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LISANDRA SERRA DAMASCENO

AVALIAÇÃO DO PERFIL FENOTÍPICO E GENOTÍPICO DE ISOLADOS CLÍNICOS DE *Histoplasma capsulatum* NO CEARÁ, E SUA RELAÇÃO COM OS ASPECTOS CLÍNICO-EPIDEMIOLÓGICOS

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Tese apresentada como requisito para obtenção do título de Doutor do Programa de Pós-graduação *Stricto Sensu* em Pesquisa Clínica em Doenças Infecciosas da Fundação Oswaldo Cruz.

Orientadora: Profa. Dra. Rosely Maria Zancopé Oliveira
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À minha eterna e saudosa inspiração, minha
avó Osmarina, meu exemplo de mãe, mulher,
ser humano, dignidade e caráter.
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“Não há conquistas sem renúncias e sacrifícios”.

(Mosteiro de São Bento)

“Só sei que nada sei”.

(Sócrates)

Damasceno, L S. **Avaliação do perfil fenotípico e genotípico de isolados clínicos de *Histoplasma capsulatum* no Ceará, e suas relações com os aspectos clínico-epidemiológicos.** Rio de Janeiro, 2016. 239 f. Tese [Doutorado em Pesquisa Clínica em Doenças Infecciosas] – Instituto Nacional de Infectologia Evandro Chagas.

RESUMO

Histoplasma capsulatum, agente da histoplasmose clássica, é encontrado com grande frequência em toda América. Diferenças genéticas previamente observadas entre *H. capsulatum* isolados de diferentes regiões geográficas do mundo permitiram especular que genótipos distintos estão associados com as formas clínicas da enfermidade. O objetivo deste estudo foi caracterizar a variabilidade fenotípica e genética de isolados de *H.capsulatum* do Ceará, e avaliar a relação destas características com os aspectos clínico-epidemiológicos dos casos de histoplasmose diagnosticados no Hospital São José de Doenças Infecciosas (HSJ), Ceará, no período de 2011-2014. Dados clínicos e epidemiológicos foram obtidos através da revisão de prontuários médicos no HSJ. A realização dos testes fenotípicos e estudos moleculares foram conduzidos no Instituto Nacional de Infectologia Evandro Chagas, da Fundação Oswaldo Cruz (INI/Fiocruz) e Universidade Autônoma do México (UNAM). Características fenotípicas dos isolados fúngicos foram identificadas após 21 dias de cultivo no meio Agar Batata Glicosado, sendo descritos aspectos relativos à macro (textura, pigmentação) e micromorfologia. Teste de conversão dimórfica foi realizado no meio ML-Gema. Teste de exoantígenos foi avaliado através de imunodifusão radial dupla e *Western Blot* (WB). Estudos moleculares foram realizados através de reação em cadeia da polimerase (PCR), utilizando os seguintes marcadores moleculares: lócus *MAT1,* região ITS, microsatélite (GA)n e sequenciamento multilocus (MLST) de quatro genes nucleares (*arf, H-anti, ole1,* e *tub1*) em todos os isolados fúngicos. Análises estatísticas foram realizadas no STATA 11.2, e análises filogenéticas através de diversos softwares (MEGA 6, PAUP, Phylm). Durante o período do estudo, um total de 43 hospitalizações ocorreram em 40 pacientes, sendo identificados 51 isolados fúngicos. A maioria das colônias apresentou pigmentação branca e branco-bege (70,6%), e tinham textura cotonosa (82%). A presença de todas as estruturas de esporulação nas colônias filamentosas foi observada em 74,5% dos isolados. Conversão dimórfica ocorreu em 98% das cepas. Ambos os antígenos H e M foram identificados em 54,9% dos isolados através de WB. Dentre os 51 isolados fúngicos estudados, 53% eram *MAT1-2,* e 47% *MAT1-1*. Colônias pulverulentas foram associadas com a presença de manifestações hemorrágicas nos pacientes, e colônias de pigmentação branca e branco-bege foram relacionadas à insuficiência renal aguda. Óbito foi associado com a presença de isolados que continham todas as estruturas de esporulação. Análise filogenética identificou uma população genética restrita ao Ceará/Brasil. Além disso, observaram-se ainda quatro haplótipos diferentes ao grupo de isolados do Ceará, sendo dois haplótipos descritos pela primeira vez no mundo. Através de análise de MLST foi possível confirmar a presença de uma nova população genética de *H. capsulatum* circulante no Ceará, com dois subgrupos genéticos diferentes entre os isolados fúngicos, com significante taxa de recombinação genética. Diferentes fenótipos, e genótipos, incluindo a presença de *MAT1-2*, que ainda não havia sido identificado no Brasil, foram observados neste estudo. Além disso, isolados de *H. capsulatum* procedentes do Ceará constituíram um novo grupo genético, com diferentes subpopulações.

Palavras-chaves: 1. Histoplasmose. 2. *Histoplasma capsulatum*. 3. Fenótipo. 4. Diversidade genética. 5. Tipo de compatibilidade sexual. 6. Sequenciamento multilocus.

Damasceno, L S. **Evaluation of phenotypic and genotypic profile of *Histoplasma capsulatum* clinical isolates in Ceará, and the correlation with clinical and epidemiological aspects.** Rio de Janeiro, 2016. 239 f. Thesis [Doctoral in Clinical Research in Infectious Disease] – Instituto Nacional de Infectologia Evandro Chagas.

ABSTRACT

Histoplasma capsulatum is the etiologic agent of histoplasmosis. It is found in endemic areas around the world, mainly in the Americas. A high genetic variability has been observed among *H. capsulatum* isolates from different regions, and some genotypes have been associated to pathogenesis of histoplasmosis. The aim of this study was to characterize the phenotipc and genetic variability of *H.capsulatum* isolates from Ceará/Brazil. In addition, the relationship between the phenotype and genotype with epidemiologic and clinical aspects of histoplasmosis-patients diagnosed at Hospital São José de Doenças Infecciosas (HSJ), Ceará, during 2011 to 2014 period, was evaluated. Epidemiologic and clinical data were obtained by review of medical records. Phenotypic and molecular studies were conduceted at Instituto Nacional de Infectologia Evandro Chagas, da Fundação Oswaldo Cruz (INI/Fiocruz) and Universidade Autônoma do México (UNAM). Phenotypic characteristics were observed after 21 days of culture in Potato Dextrose Agar. The macromorphological aspects about texture, pigmentation, and micromorfology were identified. Dimorphic conversion was performed in ML-Gema Agar. Exoantigens were detected by immunodifusion and Western Blot (WB). Molecular studies were performed by polymerase chain reaction (PCR). The molecular markers used were *MAT1* locus, ITS region, (GA)n microsatellite and multilocus sequence type (MLST) of 4 nuclears genes (*arf, H-anti, ole1,* e *tub1*). Statistical analyses were performed in STATA 11.2, and phylogenetic analysis by different softwares (MEGA 6, PAUP, Phylm). A total of 43 hospitalizations occurred in 40 patients. Fifty-one fungal isolates were identified. Albino and albino-beige colonies were observed in 70.6% of isolates and 82% of strains had cottony appearance. The presence of all characteristic structures of mycelia-form was observed in 74.5% of fungal isolates. Dimorphism occurred in 98% of strains. All fungal isolates were immunoidentificated by WB, Both H and M antigens were observed in 54.9% of fungal isolates by WB in the exoantigen test. Among the 51 isolates included in this study, 53% were *MAT1-2* and 47% were *MAT1-1*. Powdery colonies were associated with hemorrhagic manifestation, and pallid pigmentation with acute renal failure. Colonies that contained macroconidia were associated with death. The phylogenetic analyses of fungal isolates identified one restrict genetic population in Ceará/Brazil, and four distinct haplotypes were presented in the fungal isolates from Ceará. Two news unprecedented haplotypes (GA)n sequence length in that population were also highlighted. MLST analysis also confirmed the detection of a new genetic population, composed mainly for fungal isolates from Ceará. Two different clusters were identified within the study population, with a significant genetic recombination rate. Distinct phenotypes and genotypes, as MAT1-2 idiomorph that there are not been described in Brazil, were identified in this study. In addition, fungal isolates from Ceará constituted a new genetic population of *H. capsulatum*, with different genetic subpopulations.

Key-words: 1. Histoplasmosis. 2. *Histoplasma capsulatum*. 3. Phenotype. 4. Genetic diversity. 5. Mating type. 6. Multilocus sequence type.

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LISTA DE ABREVIATURAS

ARF	*ADP – Ribosylation Factor* (Fator de ribosilação do ADP)
BHI	Brain Heart Infusion Agar (Agar infusão cérebro-coração)
CBP	Calcium-Binding Protein (Proteína ligante de cálcio)
EIA	*Enzyme Immunoassay* (Ensaio imunoenzimático)
ELISA	*Enzyme-Linked Immunosorbent Assay* (Ensaio de imunoabsorção enzimática)
HAART	Highly Active Antiretroviral Therapy (Terapia Antirretroviral de Alta Potência)
H-anti	*H – antigen precursor* (Precurssor do antígeno-H)
HD	Histoplasmose Disseminada
HDA	Histoplasmose Disseminada Aguda
HDC	Histoplasmose Disseminada Crônica
HDS	Histoplasmose Disseminada Subaguda
HPA	Histoplasmose Pulmonar Aguda
HPC	Histoplasmose Pulmonar Crônica
ID	Imunodifusão radial dupla
ITS	*Internal Transcribed Spacer* (ITS1-5.8-ITS2)
MLST	Multilocus Sequencing Typing (Sequenciamento multilocus)
mtDNA	DNA mitocondrial
OLE1	Δ-*9-Fatty Acid desaturase* (delta-9 desaturase do ácido graxo)
PAMP	Pathogen-Associated Molecular Patterns (Padrões moleculares associados ao patógeno)
PAS	Periodic Acid -Schiff (Ácido Periódico-Schiff)
PCR	Polymerase Chain Reaction (Reação em Cadeia da Polimerase)
PDA	Potato Dextrose Agar (Agar Batata Glicosado)
PFGE	Pulsed-Field Gel Electrophoresis (Eletroforese em gel de campo pulsado)
PRRs	Pattern Recognition Receptors (Receptores de reconhecimento de padrão)
PVHA	Pessoas Vivendo com HIV/aids
RAPD	Random Amplified Polymorphic DNA (Polimorfismos de DNA amolificados randomicamente)
rRNA	RNA ribossômico
rDNA	DNA ribossomal
RFLP	Restriction Fragment Length Polymorphisms (Fragmentos de DNA gerados por enzimas de restrição)
SNC	Sistema Nervoso Central
TPFF	Tecido parafinado fixado em formaldeído
TUB1	α-tubulin (α-tubulina)
WB	*Western Blot*

SUMÁRIO

1 INTRODUÇÃO

Histoplasma capsulatum é um fungo dimórfico, que se encontra na natureza em sua fase infectante multicelular, a qual é sapróbia-geofílica. Em hospedeiros suscetíveis cresce em sua fase leveduriforme, que é a forma parasitária unicelular, e que pode causar infecções tanto no homem como em diversos animais (ZANCOPÉ-OLIVEIRA; MUNIZ; WANKE, 2005). Existem três variedades biológicas patogênicas, *H. capsulatum* var. *capsulatum,* de distribuição universal, responsável pela maioria das infecções clássicas; *H. capsulatum* var. *duboisii*, responsável pela histoplasmose africana, sendo restrita a esse continente; e, *H. capsulatum* var. *farciminosum*, causador da linfangite epizoótica dos equinos (ZANCOPE-OLIVEIRA; MUNIZ; WANKE, 2005). *H. capsulatum,* agente etiológico da histoplasmose, já foi isolado em praticamente todos os continentes, com exceção da Antártida, entretanto, tem um nítido predomínio na América e na África. A doença causada por este fungo depende do tempo e da intensidade da exposição à micronichos do patógeno, da quantidade do inóculo, da imunidade celular mediada por células T CD4+, dos fatores de virulência e dos genótipos das linhagens isoladas (FERREIRA; BORGES, 2009).

