequently found in the SE/CW region (presented in blue in Fig 4). Imported cases between these populations and within each State were also found (Fig 4).

The subtypes VGIIa and VGIIb, responsible for the outbreak on Vancouver Island, Canada [13, 34] and the subsequent spread to the Pacific Northwest of the USA [9, 40] have been identified in the North of Brazil (Fig 2). The sequence type ST20 (VGIIa) shows a large scattered distribution pattern in the Amazon region, with eight clinical isolates from the states of Pará, Amazonas and Roraima, and one environmental isolate from the state of Amazonas. In addition, three STs (ST122, ST266, ST267) linked to the clonal complex 20, mainly represented by ST20 (VGIIa), were also found in the Amazon region. The high frequency of this complex in the North may be related to a better adaptation/and or microevolution of these isolates to the environment, although one isolate of ST20 and the only isolate of ST252 were found in the city of São Paulo, which are most likely related to human migration processes. Imported cases caused by this sequence type have also been described in patients who had visited Vancouver Island from Denmark, Germany, Switzerland and the Netherlands [48, 49, 50] (presented in yellow in Fig 4).

The sequence type ST7 (VGIIb) has been found all over the world, including: Australia, Canada, China, Korea, Thailand and the USA [9, 17, 39, 45, 51, 52]. The Brazilian isolates of the sequence type ST7 (VGIIb) were found in the state of Amazonas. Besides these two outbreak associated sequence types, three additional sequence types from other countries have now been identified amongst Brazilian VGII strains, indicating further intercontinental spread as had been previously described [52, 53]. These include ST5, which had been reported from Australia [39], ST19, present in Greece [18, 54], and ST182, which has been found in France and China [55]. MLST analysis provided further evidence for close relationships between many Brazilian sequence types and the sequence types globally present (Fig 1). The sequence types ST7 (VGIIb), ST20 (VGIIa) and ST5, are the three sequences types identified in the current study which were also previously detected in dwelling dust samples and clinical specimens in the Amazonas state (North of Brazil), reinforcing the possibility of indoor infection, especially in wooden houses, very common in the northern part of Brazil, which was originally suggested by Brito-Santos *et al.* [56].

In the North and Northeast of Brazil, *C. gattii* behaves as an endemic fungal pathogen that causes infection in apparently healthy individuals categorized as immunocompetent patients, and the predominant VGII genotype has been recognized for at least the last 20 years among clinical and environmental strains from those large regions [57]. The results here reinforce

recent findings supported by MLST and whole-genome SNP analysis indicating that the North American outbreak lineages, including the VGIIc genotype, which has only been found in the Pacific Northwest of the USA [12] but is closely related to South American strains [21], have most likely arisen from a highly recombining *C. gattii* population from South America, probably from the Amazon rainforest [12, 16, 18].

The detection of high genetic diversity amongst Brazilian *C. gattii* VGII isolates in the current study strongly supports the possibility of the emergence of highly virulent strains in the N and NE regions of Brazil, associated with different biotopes, one with extremely humid forest in the North (the Amazon Forest) and the other with open and predominantly dry savanna formations in Northeast (brushwood known as "caatinga"). Between them, there is a transitional region, with overlapping areas of humid forest, less humid tropical savannah (known as "cerrado") then the dry caatinga, best observed in the states of Piauí and Maranhão.

An important finding of the current study is the central role of the ST278, which is associated with a clinical isolate (CFP 243) from the state Piauí and other closely associated STs from the same area. It shifts the global origin of *C. gattii* VGII, which was previously placed in the Amazon region in the state Roraima (CFP 439/LMM645 from 1998), North of Brazil, by Hagen *et al.* [18, 57] to the transitional ecological area in the Brazilian Northeast. Another very close lineage to ST278 is the ST124 from Piauí, isolated from clinical samples and decaying wood in tree hollows. One clinical sample was isolated from a case with cryptococcal meningitis and the other was isolated from the spleen of an armadillo without any evidence of disease. Thus, VGII has a potential wide host range, behaving as a multi-host pathogen. Protected microenvironments, such as tree hollows or armadillo burrows in Piauí state, probably play an important role in the *C. gattii* life cycle under such variable climatic conditions. These findings show the ecological adaptability of VGII to spread to new habitats, allowing it to survive in dry and humid warm or cold climate.

The Brazilian Northeast and North are large geographical regions, which have been subject to extensive deforestation, leading to enormous landscape changes and the establishment of new settlements, disturbing original communities and related habitats, and causing a large-scale biodiversity loss. These habitat changes, shifts in species composition and other stress factors may affect the profile of *C. gattii* populations, inducing recombination events (and/or hybridization). Human-induced land use and extensive trade of native wood from the Amazon rainforest are also possible drivers of geographical dispersal of propagules, and consequently, disease emergence events.

Taking into account that the present study has, like all previous studies, a possible sampling bias, over-representing some STs while others are underrepresented, it is necessary to suggest further studies investigating other ecological niches, such as a variety of human inhabited places as well as environmental samples from other tropical countries.

The results indicate that the isolates from the transitional ecological area in Northeast Brazil are the most likely ancestor lineages, translocating from caatinga/cerrado by adapting progressively throughout Amazonia in South America, and spread to the North American Pacific Northwest regions and other parts of the world on multiple occasions. This picture is intrinsically related to climatic changes and devastating human activities globally. Therefore, a multifocal origin for the outbreak lineages of cryptococcal infections must be considered.

Supporting Information

S1 Table. Data of 194 strains analysed in the study.
(XLSX)

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Author Contributions

Conceived and designed the experiments: LT MSCM WM BW MSL.

Performed the experiments: ACPS LXB MM CDP DCSS LT.

Analyzed the data: LT KFP MRA.

Contributed reagents/materials/analysis tools: MSCM SRL LM MC FBS WdS SF WM BW MSL.

Wrote the paper: ACPS LXB LT SRL WM BW MSL KFP.