1.1 REVISÃO DA LITERATURA

1.1.1 Histórico

A descoberta do fungo *H. capsulatum* foi realizada em 1905, por Samuel Taylor Darling, patologista americano que trabalhou no *Ancon Hospital* no Panamá, durante a construção do Canal do Panamá (DARLING, 1906;1909). Darling, ao realizar necropsia de um homem negro, adulto, nativo da Martinica, que havia falecido de uma doença febril, originalmente atribuída à tuberculose miliar, verificou a presença de numerosos corpos pequenos intracelulares com "cápsulas", no interior dos alvéolos pulmonares e de macrófagos e histiócitos de baço, fígado, linfonodos e medula óssea, semelhantes a protozoários da espécie *Leishmania sp.*, descritas por Leishman e Donovan, porém sem cinetoplasto. Em

razão desse fato, pensava tratar-se de um novo protozoário intracelular encapsulado, o qual denominou de *H. capsulatum* (DARLING, 1906;1909). Posteriormente, entre 1905-1908, Darling realizou mais duas necrópsias em dois indivíduos que haviam desenvolvido emaciação, esplenomegalia, febre, leucopenia e anemia. Os achados histopatológicos evidenciaram invasão de células endoteliais de vasos sanguíneos por *H. capsulatum,* causando necrose hepática com cirrose, pseudogranuloma em pulmões e intestinos, e necrose de linfonodos (DARLING, 1906;1909).

Em 1912, o patologista e microbiologista brasileiro, Henrique da Rocha Lima, após comparar o material examinado por Darling, com material de necropsia de um paciente com leishmaniose visceral, concluiu que *H. capsulatum* estava mais intimamente relacionado com fungos do que com protozoários (DA ROCHA-LIMA, 1912).

Até 1933, o fungo ainda não havia sido isolado em laboratório. Entretanto, em maio desse mesmo ano, Hansmann e Schenken, na reunião da Associação Americana de Patologistas e Microbiologistas em Washington/EUA, relataram a identificação de leveduras em um paciente, a partir de cultivos de biópsias de pele, linfonodos inguinais e mucosa oral. Após o óbito deste paciente, exame histopatológico confirmou a presença de leveduras em tecidos pulmonares, adrenais, pele e linfonodos. Cultivos à temperatura ambiente foram realizados, sendo observado o desenvolvimento de micélio aéreo com clamidósporos tuberculados. Apesar disso, este caso foi reportado como uma "nova" doença causada por um fungo, o qual foi descrito como sendo pertencente ao gênero *Sepedonium*, devido *H. capsulatum* não ter sido encontrado no baço (HANSMANN; SCHENKEN, 1934).

Em novembro de 1933, Dodd e Tompkins, na reunião da Sociedade Americana de Medicina Tropical, reportaram um caso de histoplasmose diagnosticado ainda em vida. Os autores relataram o encontro de estruturas ovais dentro de células mononucleares em esfregaços de sangue periférico (DODD; TOMPKINS, 1934). Após cultivar o sangue desse paciente em Agar Sangue de coelho 10% a 37ºC, De Monbreun verificou o crescimento de leveduras ovais. Em cultivos em Agar Sangue e Agar Sabouraud com glicose à temperatura ambiente, o mesmo autor observou o desenvolvimento de hifas aéreas de caráter cotonoso, e descreveu o fungo como *H. capsulatum* de Darling, comprovando assim a natureza fúngica e o caráter dimórfico deste patógeno (DE MONBREUN, 1934).

Ciferri e Redaelli (1934) demonstraram a conversão micélio-levedura em laboratório, após cultivar os isolados dos dois casos descritos anteriormente, em sua fase micelial em Agar Sangue a 37ºC, identificando o crescimento de leveduras ovais em brotamento (CIFERRI; REDALLI, 1934). Conant (1940) descreveu o ciclo biológico e todas as características

morfológicas de *H. capsulatum*, após sucessivos cultivos em meios de cultura artificiais (CONANT, 1940).

No Brasil, o primeiro isolamento de *H. capsulatum* foi realizado por Almeida e Lacaz em 1939, a partir de um cultivo de fragmento de biopsia de uma lesão de cromoblastomicose (ALMEIDA; LACAZ, 1939). Os mesmos autores descreveram o segundo isolamento, em 1941, em escarro de um paciente com tuberculose pulmonar (ALMEIDA; LACAZ, 1941). O primeiro caso de histoplasmose diagnosticado no Brasil ocorreu em 1941, em uma criança de três anos de Minas Gerais, a qual desenvolveu a forma juvenil desta micose (VILELA; PARÁ, 1941).

O isolamento de *H. capsulatum* na natureza ocorreu em 1948, em amostras de terra obtidas de solo de um galinheiro na entrada de uma toca de camundongos localizada nos EUA (EMMONS, 1949). Em 1960, o fungo voltou a ser identificado em laboratório em amostras de solo de um parque urbano no centro de Washington/EUA (EMMONS, 1961). Os principais fatos históricos sobre o patógeno *H. capsulatum* estão resumidos na figura 1.

Figura 1 – *H. capsulatum* – fatos históricos mais importantes
Elaborado pelo autor

1.1.2 O FUNGO *H. capsulatum*

1.1.2.1 Morfotipos

Histoplasma capsulatum é encontrado na natureza na fase saprofítica, onde vive sob a forma de micélio haplóide. Nesse substrato natural, bem como em cultivos mantidos entre 25-28ºC, apresenta-se como fungo filamentoso (morfotipo-M), e que macroscopicamente podem ser de dois tipos de colônias: as do tipo A de coloração branca e aspecto cotonoso, que crescem mais rapidamente em cultura; e as colônias do tipo B de coloração acastanhada, geralmente de aspecto pulverulenta, com crescimento mais lento (BERLINER, 1968; EISSENBERG; GOLDMAN, 1991). Em geral, as colônias levam aproximadamente duas semanas para se tornarem evidentes, após a conversão levedura-micélio, e três a quatro semanas para alcançarem a maturidade (NEGRONI, 2005). As estruturas microscópicas do morfotipo-M são representadas por hifas hialinas, delgadas, septadas e ramificadas (Figura 2). O micélio de reprodução é constituído por dois tipos de conídios: os microconídios que são esporos ovalados de paredes lisas, medindo de 2-5µm de diâmetro, localizados na extremidade de curtos conidióforos em ângulo reto com a hifa vegetativa; e os macroconídios que são esporos esféricos ou piriformes, largos (8 a 15µm de diâmetro), com uma parede celular espessa, os quais geralmente apresentam-se com expansões digitiformes semelhantes a tubérculos, sendo conhecidos como macroconídios tuberculados (DEEPE JR, 2005; ZANCOPE-OLIVEIRA; MUNIZ; WANKE, 2005; KAUFFMAN, 2007).

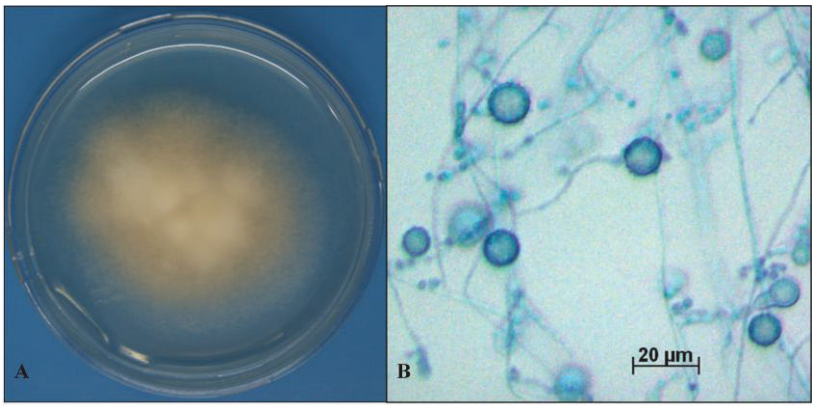

Figura 2 – Morfologia do morfotipo-M de *H. capsulatum:* (A) Colônias de coloração acastanhada em cultivo no meio Agar batata-glicosado (PDA); (B) Hifas hialinas, septadas com micro e macroconídios tuberculados corados por lactofenol azul-algodão.
Elaborado pelo autor

A fase leveduriforme (morfotipo-L) é encontrada em parasitismo no interior de macrófagos ou em células gigantes, raramente em polimorfonucleares, e quando cultivados a 37ºC em meios de cultura ricos em aminoácidos e proteínas, como Agar Sangue de coelho a 10%, Agar infusão cérebro-coração (BHI) com glicose 1% e cisteína 0,1%, Agar ML-Gema (FRESSATTI *et al.*, 1992) e Tewari (TEWARY; KEGEL, 1971). São encontrados sob a forma de corpos ovais, unibrotantes, uninucleadas, com diâmetro de 2 a 5µm (Figura 3) e parede celular relativamente larga (CONANT, 1940; ZANCOPE-OLIVEIRA; MUNIZ; WANKE, 2005). Levam cerca de dez dias a duas semanas para serem visualizadas após a conversão micélio-levedura (CONANT, 1940).

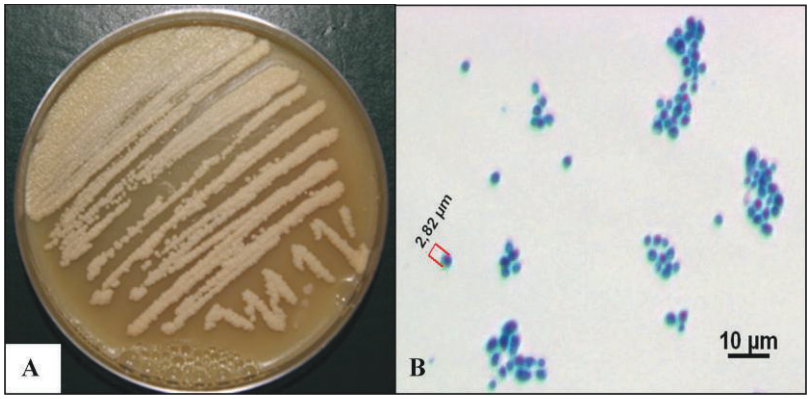

Figura 3 – Morfologia do morfotipo-L de *H. capsulatum:* (A) colônias úmidas, de coloração creme em cultivo no meio ML-Gema; (B) estruturas ovais, unibrotantes coradas por lactofenol azul-algodão.
Elaborado pelo autor

Nas preparações coradas com Giemsa ou Wright, a parede celular não capta corante. Aparece como um halo claro, com uma massa cromática polar azul e na forma de meia lua. Na coloração de Gram, mostra-se como um Gram positivo, e nas preparações coradas com ácido periódico de Shiff (PAS) tem coloração arroxeada. Tinge-se de marrom ou negro com a metanamina prata de Grocott (WANKE; LAZÉRA, 2004; ZANCOPE-OLIVEIRA; MUNIZ; WANKE, 2005). No morfotipo-M, *H. capsulatum* var. *capsulatum* e *H. capsulatum* var. *duboisii* são indistinguíveis, mas diferem quanto ao morfotipo-L: na var. *duboisii*, as leveduras são maiores, medindo até 20µm, e têm paredes mais espessas do que na var. *capsulatum* (EISSENBERG; GOLDMAN, 1991; FERREIRA; BORGES, 2009).

1.1.2.2 Composição da parede celular

A parede celular dos fungos usualmente é constituída por carboidratos, lipídios e proteínas. Os polissacarídeos representam 80% desta composição (BERNARD; LATGE, 2001; GUIMARÃES; DE CERQUEIRA; NOSANCHUK, 2011). Em *H. capsulatum*, os polissacarídeos mais abundantes são as glucanas, que são polímeros de D-glicose ligados por pontes α ou β-glicosídicas. Ambas, α e β-glucanas, estão presentes na parede celular tanto do morfotipo-M como do morfortipo-L (DOMER; HAMILTON; HARKIN, 1967). Outros polissacarídeos encontrados na parede celular do fungo são: quitina, composta por β-1,4 N-acetil-D-glucosamina; mananas que são polímeros da manose usualmente ligados a proteínas (manoproteinas); e componentes semelhantes à lecitina (GUIMARÃES; DE CERQUEIRA; NOSANCHUK, 2011).

Dependo do morfotipo, a distribuição e a organização dos polissacarídeos na parede celular do fungo são diferentes. O morfotipo-L apresenta grande quantidade de α-1,3-glucana, quitina e copolímeros da galactomana, enquanto que o morfotipo-M contém elevada quantidade de β-1,3-glucana e galactomanana (LARA-LEMUS *et al.*, 2014). De acordo com a solubilidade dos polissacarídeos e quantidade de quitina na parede celular do fungo, o morfotipo-L é classificado em quimiotipos I e II. Leveduras com parede celular quimiotipo I caracterizam-se por conter menor quantidade de material solúvel e abundância em quitina, do que o quimiotipo II, o qual apresenta elevadas quantidades de α-1,3-glucana (DOMER, 1971; EDWARDS; ALORE; RAPPLEYE, 2011; LARA-LEMUS *et al.*, 2014).

Os lipídios constituem 3-10% da parede celular de *H. capsulatum*. São representados pela monohexosidase ceramida, importantes para a sobrevida do fungo. Em geral, são carreados por vesículas extracelulares que transportam lipídios, proteínas, polissacarídeos e pigmentos. Estas, estão envolvidas em diversos processos do metabolismo, reciclagem celular, sinalização e virulência do patógeno (ALBUQUERQUE *et al.*, 2008; GUIMARÃES; DE CERQUEIRA; NOSANCHUK, 2011).

Diversas proteínas podem ser encontradas na parede celular de *H. capsulatum,* entretanto, mudanças na composição desta parede ocorrem naturalmente para adaptação ao ambiente, devido às modificações da interação patógeno-hospedeiro, e como defesa do fungo contra o estresse oxidativo. Dentre as proteínas que participam dos mecanismos citados anteriormente destacam-se: HsP60, HsP70, antígeno M, antígeno H, histonas 2B, YPS3 e melanina (GUIMARÃES; DE CERQUEIRA; NOSANCHUK, 2011). A proteína YPS3 é

expressa somente no morfotipo-L (SHEARER *et al.*, 1987; BOHSE; WOODS, 2005;2007; GUIMARÃES *et al.*, 2011). Todas essas moléculas têm importantes papéis biológicos, seja em manter a integridade e rigidez da célula fúngica, como também, modular e participar da reposta imune na interação patógeno-hospedeiro, atuando como fatores de virulência para o mecanismo da doença (GUIMARÃES; DE CERQUEIRA; NOSANCHUK, 2011).

Diferenças na composição bioquímica e molecular na parede celular do morfotipo-L de *H. capsulatum* foram observadas em três diferentes grupos filogenéticos (Figura 4). Na cepa americana G-217B (classificada filogeneticamente como NAm 2) foi verificado ausência de α-glucano e importante expressão do gene *Yps3*. Já na cepa americana Downs (classificada filogeneticamente como NAm 1) e na panamenha G-186A (classificada filogeneticamente como linhagem) observou-se presença de α-glucano na parede celular e ausência de excreção da proteína yps3. Somente na cepa Downs identificou-se a secreção uma de serino-protease. A presença das proteínas HSP60 e H2B na parede celular do morfotipo-L, assim como, a secreção da proteína ligadora de cálcio (CBP) e da ferro-redutase dependente de glutationa, estão presentes em todos os grupos filogenéticos estudados (KEATH *et al.*, 1989; HOLBROOK; RAPPLEYE, 2008).

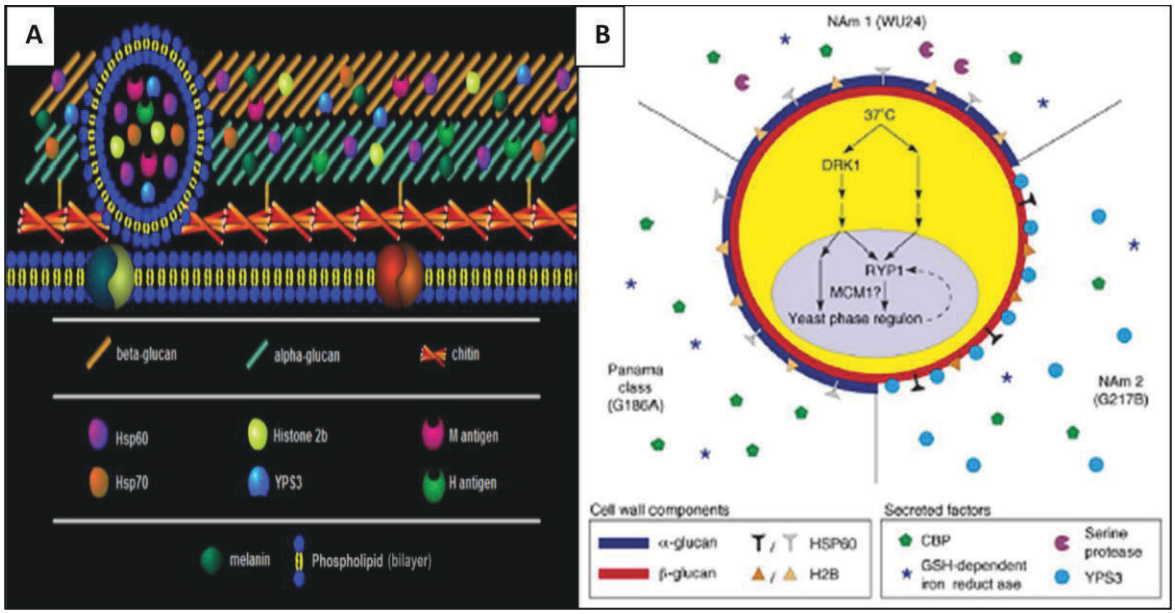

Figura 4 – Parede celular de *H. capsulatum:* (A) Esquema representativo (B) esquema ilustrativo demonstrando a diversidade bioquímica e molecular na parede celular do morfotipo-L de *H. capsulatum* em três diferentes clados filogenéticos.
Fontes: (A) GUIMARÃES; DE CERQUEIRA; NOSANCHUK, 2011, p. 3.; (B) HOLBROOK; RAPPLEYE, 2008, p. 319.

1.1.2.3 Reprodução

A reprodução é o processo no qual uma célula inicial ou célula-mãe dá origem a novas células. Nos fungos, a reprodução pode ocorrer de forma sexuada, assexuada e parasexuada. *H. capsulatum* se reproduz de forma assexuada e sexuada. Na reprodução assexuada as células são originadas através de mitose, e os indivíduos gerados apresentam material genético idêntico ao do seu progenitor. A mitose se realiza várias vezes dentro do ciclo de vida fúngico, produzindo dessa maneira grande quantidade de propágulos, que permite a preservação e propagação de organismos genomicamente bem adaptados, assim como a rápida e eficiente colonização de novos ambientes. Esse tipo de reprodução denomina-se conidiogênese, onde os esporos se formam nas extremidades de hifas especiais denominadas conidióforos, os quais dão origem aos conídios (GAMBALE, 2010; CHIU, 2012).

Na reprodução sexuada, as células se dividem através de meiose. Células fúngicas especializadas são capazes de realizar pareamentos, com consequente troca de material genético, originando indivíduos geneticamente distintos, contribuindo para a variabilidade genética deste patógeno (CHIU, 2012). Esporos de origem sexuada são denominados ascósporos e são formados no interior de células especiais denominadas ascos. Os ascos com ascósporos são encontrados no interior de receptáculos, denominados ascocarpos (GAMBALE, 2010).

Histoplasma capsulatum na forma assexuada ou anamorfa é um organismo eucarioto heterotálico, que se apresenta como micélio haploide associado a dois tipos de compatibilidade sexual ou "*mating types*": (+) ou "major" e (-) ou "minor". *H. capsulatum*, com suas três variedades biológicas, apresenta um estado sexual ou perfeito (forma teleomorfa) que resulta do pareamento de seus dois tipos de compatibilidade sexual, constituindo a espécie *Ajellomyces capsulatus*. Estas duas espécies *H. capsulatum* e *A. capsulatus* constituem o mesmo fungo holomorfo (KWON-CHUNG, 1972a;b; KWON-CHUNG; WEEKS; LARSH, 1974; KWON-CHUNG; BARTLETT; WHEAT, 1984).

Na década de 1970, identificação *in vitro* dos tipos de compatibilidade sexual era realizada através do pareamento de isolados fúngicos. Entretanto, esta é uma técnica de difícil manuseio devido à rápida perda de fertilidade do fungo. Estudo realizado com isolados clínicos e ambientais de *H. capsulatum,* através de pareamentos em laboratório, verificou que o tipo de compatibilidade (-) foi predominante em isolados clínicos (7:1), enquanto que em

isolados ambientais, observou-se a presença de igual proporção de ambos os tipos de compatibilidade sexual (KWON-CHUNG; WEEKS; LARSH, 1974).

Em 2007, o lócus *MAT1* foi determinado através de amplificação por reação em cadeia da polimerase (PCR), utilizando iniciadores específicos para os idiomorfos *MAT1-1* e *MAT1-2* que definem os tipos de compatibilidades sexuais (+) e (-) de *H. capsulatum*, respectivamente (BUBNICK; SMULIAN, 2007). Estudos moleculares recentes têm evidenciado que as frequências dos dois tipos de compatibilidade sexual deste patógeno se relacionam com a origem geográfica de procedência do isolado fúngico (RODRIGUEZ-ARELLANES *et al.*, 2013; MUNIZ *et al.*, 2014). No México e EUA, ambos os tipos de compatibilidade sexual já foram encontrados, tanto em isolados fúngicos ambientais quanto em isolados humanos. Entretanto, no Brasil, até o momento, o tipo de compatibilidade sexual (+) ou MAT1-1 tem sido o único observado em isolados clínicos e ambientais (RODRÍGUEZ-ARELLANES *et al.*, 2013).

1.1.2.4 Taxonomia

Histoplasma capsulatum, é um eucarioto que pertence ao reino *Fungi*, subreino *Dikaria*, filo *Ascomycota*, subfilo *Pezizomycotina*, classe *Eurotyomicetes*, subclasse *Eurotiomycetidae*, ordem *Onygenales*, família *Ajellomycetaceae*, gênero *Histoplasma* (anamorfo) ou *Ajellomyces* (teleomorfo), espécies *H. capsulatum* ou *A. capsulatus*, respectivamente (HIBBETT *et al.*, 2007; LUMBSCH; HUHNDORF, 2007; LUMBSCH *et al.*, 2007; KWON-CHUNG, 2012).

Apesar de existirem três variedades biológicas de *H. capsulatum* (*capsulatum, duboisii* e *farciminosum*), atualmente *H. capsulatum* tem sido considerado um complexo de espécies, baseado na definição de espécie filogenética, contrariando o conceito de espécie biológica, a qual se baseia no isolamento reprodutivo. O conceito de clado e espécie filogenética em fungos foi proposto por Dettman et al., (2003a), o qual avaliou a reorganização de fungos filamentosos do gênero *Neurospora*. Clado filogenético é reconhecido como uma linhagem evolutiva independente, através da reorganização das espécies, e está baseado na concordância genealógica, se satifeito um dos dois critérios abaixo (DETTMAN et al., 2003a):

- ✓ Concordância genealógica: o clado deve estar presente na maioria das genealogias de lócus único.
- ✓ Genealogia não-discordante: o clado é bem suportado em pelo menos, uma genealogia de lócus único, suportado por ambos os parâmteros, proporção de bootstrap (≥ 70%) através da análise de máxima parcimônia, e probabilidade posterior Bayseana (≥ 0.95). Além disso, o clado não pode ser contrariado em nenhuma outra genealogia de lócus único no mesmo nível de apoio.

Para definição de espécie filogenética, dois critérios de classificação adicionais devem ser aplicados (DETTMAN et al., 2003b):

- ✓ Diferenciação genética: espécies filogenéticas têm que ser relativamente distintas e bem diferenciadas de outras espécies.
- ✓ Subdivisão exaustiva: todos os indivíduos tem que ser alocados dentro de uma espécie filogenética, e nenhum indivíduo pode ficar como não classificado.

Estudos baseados no sequenciamento multilocus (MLST) de quatro genes codificadores de proteínas nucleares, funcionalmente e geneticamente relacionados (*ole1*- delta-9 desaturase do ácido graxo; *tub1*- α-tubulina; *arf* - fator de ribosilação do ADP; e *H-anti* - precursor do antígeno H), constitutivos do genoma de *H. capsulatum*, têm sido realizados para identificação de diferentes espécies filogenéticas de *H. capsulatum*. Estes genes foram escolhidos por serem lócus que evoluíram independentemente no genoma de *H. capsulatum* (KASUGA et al., 1999;2003). A seguir estão descritos os genes e as principais funções relacionadas com eles:

- ✓ O gene *arf* (fator de ribosilação do ADP) codifica um cofator proteico (proteína G ou ligante de GTP) que é fundamental para a fisiologia celular durante a ribosilação do ADP. Este gene está envolvido no transporte de proteínas e vesículas (LODGE *et al.*, 1994).
- ✓ O gene *H-anti* codifica para o precursor do antígeno H, uma β-glicosidase, o qual está envolvido na aquisição de nutrientes e na modulação da arquitetura da parede celular fúngica (DEEPE; DUROSE, 1995).
- ✓ O gene *ole1* (delta-9 desaturase do ácido graxo) codifica para uma enzima, delta-9 ácido graxo desaturase, que converte ácidos graxos saturados em insaturados (principalmente em ácido oleico), os quais são importantes moléculas que participam na modulação de fluidos pela membrana celular do fungo. É considerado um gene do choque térmico, já que se expressa

preferencialmente em altas temperaturas (34-40ºC) durante a fase de transição micélio-levedura (GARGANO *et al.*, 1995).