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. doi: 10.1097/QAD.0b013e328322ffac PMID: 19182676
2. Chen SC, Slavin MA, Heath CH, Playford EG, Byth K, Marriott D, Kidd SE, Bak N, Currie B, Hajkowicz K, Korman TM, McBride WJ, Meyer W,Murray R, Sorrell TC; Australia and New Zealand Mycoses Interest Group (ANZMIG)-*Cryptococcus* Study. Clinical manifestations of *Cryptococcus gattii* infection: determinants of neurological sequelae and death. Clin Infect Dis. 2012; 55(6):789–798. PMID: 22670042
3. Saijo T, Chen J, Chen SC, Rosen LB, Yi J, Sorrell TC, Bennett JE, Holland SM, Browne SK, Kwon-Chung KJ. Anti-granulocyte-macrophage colony-stimulating factor autoantibodies are a risk factor for central nervous system infection by *Cryptococcus gattii* in otherwise immunocompetent patients. MBio. 2014;18; 5(2):e00912–14. doi: 10.1128/mBio.00912-14 PMID: 24643864
4. Trilles L, Lazera MS, Wanke B, Oliveira RV, Barbosa GG, Nishikawa MM, et al. Regional pattern of the molecular types of *Cryptococcus neoformans* and *Cryptococcus gattii* in Brazil. Mem Inst Oswaldo Cruz. 2008; 103: 455–462. PMID: 18797758
5. Santos WR, Meyer W, Wanke B, Costa SP, Trilles L, Nascimento JL, Medeiros R, Morales BP, Bezerra Cde C, Macêdo RC, Ferreira SO, Barbosa GG, Perez MA, Nishikawa MM, Lazéra Mdos S. Primary endemic Cryptococcosis *gattii* by molecular type VGII in the state of Pará, Brazil. Mem Inst Oswaldo Cruz. 2008; 103(8):813–818. PMID: 19148422
6. Martins LM, Wanke B, Lazéra Mdos S, Trilles L, Barbosa GG, Macedo RC, Cavalcanti Mdo A, Eulálio KD, Castro JA, Silva AS, Nascimento FF, Gouveia VA, Monte SJ. Genotypes of *Cryptococcus neoformans* and *Cryptococcus gattii* as agents of endemic cryptococcosis in Teresina, Piauí (northeastern Brazil). Mem Inst Oswaldo Cruz. 2011; 106(6):725–730. PMID: 22012227
7. Alves GS, Freire AK, Bentes AD, Pinheiro JF, de Souza JV, Wanke B, Matsuura T, Jackisch-Matsuura AB. Molecular typing of environmental *Cryptococcus neoformans/C. gattii* species complex isolates from Manaus, Amazonas, Brazil. Mycoses. 2016; doi: 10.1111/myc.12499
8. Hagen F, Khayhan K, Theelen B, Kolecka A, Polacheck I, Sionov E, Falk R, Parnmen S, Lumbsch HT, Boekhout T. Recognition of seven species in the *Cryptococcus gattii/Cryptococcus neoformans* species complex. Fungal Genet Biol. 2015; 78: 16–48. doi: 10.1016/j.fgb.2015.02.009 PMID: 25721988
9. Bymes EJ, Bildfell RJ, Frank SA, Mitchell TG, Marr KA, Heitman J. Molecular evidence that the range of the Vancouver Island outbreak of *Cryptococcus gattii* infection has expanded into the Pacific Northwest in the United States. J Infec Dis. 2009; 199: 1081–1086.
10. Chaturvedi S, Ren P, Narasipura SD, Chaturvedi V. Selection of optimal host strain for molecular pathogenesis studies on *Cryptococcus gattii*. Mycopathologia. 2005; 160(3):207–215. PMID: 16205969

11. Engelthaler DM, Hicks ND, Gillece JD, Roe CC, Schupp JM, Driebe EM, et al. *Cryptococcus gattii* in North American Pacific Northwest: whole-population genome analysis provides insights into species evolution and dispersal. mBio. 2014; 5: e01464–01414. doi: 10.1128/mBio.01464-14 PMID: 25028429

12. Bymes EJ, Li W, Lewit Y, Ma H, Voelz K, Ren P, et al. Emergence and pathogenicity of highly virulent *Cryptococcus gattii* genotypes in the northwest United States. PLoS pathogens. 2010; 6: e1000850. doi: 10.1371/journal.ppat.1000850 PMID: 20421942

13. Kidd SE, Hagen F, Tscharke RL, Huynh M, Bartlett KH, Fyfe M, et al. A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). Proc Natl Acad Sci U S A. 2004; 101: 17258–17263. PMID: 15572442

14. Meyer W, Aanensen DM, Boekhout T, Cogliati M, Diaz MR, Esposto MC, et al. Consensus multi-locus sequence typing scheme for *Cryptococcus neoformans* and *Cryptococcus gattii*. Med Mycol. 2009; 47: 561–570. doi: 10.1080/13693780902953886 PMID: 19462334

15. Ngamskulrungroj P, Gilgado F, Faganello J, Litvintseva AP, Leal AL, Tsui KM, et al. Genetic diversity of the *Cryptococcus* species complex suggests that *Cryptococcus gattii* deserves to have varieties. PloS one. 2009; 4: e5862. doi: 10.1371/journal.pone.0005862 PMID: 19517012

16. Billmyre RB, Croll D, Li W, Mieczkowski P, Carter DA, Cuomo CA, et al. Highly recombinant VGII *Cryptococcus gattii* population develops clonal outbreak clusters through both sexual macroevolution and asexual microevolution. mBio. 2014; 5: e01494–01414. doi: 10.1128/mBio.01494-14 PMID: 25073643

17. Fraser JA, Giles SS, Wenink EC, Geunes-Boyer SG, Wright JR, Diezmann S, et al. Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. Nature. 2005; 437: 1360–1364. PMID: 16222245

18. Hagen F, Ceresini PC, Polacheck I, Ma H, van Nieuwerburgh F, Gabaldon T, et al. Ancient dispersal of the human fungal pathogen *Cryptococcus gattii* from the Amazon rainforest. PloS one. 2013; 8: e71148. doi: 10.1371/journal.pone.0071148 PMID: 23940707

19. Meyer W, Castaneda A, Jackson S, Huynh M, Castaneda E, and IberoAmerican Cryptococcal Study Group. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. Emerg Infect Dis. 2003; 9:189–195. PMID: 12603989

20. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013; 30: 2725–2729. doi: 10.1093/molbev/mst197 PMID: 24132122

21. Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods. 2012; 9: 772.

22. Guindon S, Gascuel O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst bio. 2003; 52: 696–704.

23. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 1980; 16: 111–120. PMID: 7463489

24. Ferreira-Paim K, Ferreira TB, Andrade-Silva L, Mora DJ, Springer DJ, Heitman J, et al. Phylogenetic analysis of phenotypically characterized *Cryptococcus laurentii* isolates reveals high frequency of cryptic species. PloS One. 2014; 9: e108633. doi: 10.1371/journal.pone.0108633 PMID: 25251413

25. Francisco AP, Vaz C, Monteiro PT, Melo-Cristino J, Ramirez M, Carrico JA. PHYLOViZ: phylogenetic inference and data visualization for sequence based typing methods. BMC Bioinf. 2012; 13: 87.

26. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. Genetics. 2000; 155; 945–959. PMID: 10835412

27. Falush D, Stephens M, Pritchard JK. Inference of population structure using multilocus genotype data: dominant markers and null alleles. Mol Ecol Notes. 2007; 7: 574–578. PMID: 18784791

28. Earl DA, Vonholdt BM. (2012). STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv Genet Resour. 2012; 4: 359–361.

29. Jakobsson M, Rosenberg NA. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics. 2007; 23: 1801–1806. PMID: 17485429

30. Ramasamy RK, Ramasamy S, Bindroo BB, Naik VG. STRUCTURE PLOT: a program for drawing elegant STRUCTURE bar plots in user friendly interface. Springerplus. 2014; 13; 3:431. doi: 10.1186/2193-1801-3-431 PMID: 25152854

31. Librado P, Rozas J. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics. 2009; 25: 1451–1452. doi: 10.1093/bioinformatics/btp187 PMID: 19346325

32. Huson DH, Bryant D. Application of phylogenetic networks in evolutionary studies. Mol Biol Evol. 2006; 23: 254–267. PMID: 16221896

33. Haubold B, Hudson RR. (2000). LIAN 3.0: detecting linkage disequilibrium in multilocus data. Linkage Analysis. Bioinformatics. 2000; 16: 847–848. PMID: 11108709

34. Fraser JA, Subaran RL, Nichols CB, Heitman J. Recapitulation of the sexual cycle of the primary fungal pathogen *Cryptococcus neoformans* var. *gattii*: implications for an outbreak on Vancouver Island, Canada. Eukaryotic cell. 2003; 2: 1036–1045. PMID: 14555486

35. Evanno G, Regnaut S, Goudet J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol ecol. 2005; 14: 2611–2620. PMID: 15969739

36. Chen SC, Meyer W, Sorrell TC. *Cryptococcus gattii* infections. Clin Microbiol Rev. 2014; 27: 980–1024. doi: 10.1128/CMR.00126-13 PMID: 25278580

37. Carter D, Campbell LT, Saul N, Krockenberger M. Sexual Reproduction of *Cryptococcus gattii*: a Population Genetics Perspective. In Heitman J, Kozel TR, Kwon-Chung KJ, Perfect JR, Casadevall A edtors. *Cryptococcus*. Washington, DC: ASM Press; 2011.

38. Campbell LT, Currie BJ, Krockenberger M, Malik R, Meyer W, Heitman J, et al. Clonality and recombination in genetically differentiated subgroups of *Cryptococcus gattii*. Eukaryotic cell. 2005; 4: 1403–1409. PMID: 16087745

39. Carriconde F, Gilgado F, Arthur I, Ellis D, Malik R, van de Wiele N, et al. Clonality and alpha-a recombination in the Australian *Cryptococcus gattii* VGII population—an emerging outbreak in Australia. PloS one. 2011; 6: e16936. doi: 10.1371/journal.pone.0016936 PMID: 21383989

40. Lockhart SR, Iqbal N, Harris JR, Grossman NT, DeBess E, Wohrle R, et al. *Cryptococcus gattii* in the United States: genotypic diversity of human and veterinary isolates. PloS one. 2013; 8: e74737. doi: 10.1371/journal.pone.0074737 PMID: 24019979

41. Tibayrenc M, Ayala FJ. Reproductive clonality of pathogens: a perspective on pathogenic viruses, bacteria, fungi, and parasitic protozoa. Proc Natl Acad Sci U S A. 2012; 109: E3305–3313. doi: 10.1073/pnas.1212452109 PMID: 22949662

42. Caugant DA, Maiden MC. Meningococcal carriage and disease—population biology and evolution. Vaccine. 2009; 27 Suppl 2I:B64–70.

43. Sibley LD, Ajioka JW. Population structure of *Toxoplasma gondii*: clonal expansion driven by infrequent recombination and selective sweeps. Annu rev microbiol. 2008; 62: 329–351. doi: 10.1146/annurev.micro.62.081307.162925 PMID: 18544039

44. Kasuga T, White TJ, Koenig G, McEwen J, Restrepo A, Castaneda E, et al. Phylogeography of the fungal pathogen *Histoplasma capsulatum*. Mol Ecol. 2003; 12: 3383–3401. PMID: 14629354

45. Kaocharoen S, Ngamskulrungroj P, Firacative C, Trilles L, Piyabongkarn D, Banlunara W, et al. Molecular epidemiology reveals genetic diversity amongst isolates of the *Cryptococcus neoformans*/*C. gattii* species complex in Thailand. PLoS Neg Trop Dis. 2013; 7: e2297.

46. Michod RE, Bernstein H, Nedelcu AM. Adaptive value of sex in microbial pathogens. Inf Gen Evol. 2008; 8: 267–285.

47. Taylor JW, Hann-Soden C, Branco S, Sylvain I, Ellison CE. Clonal reproduction in fungi. Proc Natl Acad Sci U S A. 2015; 112: 8901–8908. doi: 10.1073/pnas.1503159112 PMID: 26195774

48. Georgi A, Schneemann M, Tintelnot K, Calligaris-Maibach RC, Meyer S, Weber R, et al. *Cryptococcus gattii* meningoencephalitis in an immunocompetent person 13 months after exposure. Infection. 2009; 37: 370–373. doi: 10.1007/s15010-008-8211-z PMID: 19390780

49. Hagen F, van Assen S, Luijckx GJ, Boekhout T, Kampinga GA. Activated dormant *Cryptococcus gattii* infection in a Dutch tourist who visited Vancouver Island (Canada): a molecular epidemiological approach. Med Mycol. 2010; 48:528–531. doi: 10.3109/13693780903300319 PMID: 19824880

50. Lindberg J, Hagen F, Laursen A, Stenderup J, Boekhout T. *Cryptococcus gattii* risk for tourists visiting Vancouver Island, Canada. Emerg Infect Dis. 2007; 13: 178–179. PMID: 17370544

51. Feng X, Wu J, Ling B, Ren D, Yao Z. [Molecular and phenotypic characterization of a VGII genotype *Cryptococcus gattii* XH91 isolated in China]. Wei sheng wu xue bao = Acta Microbiol Sinica. 2010; 50: 1460–1465.

52. Firacative C, Ferreira-Paim KB, Trilles L, Engelthaler DM, Meyer W. Australia in the global picture of the molecular epidemiology of *Cryptococcus gattii* molecular type VGII. Microbiol Aust. 2015; 36: 67–70.

53. Meyer W. *Cryptococcus gattii* in the Age of Whole-Genome Sequencing. MBio. 2015;17; 6(6): e01761–15. doi: 10.1128/mBio.01761-15 PMID: 26578680

54. Velegraki A, Kiosses VG, Pitsouni H, Toukas D, Daniilidis VD, Legakis NJ. First report of *Cryptococcus neoformans* var. *gattii* serotype B from Greece. Med Mycol. 2001; 39: 419–422. PMID: 12054052

55. Dou HT, Xu YC, Wang HZ, Li TS. Molecular epidemiology of *Cryptococcus neoformans* and *Cryptococcus gattii* in China between 2007 and 2013 using multilocus sequence typing and the DiversiLab system. Eur J Clin Microbiol. 2015; 34: 753–762.

56. Brito-Santos F, Barbosa GG, Trilles L, Nishikawa MM, Wanke B, Meyer W, 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. 2015; 10: e0115866. doi: 10.1371/journal.pone.0115866 PMID: 25688971

57. Fortes ST, Lazera MS, Nishikawa MM, Macedo RC, Wanke B. First isolation of *Cryptococcus neoformans* var. *gattii* from a native jungle tree in the Brazilian Amazon rainforest. Mycoses. 2001; 44: 137–140. PMID: 11486449

Apêndice B - Artigo 2 "Cryptococcosis due to *Cryptococcus gattii* VGII in Southeast Brazil: The One Health approach revealing a possible role for domestic cats"

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Cryptococcosis due to *Cryptococcus gattii* VGII in southeast Brazil: The One Health approach revealing a possible role for domestic cats

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ABSTRACT

Two cats infected by *C. gattii*, presented lesions on the nasal region and respiratory signs. Strains were typed as molecular type VGII, mating type alpha, MLST subtypes ST442 and ST185. Since Rio de Janeiro is known as an endemic area for *C. neoformans* VNI, these cases might be a warning for a possible emergence of *C. gattii* VGII in southeast Brazil.

1. Introduction

Cryptococcosis is a worldwide-distributed fungal disease of humans and animals caused by members of the *Cryptococcus neoformans* and *Cryptococcus gattii* complexes [1]. Both species have been reported to infect domestic pets, livestock, as well as birds and aquatic mammals [2]. Since 1999, the *C. gattii* molecular type VGII, has emerged as an important pathogen of humans and animals as result of the first multi-species outbreak of cryptococcosis in British Columbia, Canada [3]. In the North (N) and Northeast (NE) regions of Brazil, *C. gattii*, mainly the molecular type VGII, prevails as the cause of cryptococcal meningitis in children and young adults, as well as in several environments, like domiciliary dust, soil, and trees. However, in the southern regions of the country, *C. neoformans* predominates, and infections by *C. gattii* VGII are rare and usually considered as imported cases [4].

Cryptococcosis is the most common systemic mycosis in cats. These animals were found to be particularly more susceptible to infections by *C. neoformans* than dogs in studies conducted in Western Australia [5]. Feline cryptococcosis is frequently reported in Australia, Canada and the United States [6].

In Brazil, very few animal cases of cryptococcosis have been documented [7,8] and most of them do not discriminate the causative species complexes: *C. neoformans* from *C. gattii*. We describe herein two cases of feline cryptococcosis due to *C. gattii* in the state of Rio de Janeiro, southeast Brazil, focusing on the differential diagnosis, treatment and clinical outcome, along with the phenotypic identification, molecular typing and *in vitro* antifungal susceptibility of the agents. Moreover, a role for these animals in cryptococcosis within the concept of One Health is discussed.

2. Case presentations

Two cats with suspicion of cryptococcosis based on clinical signs and positive cytopathological examination, were referred to a reference center for fungal diseases in animals in Rio de Janeiro, southeast Brazil. The cat from case 1 was a 4-year-old female cross-breed cat, castrated, weighing 3.3 kg. At the clinical examination, this cat presented a firm subcutaneous swelling over the forehead, bilateral ocular seropurulent secretion, and polyp-like mass in the nostrils. Dyspnea and nasal discharge were also observed (Fig. 1a). Exudate from the lesion located on the forehead obtained by aspiration was collected for cytopathological examination. Yeasts suggestive of *Cryptococcus* spp. were observed in Panotic-stained smears. Exudate from the same lesion and secretion from the nasal cavities obtained with a swab were seeded on to Sabouraud-dextrose agar. The physiologic characterization was performed as follows: the isolated yeasts were tested for thermotolerance

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Fig. 1. Case 1. A) Cat presenting a firm subcutaneous swelling over the forehead, bilateral ocular seropurulent secretion, and polyp-like mass in the nostrils. B) Case 1 after the disappearance of all the clinical signs initially presented, which occurred after six months of fluconazole therapy.

at 37 °C, melanin production on Niger seed agar, and assimilation of carbon and nitrogen compounds. The species complexes *C. neoformans* and *C. gattii* were differentiated via incubation on canavanine-glycine-bromothymol blue (CGB) medium. The mating type was determined applying polymerase chain reaction using specific primers for the pheromone genes. Subtyping was performed according to the ISHAM consensus multi-locus sequence typing scheme for *C. neoformans* and *C. gattii* [9]. The sequences were manually edited using the software Sequencer 4.10.1, and the allele types (AT) and the sequence types (ST) were identified via the MLST webpage (http://mlst.mycologylab.org/). The results revealed the presence of *C. gattii* VGII, mat *alfa*, sequence type ST442. Antifungal susceptibility testing was performed according to the CLSI M27-A3 guideline. The drugs tested were fluconazole, voriconazole, itraconazole, flucytosine, and amphotericin B. The minimal inhibitory concentrations (MICs) were evaluated by means of the epidemiologic cutoff values (ECVs) suggested for the specific molecular types of *C. gattii* [10,11]. These ECVs allow the classification of strains as Wild Type (WT) or Non-Wild Type (Non-WT). The MICs in μg/ml were as follows: 4.0 for fluconazole, 0.12 for voriconazole, 2.0 for flucytosine, and 0.12 for amphotericin B, all of them were consistent with the WT population. MIC of 1 $\mu g \cdot ml^{-1}$ for itraconazole was consistent with the non-WT population (Table 1).