- ✓ O gene *tub1* está envolvido na morfogênese do fungo e, portanto, tem um papel fundamental no processo de diferenciação do *H. capsulatum*. Codifica a proteína α-tubulina na fase leveduriforme, que quando submetida a 25ºC sofre ação do AMPc que promove a polimerização da proteína, levando à transição levedura-micélio (MEDOFF; JACOBSON; MEDOFF, 1981; HARRIS; KEATH; MEDOFF, 1989).

Em 2003, análise filogenética através de MLST foi realizada com uma matriz combinada dos quatros genes, onde 137 isolados de *H. capsulatum* de diferentes fontes, e procedentes de 25 países identificou oito clados filogenéticos: dois clados restritos à América do Norte (Norte-América classe 1 - NAm 1, e Norte-América classe 2 - NAm 2), dois clados encontrados predominantemente na América Latina (Latino-América A - LAm A, e Latino-América B - LAm B), um clado restrito à Austrália, um clado encontrado entre as regiões da Holanda e Indonésia, um clado identificado entre as regiões da Europa e Ásia (Euroasiano), e um clado restrito à África. Sete desses oito clados são considerados espécies filogeneticamente relacionadas. A única exceção foi o clado Eurasiano que se agrupou com o clado LAm A (KASUGA; TAYLOR; WHITE, 1999; KASUGA *et al.*, 2003). *Histoplasma capsulatum* var. *capsulatum* foi observado em todos os clados; *H. capsulatum* var *duboisii* encontra-se restrito ao clado Africano; e, *H. capsulatum* var *farciminosum* encontra-se distribuído nos clados Nam 2, Africano e Eurasiano (KASUGA *et al.*, 2003; VITE-GARIN *et al.*, 2014).

Novas análises usando MLST em isolados fúngicos de gatos, procedente de área não endêmica dos EUA, e isolados de *H. capsulatum* oriundos de morcegos do México sugeriram a presença novos clados e linhagens filogenéticas (TAYLOR; CHÁVEZ-TAPIA *et al.*, 2005; ARUNMOZHI BALAJEE *et al.*, 2013; VITE-GARIN *et al.*, 2014). Mais recentemente, um estudo mais robusto, baseado análises filogenéticas e estrutura de populações, com maior número de isolados de *H. capsulatum* (n= 234), identificou uma grande diversidade genética dentro do grupo das espécies filogenéticas oriundas da América Latina, sendo propostas cinco novos espécies dentro dessa região (Figura 5). O antigo clado LAm A foi subdividido em: LAm A1 (contendo principalmente isolados do México, Colômbia, Guatemala e Honduras), LAm A2 (composto por isolados do México, Guatemala e Colômbia) e RJ (composto por isolados do Rio de Janeiro e São Paulo); o antigo clado LAm B foi reagrupado em LAm B1 (contendo isolados da Argentina, Colômbia e Brasil) e LAm B2 (contendo isolados do Rio

Grande do Sul e Espírito Santo – Brasil). Os autores ainda propuseram uma nova espécie formada por isolados fúngicos oriundos de morcegos (*Tadarida brasiliensis e Mormoops megalophylla*) do México, denominado BAC-1. No Brasil, o mesmo estudo ainda identificou quatro clados monofiléticos diferentes: BR1 (composto por isolados de São Paulo), BR2 (composto por isolados do Rio de Janeiro, São Paulo e Ceará), BR3 (composto por isolados do Rio de Janeiro, São Paulo e Espírito Santo), e BR4 (composto por isolados de Pernambuco e outro isolado sem identificação) (TEIXEIRA *et al.*, 2016).

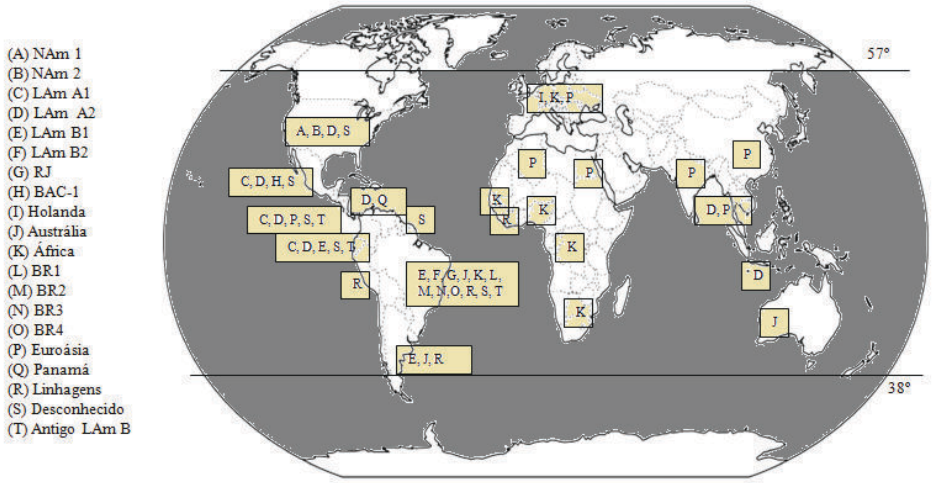

Figura 5 – Distribuição geográfica dos clados genéticos de *H. capsulatum*
Elaborado pelo autor, baseado em TEIXEIRA *et al*., 2016.

1.1.2.5 Diversidade genética

A biologia molecular tem sido de grande importância para a identificação e análise do comportamento e patogenicidade de *H. capsulatum*, assim como na diferenciação de indivíduos e no estudo filogenético desses organismos (WOODS, 2002). Fornece informações sobre aspectos epidemiológicos, caracterização das linhagens, distinção entre cepas, e dados importantes para determinar recidivas ou novas infecções. Além disso, a aplicação das técnicas moleculares tem possibilitado a identificação de diferentes fontes de infecção e a determinação de uma relação genotípica entre isolados clínicos de pacientes procedentes de diferentes regiões geográficas, o que tem contribuído para o conhecimento de áreas endêmicas de histoplasmose (CARTER *et al.*, 2001; TAYLOR; CHÁVEZ-TAPIA *et al.*, 2005).

Um grande número de técnicas moleculares, baseadas na detecção de DNA, tem sido usadas para caracterização genética de *H. capsulatum*. Essas técnicas diferem de acordo com o alvo acessado e a metodologia aplicada. Os primeiros estudos sobre a diversidade do genoma do fungo foram realizados na década de 1980, através da análise de fragmentos de DNA gerados por enzimas de restrição (RFLP), a qual identificou diferenças intraespécie de isolados de *H. capsulatum* (VINCENT *et al.*, 1986; SPITZER *et al.*, 1989; KEATH; KOBAYASHI; MEDOFF, 1992; IGNATOV; KEATH, 2002). Este método consiste na digestão total do DNA genômico com endonucleases de restrição, seguida de *Southern blot* e hibridização dos fragmentos com sondas marcadas (BOTSTEIN *et al.*, 1980). As principais regiões analisadas através desse método são: DNA mitocondrial (mtDNA), DNA ribossomal (rDNA), e o lócus do gene nuclear da proteína yps-3 complementado com subtipagem do mtDNA.

Análises por RFLP do mtDNA e rDNA com a enzima de restrição *Eco*RI agrupou inicialmente *H. capsulatum* em três classes, com base na fonte e origem geográfica: classe 1 – representada pela cepa Downs (isolado clínico de Illinois/EUA); classe 2 – representada por cepas da América do Norte (isolados clínicos de Louisiana/EUA – G-217 [A e B] e G-222) e da África; classe 3 – inclui cepas da América Central (isolados clínicos do Panamá - G184 [A e B], e G-186 [A e B]) (VINCENT *et al.*, 1986). Em 1989, Spitzer *et* al. (1989) reproduziu esta mesma técnica com a endonuclease de restrição *Hae*III, com alguns isolados clínicos do estudo anterior, além de novos isolados fúngicos ambientais. Os autores identificaram uma quarta classe, representada por um único isolado ambiental (FSL1 – Flórida/EUA) (SPITZER *et al.*, 1989). Posteriormente, análise do lócus *Yps*-3 com as enzimas de restrição *Ava*I e *Hind*III, com as mesmas cepas do estudo de Sptizer *et* al. (1989), e novos isolados clínicos de *H. capsulatum* procedentes da América Latina, identificou duas novas classes: RFLP classe 5 e 6 obtidas de pacientes com aids. Através de subtipagem do mtDNA, a classe 5 foi classificada em quatro novas subclasses (KEATH; KOBAYASHI; MEDOFF, 1992).

Outra técnica utilizada para discriminar cepas de *H. capsulatum* se dá através da detecção de fragmentos de DNA amplificados ao acaso (RAPD – *Random Amplified Polymorphic* DNA). Esta ferramenta é baseada na amplificação de fragmentos do DNA usando iniciadores com sequências arbitrárias curtas (WOODS *et al.*, 1993). Análise por RAPD demonstrou um maior grau de polimorfismo em cepas de *H. capsulatum* analisadas por RFLP (KERSULYTE *et al.*, 1992; KARIMI *et al.*, 2002). Além disso, essa ferramenta tem possibilitado identificar uma estreita associação do genótipo encontrado com a origem da fonte e área geográfica dos isolados fúngicos (POONWAN *et al.*, 1998; REYES-MONTES *et*

al., 1999; TAYLOR; CHÁVEZ-TAPIA; REYES-MONTES, 2000; PERROTTA *et al.*, 2001), assim como demonstra haver grande diferença genética entre isolados do fungo procedentes dos EUA e América Latina (MUNIZ *et al.*, 2001; PERROTTA *et al.*, 2001).

No Rio de Janeiro/Brasil, isolados clínicos, animais e ambientais foram avaliados por RAPD. Os isolados fúngicos deste estudo apresentaram 80-100% de similaridade genética. Apesar disso, após a análise dos padrões de bandas de DNA encontrados, os isolados fúngicos foram agrupados em quatro a seis diferentes genótipos. Entretanto, os perfis genéticos dos isolados de *H. capsulatum* apresentaram baixa similaridade genética (menor que 70%) com as cepas de referências usadas neste estudo oriundas dos EUA (Downs, G-222B, e FLS-1) e Panamá (G-186B) (MUNIZ *et al.*, 2001). Outros autores, utilizando análise por RAPD em isolados de *H. capsulatum* de diversas fontes e diferentes regiões geográficas do Brasil, revelaram não existir um genótipo restrito para cada região deste país, pois isolados de diversas áreas geográficas foram agrupados em um mesmo clado (ZANCOPE-OLIVEIRA; TAVARES; MUNIZ, 2005; BRILHANTE; RIBEIRO *et al.*, 2012).

A amplificação e sequenciamento da região ITS1-5.8S-ITS2 (ITS - *Internal Transcribed Spacer region*) do rRNA utilizando PCR têm sido usados mundialmente para avaliar polimorfismos inter e intraespecíficos em vários fungos, já que a região ITS é uma região não codificante e altamente conservada do DNA fúngico (ESTRADA-BÁRCENAS *et al.*, 2014). Elevada variabilidade genética tem sido observada em isolados fúngicos procedentes de uma mesma região geográfica através desta técnica, sendo observado 20 diferentes padrões de sequências em 24 isolados de *H. capsulatum* oriundos de oito cidades dos EUA. A maior variabilidade genética foi observada em Indianápolis, onde 12 isolados fúngicos foram classificados em quatro genótipos diferentes (JIANG *et al.*, 2000). Apesar disso, alguns estudos encontraram associação entre o perfil polimórfico da região ITS com a origem geográfica e a fontes ambientais dos isolados de *H. capsulatum* (TAMURA *et al.*, 2002; ESTRADA-BÁRCENAS *et al.*, 2014; LANDABURU *et al.*, 2014).

Estudos sobre a variabilidade genética da região ITS demonstraram que *H. capsulatum* var. *farciminosum* não está restrita apenas a uma região geográfica ou somente causa infecções em equinos, já que alguns autores têm encontrado alta similaridade genética (99%) de isolados humanos e outros animais não equinos de diferentes regiões geográficas, com *A. capsulatus* var. *farciminosum* (MURATA *et al.*, 2007; EISENBERG *et al.*, 2013; LANDABURU *et al.*, 2014). É provável que *H. capsulatum* var. *farciminosum* também seja patogênica para o homem, entretanto, estudos moleculares mais robustos devem ser realizados

para uma confirmação exata da espécie filogenéticas destes isolados (LANDABURU *et al.*, 2014).

Estudo comparando os resultados de análise filogenética de três técnicas moleculares em 51 isolados fúngicos, de diferentes fontes e diversos estados do Brasil (Rio de Janeiro, São Paulo, Pernambuco, Espírito Santo, Ceará, Goiás e Mato Grosso do Sul), através de PCR-RFLP da região ITS; MLST dos quatro genes nucleares; e, M13-PCR *fingerprint,* revelaram que os métodos aplicados apresentaram 84,3% de concordância, produziram resultados complementares e agruparam todos os isolados em três grandes grupos genéticos. Na análise por PCR-RFLP da região ITS foi possível observar que os isolados fúngicos do estado do Rio de Janeiro eram geneticamente distintos dos outros isolados usados no estudo, sugerindo haver somente uma única população genética no microambiente deste estado (MUNIZ *et al.*, 2010).

Marcadores microssatélites são sequências repetitivas curtas, mundialmente aplicadas para estudar a estrutura de populações genéticas de algumas espécies eucariotas. Esses marcadores são regiões do genoma que apresentam dois a quatro nucleotídeos, e são encontradas em rearranjos de 10 a 20 ou mais repetições dentro do genoma do fungo (MCEWEN *et al.*, 2000). As primeiras análises de microssatélites em *H. capsulatum* foram realizadas por Carter *et al.* (1996, 1997). Esses autores desenvolveram dois microssatélites (lócus *L638REP* – C/A(T)n; e o lócus *L610.2CA* – (CA)n), além de encontrar outro microssatélite (lócus *HSP-TC* – (GA)n), na base de dados do GenBank (http://www.ncbi.nlm.nih.gov). O microsatélite (GA)n ocorre a montante do gene da proteína HsP60, que é a proteína do choque térmico (CARTER *et al.*, 1996; CARTER *et al.*, 1997). Estudos realizados por esses mesmos autores com esses três microssatélites demonstraram haver amplo polimorfismo em populações genéticas procedentes da América do Norte e América do Sul. Apesar disso, esta técnica foi capaz de distinguir isolados fúngicos de cada região (CARTER *et al.*, 1997; CARTER *et al.*, 2001).

McEwen *et al.* (2000) analisaram quarto microssatélites em isolados de *H. capsulatum*: (CA)n de Carter *et al*. (1997), (GA)n e dois outros microssatélites encontrados na base de dados do GenBank (http://www.ncbi.nlm.nih.gov) - ATPase-AT (AT)n e ATPase-CT (CT)n, ambos são marcadores encontrados no gene ATPase da membrana plasmática do fungo. Os resultados deste estudo revelaram que todos os microssatélites podem distinguir *H. capsulatum* de diversas origens geográficas. Entretanto, (AT)n e (CT)n falharam na amplificação de isolados fúngicos procedentes dos EUA, e (CT)n para isolados da Austrália.

Em 2012, um estudo foi conduzido usando uma sequência polimórfica de um fragmento de 240 nucleotídeos, o qual incluía o microssatélite (GA)n e regiões do gene HsP60. Os resultados demonstraram que os isolados de *H. capsulatum* recuperados de morcegos migratórios (capturados randomicamente no México, Brasil e Argentina) estão distribuídos de acordo com a rota de migração e, portanto, este método molecular pode ser utilizado como marcador biogeográfico (TAYLOR *et al.*, 2012).

Eletroforese em gel de campo pulsado (PFGE) ou cariotipagem por eletroforese é uma técnica molecular aplicada para a separação de cromossomos íntegros (ROMERO-MARTINEZ; CANTEROS; TAYLOR, 2004). Steele *et al.* (1989) usando PFGE identificou variabilidade no tamanho e no perfil de migração de bandas cromossômicas em três cepas de referência de *H. capsulatum*: Downs que apresentava sete cromossomos; G-186B – quatro cromossomos; e G-217B – três cromossomos (STEELE *et al.*, 1989). Em 1998, Car e Shearer utilizando a técnica bioquímica denominada reassociação cinética do DNA e reconstrução genômica com três cópias simples dos genes calmudolina, α-tubulina e β-tubulina, através de *Southern blot*, também identificou um genoma 40% maior na cepa Downs (EUA) e com 16 vezes mais repetição de DNA, do que na cepa G-186AS (Panamá). O genoma da cepa Downs foi determinado como sendo constituído por 32 Mpb com 8% de repetição, e o da cepa G-186AS constituído por aproximadamente 23 Mpb com menos de 0,5% de sequências repetitivas (CARR; SHEARER, 1998).

Canteros *et* al. (2005) realizaram análise por PFGE em 19 isolados clínicos de *H. capsulatum* de diferentes países (Argentina, México e Guatemala), e observaram alto grau de polimorfismo destes isolados, identificando 13 diferentes cariótipos. Além disso, os mesmos autores encontraram variações nos padrões de bandas dos cromossomos destes isolados, os quais apresentaram cinco a sete bandas (variando de 1,3 a 10 Mpb em tamanho). Entretanto, nenhuma associação foi encontrada entre os cariótipos e origem geográfica ou fonte clínica (CANTEROS *et al.*, 2005).

Atualmente, MLST é a técnica molecular mais bem padronizada para identificação de espécies e clados filogenéticas de *H. capsulatum* (KASUGA; TAYLOR; WHITE, 1999; KASUGA, T. *et al.*, 2003; TEIXEIRA *et al.*, 2016). Os estudos que utilizaram esta técnica para avaliação da variabilidade genética do fungo foram descritos na secção anterior.

1.1.2.6 Ecologia

Atualmente, existem 11 espécies filogenéticas de *H. capsulatum* difundidas mundialmente, as quais estão distribuídas em latitudes 57º Norte (BUREK-HUNTINGTON; GILL; BRADWAY, 2014) e 38º Sul (CALANNI *et al.*, 2013), sugerindo uma grande dispersão geográfica do fungo. *H. capsulatum* é um fungo encontrado em nichos ecológicos com condições especiais: temperatura do ar e solo entre 18°C a 28ºC, pluviometria entre 800 e 1.200 mm, umidade relativa do ar > 60%, e ambientes escuros que favorecem a esporulação (TEWARI, 1998; TAYLOR *et al.*, 1999; TAYLOR; REYES-MONTES; CHÁVEZ-TAPIA *et al*., 2000; FERREIRA; BORGES, 2009).

O fungo cresce bem em solos ricos em substâncias orgânicas, especialmente com altas concentrações de nitrogênio e fósforo, com pH ácido e particularmente onde há dejeções de aves de criação, de morcegos ou pássaros agregados. Situam-se a pouca profundidade, preferencialmente entre 15 – 20 cm da superfície. O alto teor de ácido úrico e outros componentes nitrogenados de baixo peso molecular, encontrados nas excretas desses animais, inibem o crescimento de grande parte da microbiota competidora e favorecem o crescimento e proliferação do fungo, que utiliza esses componentes como fonte de nitrogênio (TAYLOR *et al.*, 1999; TAYLOR; REYES-MONTES; CHÁVEZ-TAPIA *et al.*, 2000; TAYLOR; RUIZ-PALACIOS *et al.*, 2005).

Áreas de grande densidade de *H. capsulatum* são encontradas em microambientes como criadouros de galinhas, grutas ou cavernas habitadas por morcegos e cúpulas de prédios invadidas por morcegos, sendo denominados de “pontos epidêmicos” (CHICK *et al.*, 1981; DEEPE JR, 2005; ZANCOPE-OLIVEIRA *et al.*, 2011). Um estudo realizado em 1987 no Município do Rio de Janeiro, na localidade de Rio da Prata, identificou contaminação por *H. capsulatum* em 7,2% das amostras de solo coletadas de galinheiros daquela localidade (ZANCOPÉ-OLIVEIRA; WANKE, 1987). As infecções frequentemente ocorrem quando há revolvimento de solos contaminados, que gera dispersão de aerossóis com elevado número de propágulos infectantes, os quais são capazes de atingir e infectar pessoas num raio de até 32 km (LATHAM *et al.*, 1980; CHICK *et al.*, 1981).

Os morcegos estão entre os poucos mamíferos silvestres que desempenham papel importante na manutenção do ciclo biológico de *H. capsulatum* na natureza (TAYLOR; REYES-MONTES; CHÁVEZ-TAPIA *et al.*, 2000; DIAS *et al.*, 2011). A correlação entre morcegos e fungos patogênicos foi descrita primeiramente por Emmons (1956), em

Maryland/EUA, após isolar o fungo de solo contaminado pelo guano desses animais (EMMONS, 1956). Além do solo contaminado com as excretas de morcegos, *H. capsulatum* pode ser encontrado também em vísceras e sangue destes. Esses animais por terem hábito de agregação, geralmente transmitem a infecção à colônia quando excretam fungos nas fezes. São também dispersores do fungo na natureza, já que seu deslocamento pode ensejar aparição de novos focos (ZANCOPE-OLIVEIRA; MUNIZ; WANKE, 2013).

1.1.3 Histoplasmose

1.1.3.1 Epidemiologia

Casos de histoplasmose humana e em outros mamíferos são relatados em todos os continentes, com exceção da Antártida. Entretanto, há um nítido predomínio na América e na África (FERREIRA; BORGES, 2009; ZANCOPE-OLIVEIRA *et al.*, 2011; ZANCOPE-OLIVEIRA; MUNIZ; WANKE, 2013). Nas Américas, casos de histoplasmose em humanos já foram descritos desde o oeste do Canadá (ANDERSON *et al.*, 2006) até a Patagônia na Argentina (CALANNI *et al.*, 2013). Infecção por *H. capsulatum* também já foi observada tanto em animais domésticos como em selvagens (NEGRONI, 2005; BRILHANTE; COELHO *et al.*, 2012). No nordeste do Brasil, Cordeiro *et* al. (2011) após analisar 224 amostras de sangue de cães com leishmaniose visceral, observou que 1,78% desses animais tinham anticorpos contra *H. capsulatum* (CORDEIRO et al., 2011). Em 2014, no Alasca, o primeiro caso de histoplasmose em um animal selvagem (*Enhydra lutris kenyoni* - lontra marinha) foi identificado, através de exames histopatológicos (BUREK-HUNTINGTON; GILL; BRADWAY, 2014).

Na América do Norte, as zonas de maior endemicidade estão situadas nos vales dos rios Mississipi e Ohio, e na América do Sul, localiza-se na bacia do Rio Prata e na Serra do Mar (NOSANCHUK; GACSER, 2008; WHEAT *et al.*, 2016). Na América Latina, os principais países que relatam casos de histoplasmose são: México, Brasil, Argentina, Colômbia, Guiana Francesa e Venezuela (COLOMBO *et al.*, 2011).

Na África, as variedades *capsulatum* e *duboisii* coexistem. *H. capsulatum* var. *duboisii* ocorre exclusivamente nas áreas tropicais da África, principalmente nas regiões

central e oeste da África Subsaariana, com 70% dos casos provenientes da Nigéria, Zaire, Uganda e Senegal (LOULERGUE *et al.*, 2007; FERREIRA; BORGES, 2009).

As microepidemias de histoplasmose pulmonar aguda (HPA) eram as formas mais frequentemente observadas no mundo, até o surgimento da aids, quando a forma disseminada passou a predominar. Surtos de HPA são observados principalmente em indivíduos que realizaram atividades ocupacionais, como limpeza de locais abandonados, ou recreativos, como visitação de grutas ou cavernas habitadas por morcegos (NEGRONI *et al.*, 2010; ZOLLNER *et al.*, 2010; CDC, 2014; ROCHA-SILVA *et al.*, 2014).

No Brasil, a incidência da histoplasmose é pouco conhecida. Entretanto, inquéritos epidemiológicos através da intradermorreação à histoplasmina, realizados entre as décadas de 1940-1990, com diferentes diluições de histoplasmina, encontraram prevalências variáveis em diversas regiões do Brasil (FAVA; FAVA NETO, 1998), como mostra a figura 6. As maiores prevalências foram encontradas nas regiões Sudeste (93,2% na localidade de Praia Vermelha – Estado do Rio de Janeiro) e Sul (89% em Cachoeira do Sul – Rio Grande do Sul)(WANKE, 1985; ZEMBRZUSKI, 1993). No Estado do Ceará estudos sobre a prevalência da infecção por *H. capsulatum* foram realizados em residentes de regiões serranas, e em de área rural, sendo observada positividade ao teste com histoplasmina em 23,6% - 61,5% (COÊLHO; GADELHA; CÂMARA, 1986; DIÓGENES *et al.*, 1990; FAÇANHA *et al.*, 1991). A maior prevalência encontrada foi na Serra do Pereiro (61,5%) que fica localizada ao sul do estado do Ceará (DIÓGENES *et al.*, 1990).