Fluconazole (100 mg PO) was prescribed once a day (day 0), and the cat was followed up every 30 days for clinical evaluation. After the disappearance of all the clinical signs initially presented, which occurred after six months of therapy, the cat was discharged (Fig. 1b).

The cat from case 2 was a 6-year-old male cross-breed cat, castrated, positive for feline leukaemia virus (FeLV), weighing 5.2 kg. The cat had been previously treated at a private veterinary clinic with itraconazole (100 mg PO, q24h) for one month, with no clinical response. At clinical examination, a soft subcutaneous swelling over the left periocular medial region, a lesion on the conjunctiva of the left eye, polyp-like mass in the nostrils and sneezing were observed (Fig. 2a). Exudate from the conjunctival lesion and nasal secretion samples were seeded on to Sabouraud-dextrose agar. The physiologic characterization was performed and revealed the presence of *C. gattii*. The isolates were further characterized as VGII, mat *alfa*, sequence type ST185, which were identified according to the techniques described for the first case (see above). The antifungal susceptibility testing and the ECVs classification of the strain were performed as described for case 1 (see above). The MIC's (μg/ml) were as follows: 16 for fluconazole, 4.0 for flucytosine, and 0.12 for amphotericin B, values consistent with WT population, as well as 1.0 for both itraconazole and voriconazole, which were consistent with the non-WT population (Table 1).

Itraconazole was replaced by fluconazole (100 mg PO, q24h) (day 0). The cat was followed up every 30 days for clinical evaluation. The clinical cure occurred after five months of therapy, when the cat was discharged (Fig. 2b).

3. Discussion

Since the first description in Brazil in 1971, only 27 other cases of feline cryptococcosis have been reported (Supplementary Table 1). Of these, *C. gattii* was properly identified in three cases, with one case due to the molecular type VGII. Thus, animal cryptococcosis is probably underdiagnosed and under-reported in Brazil, with the real incidence

Table 1
Laboratory results of the two cryptococcosis feline cases herein reported.

Case report	Molecular ID	Mating type	MLST (sequence type)	AST (CLSI M27-A3)		
				Antifungal	MIC	Classification
Case 1	*C. gattii* VGII	*alfa*	ST442	Fluconazole	4.0	[a]WT
				Voriconazole	0.12	WT
				Itraconazole	1.0	[b]Non-WT
				Amphotericin	0.12	WT
				Flucytosine	2.0	WT
Case 2	*C. gattii* VGII	*alfa*	ST185	Fluconazole	16	WT
				Voriconazole	1.0	Non-WT
				Itraconazole	1.0	Non-WT
				Amphotericin	0.12	WT
				Flucytosine	4.0	WT

[a] Wild Type (WT).
[b] Non-Wild Type (Non-WT).

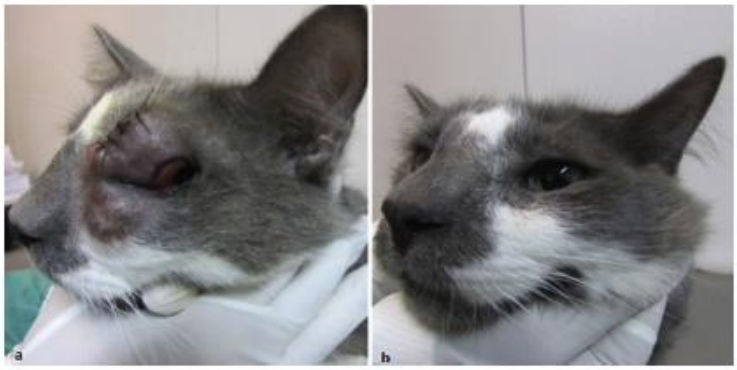

Fig. 2. Case 2. A) Soft subcutaneous swelling over the left periocular medial region, a lesion on the conjunctiva of the left eye and polyp-like mass in the nostrils. B) Cat presenting clinical cure occurred after five months of fluconazole therapy.

being severely biased. Herein, we report two cases of feline cryptococcosis due to *C. gattii* VGII in an urban area of the Southeast Brazil, largely known to be endemic for human cryptococcosis caused by *C. neoformans* VNI [4].

Environmental studies in Rio de Janeiro have isolated mainly *C. neoformans* VNI and *C. gattii* VGI, while *C. gattii* VGII has rarely been isolated [12]. Authors described the only Brazilian case of cryptococcosis in a cat whose isolate was assessed for its molecular type, and *C. gattii* VGII was identified [7]. Considering that cryptococcal infection in animals, reflects infections in human host from the same geographic area, studies about animal cryptococcosis in Rio de Janeiro reiterate the value of sentinel animal surveillance for this emerging infectious disease [13], reinforcing the need for a correct molecular identification of the agents.

Cats with localized cutaneous lesions can be treated successfully with fluconazole, which is the drug of choice for feline cryptococcosis. However, many cats with *C. gattii* VGII and VGIII infections in North America fail to respond to this azole, presenting persistent high antigen titers and frequent relapses. In these cases, itraconazole is a better option [5]. In previous reports from Brazil due to *C. gattii*, a cat presenting a nasal granuloma achieved clinical cure after six months therapy of itraconazole/5-flucytosine combination [7] and the other case, which presented a mass on the nasal region and partial obstruction of the nostrils, died 40 days after itraconazole therapy [8]. Case 2 failed to initially respond to itraconazole and the correspondent strain was a non-WT to itraconazole and voriconazole, with a non-WT organism showing reduced susceptibility to the agent being evaluated when compared to the WT population [11], as was observed in case 2. However, cases herein described were caused by strains classified as WT to fluconazole. Treatment with this drug lead to cure of both cases and no recurrence signs were observed over the following six months. Hence, more epidemiological studies are necessary to evaluate the trends in cryptococcal antifungal susceptibility to drugs and response to treatment, spread and circulation of subtypes in certain areas.

Cryptococcosis and sporotrichosis infections are thought to be acquired from the environment. However, in the Rio de Janeiro sporotrichosis epizootic, feline cases are mainly related to be acquired by scratches or bites from other infected cats [14], whereas cryptococcosis cases are thought to be acquired through inhalation of yeasts or basidiospores from the environment, with no individual to individual transmission ever been described [15]. Heteroresistance to itraconazole is intrinsic and usually increases the virulence of *C. gattii* [16]. This phenomenon may represent an additional mechanism that contributes to relapses of cryptococcosis in animals during itraconazole therapy. Itraconazole is the first choice therapy for sporotrichosis, since fluconazole is not effective *in vitro* against *Sporothrix* spp [17]. These differences in therapy of cryptococcosis and sporotrichosis reinforces the importance of differential diagnosis between these important infections.