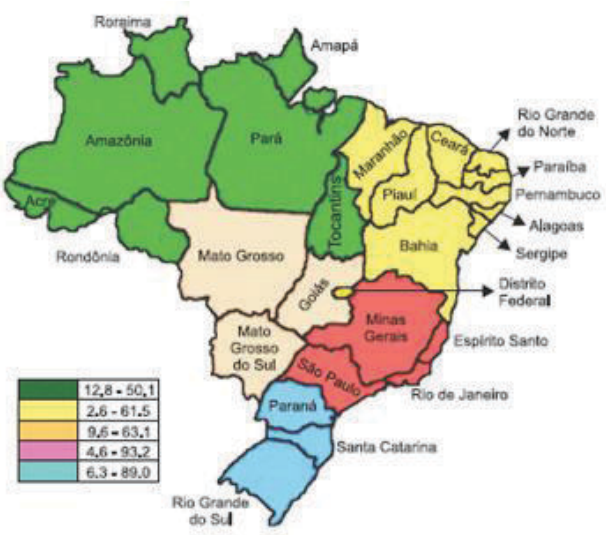

Figura 6 – Distribuição geográfica da histoplasmose no Brasil por meio de inquéritos de reatividade intradérmica à histoplasmina
Fonte: GUIMARÃES; NOSANCHUK; ZANCOPÉ-OLIVEIRA, 2006, p.3.

Na década de 1980, com o surgimento da aids, vários casos de histoplasmose, em particular na forma disseminada, foram diagnosticados mundialmente, e a partir de 1987 a histoplasmose extrapulmonar foi considerada como doença definidora de aids (CDC, 1987). No Brasil, casos de histoplasmose disseminada (HD) também passaram a ser identificados em pessoas vivendo com HIV/aids (PVHA) que apresentam grave imunossupressão, acompanhando o cenário mundial desta coinfecção (UNIS; OLIVEIRA; SEVERO, 2004; CASOTTI *et al.*, 2006; CHANG *et al.*, 2007; RIBEIRO *et al.*, 2009).

Apesar da terapia antirretroviral combinada (HAART) ter modificado drasticamente a evolução da epidemia da aids, muitos pacientes ainda se internam nos serviços de saúde com infecções oportunistas, o que reflete a avançada imussupressão associada à aids (FORD *et al.*, 2015). Estudo realizado em um hospital de referência para doenças infecciosas em São Paulo/Brasil, observou que histoplasmose foi à terceira infecção fúngica mais frequente em pacientes com aids internados naquela instituição (OLIVEIRA *et al.*, 2014).

Estima-se que somente na América do Norte e na África Subsaariana 300 mil casos de histoplasmose sejam diagnosticados anualmente, com 10.000 casos evoluindo para o óbito (ARMSTRONG-JAMES; MEINTJES; BROWN, 2014). No Brasil, poucos dados epidemiológicos sobre a histoplasmose estão disponíveis, entretanto, um estudo recente estimou a taxa de infecções fúngicas em humanos no ano de 2011, durante internações hospitalares. Histoplasmose teve uma incidência geral de 2,19 admissões hospitalares para cada 1.000 hospitalizações naquele ano, baseado em dados do Ministério da Saúde do Brasil (GIACOMAZZI *et al.*, 2015).

O Estado do Ceará vem se destacando a nível nacional, pela elevada ocorrência de HD em PVHA, com uma média de 19-38 casos diagnosticados a cada ano (DE FRANCESCO DAHER *et al.*, 2006; PONTES *et al.*, 2010; BRILHANTE; FECHINE *et al.*, 2012; DAMASCENO *et al.*, 2013).

A ocorrência de HD em PVHA foi investigada por De Franchesco Daher *et* al. (2006), no período de 1995 a 2004, quando verificou 164 casos diagnosticados dessa coinfecção em hospital de referência em doenças infecciosas e parasitárias do Estado do Ceará. Damasceno *et* al. (2013) encontraram 264 casos diagnosticados de HD/aids no período de 2002-2008 em duas instituições de saúde pública do Ceará, com alto impacto na morbimortalidade desses indivíduos. Óbito ocorreu em 30% dos pacientes no período do internamento, e durante o seguimento clínico de 126 pacientes, 23,3% dos pacientes tiveram recorrência da histoplasmose. Entretanto, estudo realizado em outra área endêmica da América Latina revelou uma queda de óbitos por histoplasmose em PVHA, após a era HAART, com

estabilização no número de casos de histoplasmose diagnosticados a partir de 1998 (ADENIS; NACHER; HANF; VANTILCKE*; et al.*, 2014).

Histoplasmose disseminada em PVHA, geralmente acomete indivíduos do sexo masculino, adulto-jovens, com linfócitos T CD4+ < 150cels/mm^3 e sem história epidemiológica de contato ou atividades em microambientes habitados por *H. capsulatum*. Pode ocorrer como primeira doença oportunista associada à aids em mais de 70% dos pacientes de áreas endêmicas (PONTES *et al.*, 2010; DAMASCENO *et al.*, 2013; ADENIS; NACHER; HANF; VANTILCKE*; et al.*, 2014). Histoplasmose é a segunda doença febril definidora de aids notificada pela Coordenação Municipal de DST-aids do Município de Fortaleza-Ceará, com um aumento da frequência dessa patologia de 1,5% para 3,7%, nos períodos de 1983-1999 e 2000-2006, em PVHA (DAMASCENO, 2011).

Mais recentemente, estudo de prevalência sobre a infecção por *H. capsulatum* em 161 indivíduos HIV positivos, residentes em Fortaleza/Ceará (com linfócitos T CD4+ acima de 350céls/mm^3), foi realizado através da intradermorreação à histoplasmina. Os resultados revelaram haver aproximadamente 12% de reatividade a histoplasmina nesses pacientes (BEZERRA *et al.*, 2013). Estudo sobre a distribuição espacial dos casos de HD/aids em indivíduos residentes em Fortaleza/Ceará identificou que a maior frequência dos casos desta coinfecção ocorreu em indivíduos que residiam em área com baixos índices de saneamento básico, e naqueles que habitavam uma região próxima a um parque ambiental, onde são realizadas atividades ecológicas (CORREIA *et al.*, 2016).

1.1.3.2 Epidemiologia molecular da histoplasmose

Estudos epidemiológicos têm revelado diferenças na apresentação das manifestações clínicas e evolução da histoplasmose entre pacientes dos EUA e da América do Sul. Elevadas taxas de mortalidade e recidiva, bem como a presença de lesões mucocutâneas são mais frequentes em pacientes do Brasil, Argentina e Colômbia, especialmente em PVHA (COUPPIE *et al.*, 2006; DAMASCENO *et al.*, 2014).

Técnicas moleculares também têm identificado importantes diferenças genéticas em isolados de *H. capsulatum* provenientes de lesões de pele da América do Sul, quando comparadas com cepas norte americanas. Análise por RFLP do gene *Yps-3* demonstrou que as

classes 5 e 6, originalmente da América Latina, são os genótipos predominantemente associados com estas manifestações clínicas (KARIMI *et al.*, 2002).

Durkin et al. (2004) estudou as diferenças clínicas entre isolados *H. capsulatum* da América Latina (RFLP classes 5 e 6) e da América do Norte (RFLP classe 2), após infecção intratraqueal do morfotipo-L em animais de laboratório. Os animais infectados com *H. capsulatum* RFLP classe 2 desenvolveram doença crônica e tiveram 100% de sobrevida, enquanto que aqueles infectados por isolados RFLP classes 5 e 6 apresentaram um grande número de mortes, com uma sobrevida de apenas 20%, após 21 dias da infecção (DURKIN *et al.*, 2004).

No Rio Grande do Sul/Brasil, isolados de *H. capsulatum* foram obtidos através de cultura de lesões de pele de oito pacientes. Estes isolados foram analisados por meio de sequenciamento da região ITS. Dois perfis genéticos diferentes foram identificados, os quais foram similares geneticamente a outros isolados de pacientes colombianos e argentinos, entretanto, diferentes de isolados dos EUA. Este estudo sugere existir uma clara diferença entre as características patogênicas dos isolados latino-americanos, em relação aos isolados fúngicos dos EUA (GOLDANI *et al.*, 2009).

Raros estudos tem relacionado o comportamento das espécies filogenéticas identificadas por MLST com a forma e evolução clínica de pacientes com histoplasmose. Na China, um estudo descreveu a presença do genótipo Australiano em um isolado de *H. capsulatum* de um indivíduo nativo daquela região, que havia desenvolvido histoplasmose disseminada crônica. O paciente evoluiu com cura clínica após seis meses de tratamento com itraconazol, e não apresentou nenhum episódio de recidiva durante o seguimento clínico de um ano (WANG *et al.*, 2014).

Técnicas moleculares também têm sido de grande importância para caracterizar mecanismo de reativação da histoplasmose, confirmar a presença de infecções mistas, e identificar fontes de surtos desta micose sistêmica. (POONWAN *et al.*, 1998; MUNIZ *et al.*, 2001).

Em 2001, análise por RAPD foi realizada em quatro isolados de *H. capsulatum* de dois pacientes, recuperados em diferentes tempos. Esta análise revelou que cada paciente abrigava a mesma população genética detectada em tempos diferentes, o que sugeriu que a infecção fúngica estivesse ocorrendo por mecanismo de reativação (MUNIZ *et al.*, 2001). Outros autores descreveram a presença de infecção mista por *H. capsulatum* em um PVHA, após detectar dois perfis genéticos distintos analisados por RAPD, obtidos de lavado broncoalveolar e sangue (POONWAN *et al.*, 1998).

Em 2005, um surto de histoplasmose pulmonar ocorreu em Acapulco, México. Sequências de DNA de isolados de *H. capsulatum* obtidos do solo de uma planta (fertilizador orgânico) do hotel X em Acapulco foram analisadas por RAPD e sequenciamento dos genes *H-anti* e *ole1,* e comparadas com as sequências genéticas de isolados fúngicos de pacientes de outros surtos do mesmo país. Padrões de bandas de DNA de isolados de *H. capsulatum* do fertilizador orgânico foram diferentes dos perfis de bandas dos outros isolados fúngicos deste estudo. Entretanto, análise das sequências gênicas detectou alta similaridade entre os isolados testados (92%-99%) com os isolados clínicos usados como referência neste estudo. Portanto, foi possível confirmar a fonte de infecção do surto somente através do sequenciamento dos genes *H-anti* e *Ole1* (TAYLOR; RUIZ-PALACIOS *et al.*, 2005). Entretanto, outros autores têm confirmado a fonte de infecção de surtos de histoplasmose usando análise por RAPD no México (MUNOZ, B. *et al.*, 2010), e na Argentina tanto em áreas endêmica e não endêmica para histoplasmose (NEGRONI *et al.*, 2010; CALANNI *et al.*, 2013).

1.1.3.3 Patogenia

A infecção por *H. capsulatum* depende de interações dinâmicas da imunidade inata com a imunidade adquirida e fatores de virulência fúngicos. Os fatores essenciais que podem resultar em infecção dependem dos mecanismos de transição do micélio para fase de levedura, da sobrevivência dos fungos nos macrófagos e da regulação dos fatores de virulência associados (EDWARDS; RAPPLEYE, 2011; MIHU; NOSANCHUK, 2012).

Após a inalação de fragmentos de hifas e microconídios aerossolizados, os quais entram em contato com as células de defesa do hospedeiro no trato respiratório superior, inicia-se a interação patógeno-hospedeiro. Macrófagos, monócitos e mais raramente neutrófilos realizam a opsonofagocitose através de receptores de complemento para moléculas do fungo. Entretanto, vias alternativas de ligação do patógeno são utilizadas como mecanismos de adaptação e consequentemente, de sobrevida do fungo nas células do hospedeiro (LOPEZ, 2006).

A interação patógeno-hospedeiro desencadeia a resposta imune inata, na tentativa de controlar a infecção. O principal mecanismo de resistência contra o fungo é representado por fagócitos, células dendríticas e linfócitos T. Os fagócitos profissionais e as células dendríticas são as primeiras linhas de defesa do hospedeiro, os quais apresentam receptores de

reconhecimento de padrão (PRRs), que estão expressos na superfície destas células. Os PRRs reconhecem padrões moleculares associados ao patógeno (PAMPs) (ROMANI, 2011). Dentre estes, destacam-se os TRLs e CRLs que ativam múltiplas vias de sinalização intracelular para a ligação de PAMPs específicas do fungo (ROMANI, 2011). Como exemplo de PAMPs, destacamos a proteína do choque térmico Hsp60 que tem a capacidade de se ligar a β2-integrinas CD18 (PRR), e β-1,3-glucano que se liga especificamente a dectina-1 (PRR), o qual está localizado na superfície de macrófagos (GOMEZ; ALLENDOERFER; DEEPE, 1995; ROMANI, 2011).

Um curto período de tempo após ser inalado, *H. capsulatum* inicia o processo de transição micélio-levedura, que pode ocorrer dentro ou fora de macrófagos (TIAN; SHEARER, 2002). Apesar da duração desse evento ser variável, e depender tanto do isolado fúngico como da célula do hospedeiro, tem-se proposto que esse processo se inicia ainda no trato respiratório superior, dentro das células dendríticas (SAHAZA *et al.*, 2014), já que o fungo é capaz de se ligar a estas células através de receptores de fibronectina (GILDEA; MORRIS; NEWMAN, 2001).

A mudança para o morfotipo-L, forma parasitária, está intimamente envolvido com as estratégias de adaptação para a sobrevivência do fungo, que faz deste, um patógeno intracelular facultativo, quase que exclusivo das células do sistema retículo-endotelial de mamíferos (TIAN; SHEARER, 2001;2002; WOODS, 2002). Os mecanismos que resultam no êxito desse processo são complexos, mas em grande parte dependem da mudança de temperatura (de 25ºC para 37ºC), da modificação dos componentes da parede celular do fungo e da expressão de genes que regulam esse evento (DEEPE JR, 2005; NOSANCHUK; GACSER, 2008).

O controle da infecção é amplamente baseado na ativação da imunidade celular em conjunto com a resposta inata, e é observada após a segunda ou terceira semanas, sendo que a doença progressiva com disseminação ocorre predominantemente na ausência de imunidade celular intacta (LAZAR-MOLNAR *et al.*, 2008). Células T CD4+ induzem a produção de citocinas do tipo Th1, particularmente IFN-γ e TNF-α, as quais ativam macrófagos que são as principais células efetoras de resistência do hospedeiro ao fungo (LAZAR-MOLNAR *et al.*, 2008), o que leva à formação de reação granulomatosa e tendência à calcificação (NOSANCHUK; GACSER, 2008; EDWARDS; RAPPLEYE, 2011; NOSANCHUK *et al.*, 2012). Nas formas disseminadas, há uma resposta imune do tipo Th2. Citocinas do tipo IL4, IL5 e IL10 são produzidas e inibem a resposta protetora Th1. O curso da doença é agudo e

grave, e os granulomas são mal formados ou não se formam (NOSANCHUK; GACSER, 2008; NOSANCHUK *et al.*, 2012).

Em nível celular, o hospedeiro utiliza um ambiente especializado para "matar" o microorganismo, como: acidificação do meio intracelular, produção de radicais tóxicos de oxigênio e de intermediários de nitrogênio, proteases e outras enzimas hidrolíticas, peptídeos antimicrobianos, e a restrição de nutrientes essenciais como cálcio e ferro (LOPEZ, 2006).

Em contrapartida, *H. capsulatum* é um patógeno que apresenta diversos mecanismos de escape, para que possa sobreviver e se replicar dentro dos fagolisossomos, e assim estabelecer a infecção. Dentre esses mecanismos destaca-se (LOPEZ, 2006):

1. Manipulação do microambiente para manter um pH menos ácido, através da exclusão da bomba H+ATPase da membrana vacuolar;
2. Inibição da fusão fagolisossomal, onde o vacúolo escapa do fagócito até o interior do citoplasma, originando uma fagocitose sem estimulação do ciclo respiratório.
3. Resistência a proteases lisossomais e enzimas hidrolíticas favorecidas pelos dois mecanismos descritos anteriormente;
4. Aquisição de ferro que pode ocorrer através de: a) produção e liberação de sideróforos; b) liberação de ferro unido à transferrina por diminuição do pH; e c) redução férrica;
5. Aquisição de cálcio através da produção de uma proteína ligante de cálcio (CBP), no qual o gene que codifica essa proteína (*CBP1*) é específico da fase leveduriforme;

Como podemos observar o estabelecimento da infecção é complexo, e depende de vários fatores de virulência do fungo e da resposta imune do hospedeiro. Vale ressaltar que existem diferenças quanto às moléculas que compõe a parede celular e a expressão gênica entre espécies filogenéticas do fungo, as quais estão intimamente relacionadas com a virulência da cepa (HOLBROOK; RAPPLEYE, 2008).

Em 2014, três cepas que diferiam quanto ao perfil genético e quimiotipo do morfotipo-L (G-217B – classe Nam2 e quimiotipo I; WU24 – classe NAm 1 e quimiotipo II; e G-186A – linhagem do Panamá e quimiotipo II) foram avaliadas em estudo de virulência *in vivo*. Os resultados revelaram que quando doses baixas de leveduras eram inoculadas nos animais, a cepa G-186A apresentou-se mais virulenta, já que persistiu mais tempo, causando mais inflamação nos pulmões do que as outras cepas do estudo. Em doses elevadas, a cepa G-217B apresentou uma carga fúngica mais elevada nos pulmões, em relação às demais cepas do

estudo, e mostrou uma resolução retardada da infecção (SEPULVEDA; WILLIAMS; GOLDMAN, 2014).

1.1.3.4 Ciclo biológico de *H. capsulatum*

Após a inalação, os microconídios atingem os alvéolos pulmonares e se transformam em leveduras gemulantes. Nessa fase, as leveduras se localizam no parênquima pulmonar e linfonodos hilomediastinais, onde se reproduzem. Em geral, os indivíduos que entram em contato com fungo não desenvolvem a doença, devido à resposta imune inata adequada, o que permite conter a infecção. Entretanto, através dos mecanismos de escape do fungo, *H. capsulatum* pode permanecer viável em áreas cicatrizadas por vários anos, e causar infecção recidivante por reativação de foco latente (NOSANCHUK; GACSER, 2008; EDWARDS; RAPPLEYE, 2011).

Caso haja falha da resposta imune ou o contato com uma carga fúngica elevada, pode ocorrer invasão de células da corrente sanguínea do sistema fagocítico-mononuclear (ZANCOPE-OLIVEIRA; MUNIZ; WANKE, 2005). Inicialmente ocorre uma fungemia assintomática, e geralmente após duas semanas, os indivíduos desenvolvem as manifestações clínicas de histoplasmose, devido à disseminação de fungos para órgãos como fígado, baço, linfonodos e medula óssea.

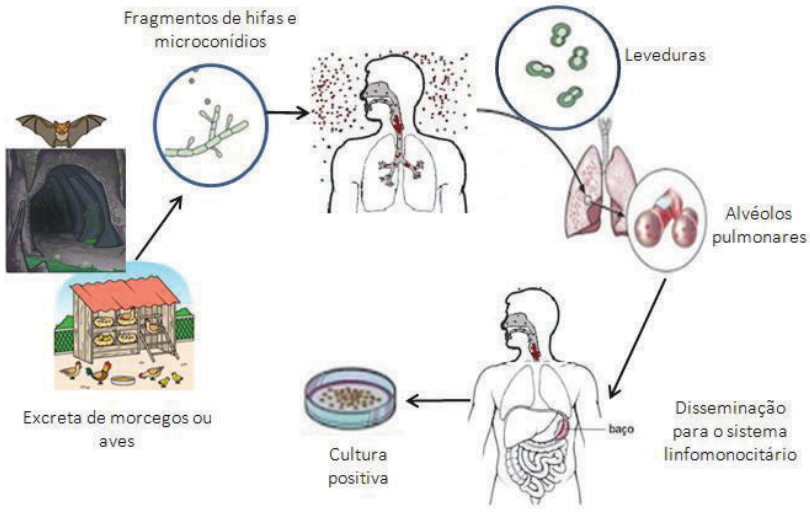

Figura 7 – Ciclo biológico de *H. capsulatum*
Fonte adaptada: HULL; HEITMAN, 2002, p. 560.

1.1.3.5 Manifestações Clínicas

Em geral, infecções causadas por *H. capsulatum* são assintomáticas ou subclínicas. Menos de 1% das pessoas apresentam manifestações clínicas após a exposição primária ao fungo, principalmente quando há inalação de grande quantidade de propágulos (KAUFFMAN, 2009). A infecção pode ficar restrita aos pulmões, ou expandir-se para órgãos adjacentes (linfonodos mediastinais) e para todo o sistema fagocítico-mononuclear, acometendo vários órgãos e sistemas. Formas progressivas e disseminadas da histoplasmose, assim como a gravidade das manifestações clínicas, geralmente decorre da reativação fúngica quando há quedas ou falhas da imunidade celular ou dos mecanismos de fagocitose e lise dos macrófagos (FERREIRA; BORGES, 2009).

Primoinfecção assintomática representa a maioria das infecções primárias, reconhecidas clinicamente pela viragem da intradermorreação à histoplasmina de negativa para positiva. Após meses ou anos, podem ser observados nódulos calcificados em pulmões, linfonodos e baço (BRADSHER, 1996).

A HPA geralmente ocorre em indivíduos que realizam atividades em grutas ou cavernas habitadas por morcegos ou aves, ou durante atividades de limpeza de locais abandonados, onde há micronichos de *H. capsulatum* (KAUFFMAN, 2007). Esta forma clínica é autolimitada, e os pacientes apresentam sintomas gerais inespecíficos como febre e cefaleia, e sintomas de infecção respiratória aguda. A histoplasmose pulmonar crônica acomete indivíduos com doença pulmonar obstrutiva crônica ou com defeito anatômico pulmonar. Geralmente acomete homens adultos (acima de 50 anos) e tabagistas. Os sintomas característicos são semelhantes aos da tuberculose pulmonar, como tosse, perda de peso, febre baixa, e escarros hemoptoicos (FERREIRA; BORGES, 2009; SANTOS *et al.*, 2009).

A HD ocorre quando a infecção acomete órgãos extrapulmonares e/ou extralinfonodais (KAPLAN *et al.*, 2009). Casos de HD aguda geralmente são observados em indivíduos com disfunção imunológica grave, como portadores de doenças neoplásicas hematológicas (leucose, linfoma), usuários de drogas imunossupressoras, e portadores de imunodeficiências celulares primárias (KAUFFMAN, 2007; FERREIRA; BORGES, 2009). As formas subaguda ou crônica da HD também podem ocorrer, e em geral, acometem indivíduos nos extremos de idade, alcoolistas crônicos, tabagistas e portadores de *Diabetes Mellitus* ou outras comorbidades crônicas (WANKE; LAZÉRA, 2004; DEEPE JR, 2005).

Nestes casos a evolução da doença é mais prolongada, e a deterioração clínica é mais lenta que na forma aguda da doença (FAIOLLA *et al.*, 2013).

No Instituto de Pesquisa Clínica Evandro Chagas, atualmente denominado Instituto Nacional de Infectologia Evandro Chagas, da Fundação Oswaldo Cruz (INI/FIOCRUZ), no Brasil, no período de 1987-2003, 74 casos de histoplasmose foram diagnosticados. As formas mais encontradas foram: disseminada (46/74), pulmonar crônica (9/74), pulmonar aguda (10/74) e mediastinite (5/74). Coinfecção com aids foi identificada em 38 pacientes, ocorrendo em todos a forma disseminada (LEIMANN *et al.*, 2005).

Na coinfecção HD/aids, os principais sintomas e sinais observados são febre prolongada (83-97%), perda de peso (62-92%), sintomas respiratórios (43-80%), astenia (63-69%), hepatomegalia (40-83%) e manifestações gastrointestinais (46-75%) (GUTIERREZ *et al.*, 2005; DE FRANCESCO DAHER *et al.*, 2006; BADDLEY *et al.*, 2008). Lesões cutâneas são observadas, principalmente em pacientes oriundos de países da América Latina, especialmente no Brasil, com frequência elevada de 35-85%, diferentemente de outras regiões endêmicas, como países da América do Norte (6,5%-10%), Guiana Francesa (13,4%) e Panamá (17,5%) (KARIMI *et al.*, 2002; COUPPIE *et al.*, 2006; BADDLEY *et al.*, 2008). As principais formas de manifestação das lesões cutâneas são pústulas com crostas, nódulos, lesões acne-*like*, úlceras e pápulas umbilicadas, podendo ocorrer de forma isolada em pacientes com aids (COUPPIE *et al.*, 2006; RAMOS, 2008; BONIFAZ *et al.*, 2009).