The emergence of *C. gattii* in urban environments is a challenge because it comes with a great potential to generate outbreaks of cryptococcosis among immunocompetent individuals and animals. Future studies on the etiological agents of cryptococcosis in domestic animals in urban environments are necessary to understand the dynamics of this mycosis in these animals.

Financial interest's declaration

The authors declare that no conflict of interests exist.

Ethical approval

The procedures were approved by the Animal Ethics Committee under the license number LW 37/12.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mmcr.2019.04.004.

References

[1] K.J. Kwon-Chung, J.E. Bennett, B.L. Wickes, W. Meyer, C.A. Cuomo, K.R. Wollenburg, T.A. Bicanic, E. Castañeda, Y.C. Chang, J. Chen, M. Cogliati, F. Dromer, D. Ellis, S.G. Filler, M.C. Fisher, T.S. Harrison, S.M. Holland, S. Kohno, J.W. Kronstad, M. Lazera, S.M. Levitz, M.S. Lionakis, R.C. May, P. Ngamskulrongroj, P.G. Pappas, J.R. Perfect, V. Rickerts, T.C. Sorrell, T.J. Walsh, P.R. Williamson, J. Xu, A.M. Zelazny, A. Casadevall, The case for adopting the "species complex" nomenclature for the etiologic agents of cryptococcosis, mSphere 2 (2017), https://doi.org/10.1128/mSphere.00357-16.

[2] M.S. Lazéra, T.G. Mitchell, K. Nielsen, E. Castañeda, B. Wanke, Environmental niches for *Cryptococcus neoformans* and *Cryptococcus gattii*, in: K.J. Kwon-Chung, T.R. Kozel, J.R. Perfect, J. Heitman, A. Casadevall (Eds.), Cryptococcus, American Society of Microbiology, 2011, pp. 237–259, , https://doi.org/10.1128/9781555816858.ch18.

[3] C. Duncan, K.H. Bartlett, S. Lester, B. Bobsien, J. Campbell, C. Stephen, S. Raverty, Surveillance for *Cryptococcus gattii* in horses of Vancouver Island, British Columbia,

Canada, Med. Mycol. 49 (2011) 734–738, https://doi.org/10.3109/13693786.2011.560196.
[4] L. Trilles, M. dos S. Lazéra, B. Wanke, R.V. Oliveira, G.G. Barbosa, M.M. Nishikawa, B.P. Morales, W. Meyer, Regional pattern of the molecular types of *Cryptococcus neoformans* and *Cryptococcus gattii* in Brazil, Mem. Inst. Oswaldo Cruz 103 (2008) 455–462.
[5] S. McGill, R. Malik, N. Saul, S. Beetson, C. Secombe, I. Robertson, P. Irwin, Cryptococcosis in domestic animals in Western Australia: a retrospective study from 1995–2006, Med. Mycol. 47 (2009) 625–639, https://doi.org/10.1080/13693780802512519.
[6] S.R. Trivedi, R. Malik, W. Meyer, J.E. Sykes, Feline Cryptococcosis Impact of current research on clinical management, J. Feline Med. Surg. 13 (2011) 163–172, https://doi.org/10.1016/j.jfms.2011.01.009.
[7] P.H.M. Cardoso, F. de A. Baroni, E.G. Silva, D.C. Nascimento, M. dos A. Martins, W. Szeszs, C.R. Paula, Feline nasal granuloma due to *Cryptococcus gattii* type VGII, Mycopathologia 176 (2013) 303–307, https://doi.org/10.1007/s11046-013-9686-4.
[8] D.A. de Paula, A.B. de Almeida, F.S. da Cruz, F.H. Furlan, E.M. Colodel, V.R. Sousa, L. Nakazato, V. Dutra, Occurrence and molecular characterization of cryptococcosis in dogs and cats in Mato Grosso, Brazil, Pesqui, Veterinária Bras 34 (2014) 167–172.
[9] F. Brito-Santos, G.G. Barbosa, L. Trilles, M.M. Nishikawa, B. Wanke, W. Meyer, F.A. Carvalho-Costa, M. dos S. Lazéra, Environmental isolation of Cryptococcus gattii VGII from indoor dust from typical wooden houses in the deep amazonas of the Rio negro basin, PLoS One 10 (2015) e0115866, , https://doi.org/10.1371/journal.pone.0115866.
[10] A. Espinel-Ingroff, A. Chowdhary, M. Cuenca-Estrella, A. Fothergill, J. Fuller, F. Hagen, N. Govender, J. Guarro, E. Johnson, C. Lass-Florl, S.R. Lockhart, M.A. Martins, J.F. Meis, M.S.C. Melhem, L. Ostrosky-Zeichner, T. Pelaez, M.A. Pfaller, W.A. Schell, L. Trilles, S. Kidd, J. Turnidge, *Cryptococcus neoformans-Cryptococcus gattii* species complex: an international study of wild-type susceptibility endpoint distributions and epidemiological cutoff values for amphotericin B and flucytosine, Antimicrob. Agents Chemother. 56 (2012) 3107–3113, https://doi.org/10.1128/AAC.06252-11.
[11] A. Espinel-Ingroff, A.I. Aller, E. Canton, L.R. Castañón-Olivares, A. Chowdhary, S. Cordoba, M. Cuenca-Estrella, A. Fothergill, J. Fuller, N. Govender, F. Hagen, M.T. Illnait-Zaragozi, E. Johnson, S. Kidd, C. Lass-Flörl, S.R. Lockhart, M.A. Martins, J.F. Meis, M.S.C. Melhem, L. Ostrosky-Zeichner, T. Pelaez, M.A. Pfaller, W.A. Schell, G. St-Germain, L. Trilles, J. Turnidge, *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, Antimicrob. Agents Chemother. 56 (2012) 5898–5906, https://doi.org/10.1128/AAC.01115-12.
[12] G. Barbosa, Cryptococcus gattii VGI and *Cryptococcus neoformans* VNI associated with wood decay in Ficus hollow trees in Rio de Janeiro, Brazil, Br. Microbiol. Res. J. 3 (2013) 106–115, https://doi.org/10.9734/BMRJ/2013/2682.
[13] P. Danesi, C. Firacative, M. Cogliati, D. Otranto, G. Capelli, W. Meyer, Multilocus sequence typing (MLST) and M13 PCR fingerprinting revealed heterogeneity amongst *Cryptococcus* species obtained from Italian veterinary isolates, FEMS Yeast Res. 14 (2014) 897–909, https://doi.org/10.1111/1567-1364.12178.
[14] I.D.F. Gremião, L.H.M. Miranda, E.G. Reis, A.M. Rodrigues, S.A. Pereira, Zoonotic epidemic of sporotrichosis: cat to human transmission, PLoS Pathog. 13 (2017) e1006077, , https://doi.org/10.1371/journal.ppat.1006077.
[15] M.S. Lazera, F.D.A. Pires, L. Camillo-Coura, M.M. Nishikawa, C.C.F. Bezerra, L. Trilles, B. Wanke, Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees, J. Med. Vet. Mycol. 34 (1996) 127–131.
[16] G.F. Ferreira, J.R.A. Santos, M.C. da Costa, R.A. de Holanda, Â.M.L. Denadai, G.J.C. de Freitas, Á.R.C. Santos, P.B. Tavares, T.A. Paixão, D.A. Santos, Heteroresistance to itraconazole alters the morphology and increases the virulence of *Cryptococcus gattii*, Antimicrob. Agents Chemother. 59 (2015) 4600–4609, https://doi.org/10.1128/AAC.00466-15.
[17] M.B. d. L. Barros, R. de Almeida Paes, A.O. Schubach, *Sporothrix schenckii* and sporotrichosis, Clin. Microbiol. Rev. 24 (2011) 633–654, https://doi.org/10.1128/CMR.00007-11.

Supplementary Table 1. Feline cryptococcosis cases in Brazil described between 1971-2019.

Year of publication	Reference	State	Number of cases	Species ID	Clinical Signs
1971	Cruz *et al*.,[1]	Rio de Janeiro	1	*C. neoformans*	Swelling over the frontal region.
2002	Mendonça *et al*.,[2]	Minas Gerais	1	*C. neoformans*	Vegetative lesion with spongeous aspect in the right mandibula.
2004	Chiesa *et al*.,[3]	São Paulo	18	*C. neoformans*	No description of the clinical presentation.
2006	Juliano *et al*.,[4]	Mato Grosso do Sul	1	*C. neoformans*	Respiratory signs and swelling over the bridge of the nose.
2011	Martins *et al*.,[5]	Rio Grande do Sul	1	*C. neoformans*	Disseminated infection: multiple cutaneous nodules, anorexia, and apathy.
2013	Cardoso *et al*.,[6]	São Paulo	1	*C. gattii*	Nasal granuloma.
2014	De Paula *et al*.,[7]	Mato Grosso	2	*C. gattii*	Case 1. Respiratory distress, apathy, anorexia, cough and sneeze. Case 2. Ulcerated mass in nasal region, secretion and partial obstruction of nose.
2018	Lima *et al*.,[8]	São Paulo	1	*C. neoformans*	Respiratory signs and swelling over the bridge of the nose.
2018	Balda *et al*.,[9]	São Paulo	1	*C. neoformans*	Skin lesions on the nasal planum and the second digit of the left thoracic limb.
2019	Present study	Rio de Janeiro	2	*C. gattii*	Case1. Swelling over the forehead, polyp-like mass in the nostrils, bilateral ocular secretion and respiratory signs. Case 2. Swelling over the left periocular medial region, polyp-like mass in the nostrils, sneezing.

References:

[1] L.C.H. da Cruz, W.A. Chagas, J.B. de Figueiredo, Cryptococcosis in a cat. First case in Brazil, Cryptococcosis Cat First Case Braz. 1 (1971) 25–28.

[2] C. Mendonça, K. Waldemarin, H. Coelho, M. Lacerda, Criptococose na cavidade oral de um gato doméstico - relato de caso., Criptococose Na Cavidade Oral Um Gato Doméstico - Relato Caso. 5 (2002) 257–263.

[3] S. Chiesa, R. Castro, M. Otsuka, N. Michalany, C. Larsson Jr, C. Larsson, Cryptococcosis in São Paulo (Brazil): clinical and epidemiological features (1992-2003), Cryptococcosis São Paulo Braz. Clin. Epidemiol. Featur. 1992-2003. 15 (2004) 46.

[4] R.S. Juliano, A. Souza, R. Scheide, Criptococose felina, Criptococose Felina. 35 (2006) 65–70.

[5] D.B. Martins, R.A. Zanette, R.T. França, F. Howes, M.I. Azevedo, S.A. Botton, C. Mazzanti, S.T.A. Lopes, J.M. Santurio, Massive cryptococcal disseminated infection in an immunocompetent cat: Letter to the Editor, Vet. Dermatol. 22 (2011) 232–234. doi:10.1111/j.1365-3164.2010.00948.x.
[6] P.H.M. Cardoso, F. de A. Baroni, E.G. Silva, D.C. Nascimento, M. dos A. Martins, W. Szezs, C.R. Paula, Feline Nasal Granuloma Due to Cryptoccocus gattii Type VGII, Mycopathologia. 176 (2013) 303–307. doi:10.1007/s11046-013-9686-4.
[7] D.A. de Paula, A.B. de Almeida, F.S. da Cruz, F.H. Furlan, E.M. Colodel, V.R. Sousa, L. Nakazato, V. Dutra, Occurrence and molecular characterization of cryptococcosis in dogs and cats in Mato Grosso, Brazil, Pesqui. Veterinária Bras. 34 (2014) 167–172.
[8] P.Q. de Lima, F. P. de Oliveira, J. A. Marciano. Cryptococcosis in a cat - Case report. Rev Cient Med Vet. n. 30 (2018).
[9] A.C. Balda, J.C. Gonçalves, R.C. Menezes, A.C.F. de Souza, G.D. Cruz. Invasive cutaneous cryptococcosis of the nasal planum in a cat. Clin. Vet. 133 (2018) 26-31.

ANEXOS

ANEXO A – PARECER CONSUBSTANCIADO DO CEP

INSTITUTO DE PESQUISA
CLÍNICA EVANDRO CHAGAS -
IPEC / FIOCRUZ

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Estudo da infecção por Cryptococcus spp em crianças e investigação de fontes ambientais de agentes da criptococose no município de Santa Isabel do Rio Negro, Amazonas, Brasil

Pesquisador: Marcia dos Santos Lazera

Área Temática:

Versão: 4

CAAE: 23238913.3.0000.5262

Instituição Proponente: Instituto de Pesquisa Clínica Evandro Chagas - IPEC / FIOCRUZ

Patrocinador Principal: Fundação Oswaldo Cruz

DADOS DO PARECER

Número do Parecer: 1.226.671

Apresentação do Projeto:

A apresentação do projeto encontra-se adequada.

Objetivo da Pesquisa:

O objetivo da pesquisa é válido. Está apresentada uma justificativa válida para o uso de um grupo vulnerável no estudo.

Avaliação dos Riscos e Benefícios:

Os riscos iniciais do estudo foram bem apresentados. Também foram incluídos os riscos referentes a coleta de sangue e líquor. De acordo com os pesquisadores, os resultados deste estudo serão fundamentais para posterior orientação dos moradores, não só das casas positivas, mas também para a comunidade no entorno, buscando medidas para eliminar a presença de C.gattii neste ambiente, ou pelo menos reduzir seu acúmulo no intra-domicílio, incluindo a remoção de focos potenciais como reparo e troca de partes em decomposição nas tábuas e pintura para proteção das superfícies expostas das mesmas.

Comentários e Considerações sobre a Pesquisa:

A pesquisa tem como objetivo estudar a infecção criptocócica em crianças residentes e investigar a ocorrência dos agentes da criptococose em fontes ambientais diversas relacionadas ao domicilio e

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peridomicilio na região do alto do rio Negro, no município de Santa Isabel do Rio Negro no estado do Amazonas. Trata-se então de uma pesquisa envolvendo um grupo vulnerável (crianças e adolescentes). Também é uma região na qual há forte presença de população indígena, mesmo em áreas urbanas.

Considerações sobre os Termos de apresentação obrigatória:

A linguagem do TCLE encontra-se clara e de fácil entendimento. Estão incluídos os riscos para coleta de sangue e líquor, deixando os pais e responsáveis pelos participantes do estudo informados sobre esses procedimentos.

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

A anuência do Hospital Municipal Irmã Edwiges Maria Sirorska foi apresentada.

Sobre a questão da população indígena os pesquisadores informam que: "esta população não tem características de Comunidade Indígena. É uma população descendente de povos indígenas do Alto Rio Negro, aculturada ao longo do século XX, em um processo de concentração demográfica que resultou na formação de uma cidade. É por este motivo que não interpretamos a população do estudo como sendo constituída por Povos Índigenas no conceito que a Comissão Nacional de Ética em Pesquisa (CONEP) tem deste grupo étnico, a saber: "povos com organizações e identidades próprias, em virtude da consciência de sua continuidade histórica como sociedades pré–colombianas" como citado na Resolução nº 304 de 09 de agosto de 2000." Desta forma caracterizando a população de estudo como não indígena.

Considerações Finais a critério do CEP:

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Projeto Detalhado / Brochura Investigador	ProjetoPhdFabioBritook.doc	01/09/2015 14:45:50	Marcia dos Santos Lazera	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	ANEXO234TCLELAZERA2015.doc	01/09/2015 14:46:19	Marcia dos Santos Lazera	Aceito
Outros	ANEXO5FICHACLINICAOK.doc	01/09/2015 14:47:27	Marcia dos Santos Lazera	Aceito
Outros	ANEXO6COLETADOMICILIAROK.doc	01/09/2015	Marcia dos Santos	Aceito

Outros	ANEXO6COLETADOMICILIAROK.doc	14:48:09	Lazera	Aceito
Declaração de Instituição e Infraestrutura	AnuenciaLucilaideAnexo7.PDF	01/09/2015 14:49:00	Marcia dos Santos Lazera	Aceito
Declaração de Instituição e Infraestrutura	AnuenciaMarcusViniciusAnexo7.PDF	01/09/2015 14:49:15	Marcia dos Santos Lazera	Aceito
Declaração de Instituição e Infraestrutura	CartaAnuenciaSIRN.pdf	01/09/2015 14:49:40	Marcia dos Santos Lazera	Aceito
Folha de Rosto	FOLHADEROSTOPLATAFORMABRASILOK.PDF	01/09/2015 14:53:30	Marcia dos Santos Lazera	Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_232389.pdf	01/09/2015 14:54:35		Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

RIO DE JANEIRO, 14 de Setembro de 2015

Assinado por:
Léa Ferreira Camillo-Coura
(Coordenador)

ANEXO B- PARTICIPAÇÃO EM EVENTOS INTERNACIONAIS

10th INTERNATIONAL CONFERENCE ON
CRYPTOCOCCUS AND CRYPTOCOCCOSIS

FOZ DO IGUAÇU, BRAZIL • 26th- 30th MARCH 2017

10TH ICCC 2017, BRAZIL

Certificate

The organizing committee of the **10th International Conference on *Cryptococcus* and Cryptococcosis** presents the young investigator award to honour the outstanding study, entitleled:"»Agents of cryptococcosis in indoor dust - New evidences and challenge in Novo Airão municipality, Rio Negro river basin, in the Brazilian Amazon» to Fabio Brito-Santos

Foz do Iguaçu, March, 30th, 2017

Prof Dr.Márcia Lazera
President of the Commitee of the 10 ICCC

Institut Pasteur

Département Mycologie

Paris, le 30 mai 2015

Fabio Brito-Santos, Esp, MSc
Mycology Laboratory, National Institute of Infectiology
Oswaldo Cruz Foundation, Av. Brasil, 4365
Brésil

Ref : Invitation aux Journées Départementales du Département Mycologie de l'Institut Pasteur, 29-30 juin 2015 – Chatenay-en-France

Cher Collègue,

J'ai le plaisir de vous inviter à participer aux Journées Départementales du Département Mycologie de l'Institut Pasteur qui se tiendront les 29 et 30 juin 2015 à La Censière, 8 rue Honoré de Mirabeau, 95190 Chatenay-en-France.

Une chambre vous a été réservée pour la nuit du 29 au 30 juin. L'ensemble des frais de participation à ces deux journées sera pris en charge par le Département Mycologie de l'Institut Pasteur. Par ailleurs, une bourse d'un montant maximum de 1000 Euros vous a été attribuée par le Département Mycologie et la Division Internationale de l'Institut Pasteur pour couvrir vos frais de voyage.

Cordialement,

Prof. Christophe d'Enfert, PhD
Directeur du Département Mycologie

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