Em geral, a HD é a primeira doença definidora de aids em aproximadamente 60% dos indivíduos com coinfecção HD/aids, os quais apresentam linfócitos T CD4+ < 150céls/mm^3 (BRILHANTE; FECHINE *et al.*, 2012; DAMASCENO *et al.*, 2013; MARUKUTIRA *et al.*, 2014). Pacientes com insuficiência renal aguda, hipoalbuminemia, elevação da desidrogenase láctica, trombocitopenia, elevação de aspartato aminotransferase e anemia, geralmente evoluem com doença grave e óbito (COUPPIE *et al.*, 2006; DE FRANCESCO DAHER *et al.*, 2006; BADDLEY *et al.*, 2008). As características clínicas e epidemiológicas das formas clínicas mais comuns de histoplasmose estão descritas na tabela 1.

Disseminação para o sistema nervoso central (SNC) ocorre em até 20% dos casos de pacientes com a forma disseminada (FERREIRA; BORGES, 2009; MYINT *et al.*, 2014). Indivíduos acometidos pelas formas pulmonares podem evoluir com complicações como fibrose mediastinal, pericardite, broncolitíase, histoplasmoma pulmonar e envolvimento pleural. Fibrose mediastínica geralmente é uma complicação grave e fatal, entretanto, raramente ocorre em indivíduos que apresentaram a forma pulmonar aguda da histoplasmose (KAUFFMAN, 2007). Formas isoladas como endocardite, infecção endovascular e meningite

também já foram descritas, e acometem indivíduos que apresentem algum dispositivo invasivo como próteses e catéteres, os quais podem colonizar-se pelo fungo e causar infecção (LEDTKE *et al.*, 2012; VEERAVAGU *et al.*, 2013).

Tabela 1 - Características clínico-epidemiológicas da histoplasmose.

Forma clínica	Características epidemiológicas	Manifestações clínicas	Sinais clínicos	Evolução
HPA	Exposição maciça a fontes de infecção Período de incubação: 7-21 dias	Febre alta Calafrios Cefaléia Tosse seca Astenia Hiporexia Dor torácica Manifestações de hiperergia (2-3 semanas, após o início dos sintomas)*	Hepatoesplenomegalia Adenomegalias superficiais Frêmitos ou crepitações pulmonares	Cura espontânea (maioria) Re-infecção: re-exposição (quadro mais agudo)
HPC	Indivíduos com DPOC Maiores de 50 anos	Febre baixa Expectoração mucopurulenta Dor torácica Dispnéia Perda de peso Astenia Sudorese noturna Hemoptise (estágios mais tardios)	Alterações próprias de enfisema pulmonar	Óbito em até 80% dos casos
HDA	Disfunção imunológica grave	Febre elevada e prolongada Perda de peso Diarreia Astenia Tosse Meningoencefalite	Hepatoesplenomegalia Adenomegalias generalizadas Lesões cutâneas de aspecto variado Sinais de irritação meníngea	Óbito se não instituir terapêutica rapidamente
HDS	Evolução mais prolongada e deterioração clínica mais lenta que na HDA	Febre (50%) Perda de peso Síndrome de Addison Meningite	Hepatoesplenomegalia Úlceras em orofaringe	Cura após tratamento clínico
HDC	Adultos maiores que 40 anos Sexo masculino Disfunção imunológica leve**	Perda de peso Febre baixa (30%)	Lesão cutaneomucosa (50-90%), em naso e orofaringe Meningite crônica	Cura após tratamento clínico

Fonte adaptada: DAMASCENO, 2011, p. 28.

HPA= histoplasmose pulmonar aguda; HPC= histoplasmose pulmonar crônica; HDA= histoplamose disseminada aguda; HDS= histoplasmose disseminada subaguda; HDC= histoplasmose disseminada crônica; DPOC= doença pulmonar obstrutiva crônica; *Eritema nodoso, conjuntivite flictenular, pleurisia fibrinosa, pericardite e artrite.**Idade avançada, alcoolismo crônico, diabetes, tumores sólidos, doses baixas e prolongadas de corticoide e linfoma crônico.

1.1.3.6 Diagnóstico da infecção por *H. capsulatum*

- Cultura

O isolamento de *H. capsulatum* em culturas de tecidos ou fluidos corporais representa o padrão ouro para o diagnóstico de histoplasmose. A realização desse método é obrigatória na complementação diagnóstica, e apresenta sensibilidade que varia de 50%-85% (GUIMARÃES; NOSANCHUK; ZANCOPE-OLIVEIRA, 2006; KAUFFMAN, 2008; WHEAT, 2009). Entretanto, o diagnóstico é laborioso já que o crescimento do fungo na fase micelial pode levar até seis semanas. Além disso, para confirmar a identificação do fungo, faz-se necessário a realização do teste de reversibilidade micélio-levedura em meios como Agar Sangue ou BHI enriquecidos com cisteína, mantidas a 37ºC, demonstrando assim o dimorfismo fúngico, que pode ocorrer entre duas a quatro semanas (LOPEZ, 2006). Apesar de demorado, é o método mais confiável para a identificação de *H. capsulatum*. Aspirados de medula óssea são os espécimes biológicos com maior positividade em culturas (70-90%). Para amostras de sangue periférico, recomenda-se a realização de lisecentrifugação que aumenta a sensibilidade e reduz a identificação tardia do fungo (ADENIS; AZNAR; COUPPIE, 2014).

- Exame direto

Esta técnica pode contribuir para identificação mais rápida do fungo, entretanto, a sensibilidade varia de acordo com o tipo de espécime biológico, severidade da doença e experiência do operador. Colorações como Giemsa e Wright podem ser usadas como diagnóstico presuntivo em aspirados de medula óssea e sangue periférico para detecção de leveduras, podendo ser positivos em até 50-75% dos pacientes. Diagnóstico diferencial deve ser realizado com outros fungos, como *Candida glabrata*, *Penicilium marneffei*, assim como com os parasitos do gênero *Leishmania* e *Trypanossoma* (ADENIS; AZNAR; COUPPIE, 2014; SCHEEL; GÓMEZ, 2014).

❖ Histopatologia

Em biópsias teciduais de pacientes imunocompetentes são observadas reações granulomatosas típicas com necrose caseosa, que, com o passar do tempo, tendem a calcificar. Entretanto, em pacientes imunossuprimidos, os granulomas são mal formados com grande quantidade de elementos leveduriformes, tanto intra quanto extracelulares. As colorações metanamina prata de Gomori-Grocott, PAS e hematoxilina-eosina permitem a observação do fungo, como leveduras de paredes celulares fortemente coradas, unibrotantes, pequenas e ovaladas (WANKE; LAZÉRA, 2004; SCHEEL; GÓMEZ, 2014).

❖ Pesquisa de anticorpos

A pesquisa de anticorpos tem sido considerada um importante instrumento no diagnóstico e monitoramento da histoplasmose. Busca identificar anticorpos contra os antígenos H e M de *H. capsulatum*. Algumas limitações podem ser observadas em relação a esses testes, como a dificuldade de diferenciar doença ativa de exposição ou contato prévio com o fungo; resultados falso negativos em infecções recentes, devido o desenvolvimento da resposta humoral ocorrer entre duas e seis semanas; e em pacientes imunossuprimidos, onde geralmente são negativos em mais de 50% dos casos (KAUFFMAN, 2008; SCHEEL; GÓMEZ, 2014).

Os antígenos H e M, presentes na parede celular do fungo, são importantes moléculas que interferem no mecanismo de resistência à infecção (NOSANCHUK *et al.*, 2012). Esses antígenos são marcadores de doença ativa em pacientes imunocompetentes, principalmente aqueles que apresentam a forma pulmonar aguda da micose (REISS *et al.*, 2000).

A imunodifusão radial dupla (ID) é um teste qualitativo de detecção de anticorpos. Baseia-se na difusão e precipitação de anticorpos e antígenos solúveis em gel de agarose, através da interação Ag-Ac, visualizados por meio de bandas de precipitinas coradas (OUCHTERLONY, 1949). Este método tem sensibilidade de aproximadamente 70-95%, e especificidade de 100%, em indivíduos com histoplasmose pulmonar aguda ou subaguda, e em quase todos os indivíduos com a forma crônica da doença (GUIMARÃES; NOSANCHUK; ZANCOPE-OLIVEIRA, 2006; KAUFFMAN, 2008; NEGRONI *et al.*, 2010;

SCHEEL; GÓMEZ, 2014). Em indivíduos com aids a sensibilidade é consideravelmente menor (57%), do que em indivíduos imunocompetentes (LEIMANN *et al.*, 2005). Anticorpos contra o antígeno M, uma catalase B, estão presentes em infecções agudas, e permanecem detectados nas formas crônicas e em infecções já resolvidas por meses ou anos (GUIMARÃES *et al.*, 2008; KAUFFMAN, 2008; WHEAT, 2009). Já os anticorpos contra o antígeno H, uma β-glicosidase, são detectados em menos de 20% dos pacientes, principalmente em indivíduos com as formas mais graves da histoplasmose. Desaparecem mais rapidamente que os anticorpos contra o antígeno M, porém podem persistir por um a dois anos após a cura clínica. A presença desses anticorpos no mesmo soro é altamente específica para o diagnóstico de histoplasmose (WHEAT *et al.*, 1982; GUIMARÃES; NOSANCHUK; ZANCOPE-OLIVEIRA, 2006; KAUFFMAN, 2008; WHEAT, 2009).

Outra técnica utilizada para detecção de anticorpos é o imunobloting ou *Western Blot* (WB) que consiste na separação de frações proteicas através de eletroforese em gel de poliacrilamida, seguida de eletrotransferência dessas proteínas para uma membrana de nitrocelulose, finalizando com uma reação imunoenzimática (que utiliza anticorpos conjugados a uma enzima) para a identificação de anticorpos contra os antígenos específicos como H e M (KURIEN; SCOFIELD, 2006). Em 1999, Pizzini et al. (1999) avaliou o uso de WB em uma microepidemia de HPA, observando uma alta sensibilidade (90%) e especificidade (100%) nestes pacientes, quando utilizou a histoplasmina purificada e deglicosilada (ptHMIN) como antígeno padrão (PIZZINI *et al.*, 1999). Leiman et al. (2005) encontrou 100% de positividade em quatro pacientes com histoplasmose (um com HPA, um com HD e dois com mediastinite) (LEIMANN *et al.*, 2005). O WB é mais sensível que a ID (WHEAT; KAUFFMAN, 2003), e geralmente amostras negativas na ID são positivas quando avaliadas por WB (REISS *et al.*, 2000).

Recentemente, WB, utilizando ptHMIN, foi realizado para avaliação do diagnóstico das diversas formas clínicas da histoplasmose, em 50 pacientes procedentes do Rio de Janeiro. Foi observada uma sensibilidade de 92% e uma especificidade de 98%. Quando comparado com ID, o WB apresentou maior especificidade para o diagnóstico da micose, já que detectou com maior frequência as bandas M e H simultaneamente. Entretanto, casos falso-negativos ocorreram em seis pacientes com aids (ALMEIDA *et al.*, 2016).

O ensaio de imunoabsorção enzimática (ELISA) ou ensaio imunoenzimático (EIA) busca a detecção de anticorpos (ELISA indireto) de *H. capsulatum* sendo usado em alguns laboratórios de referência somente "*in-house"* (SCHEEL; GÓMEZ, 2014). Desde a década de 1980, EIA tem sido utilizado para a detecção de anticorpos contra *H. capsulatum* com

variados graus de sensibilidade e especificidade (KUMAR *et al.*, 1985; RAMAN *et al.*, 1990; TORRES *et al.*, 1993). Em 2004, EIA com ptHMIN apresentou melhores resultados, com uma sensibilidade de 92% e especificidade de 95% (GUIMARÃES *et al*., 2004). Posteriormente, a mesma técnica com ptHMIN foi aplicada para o diagnóstico de pacientes com diversas formas clínicas da histoplasmose, onde observou-se uma sensibilidade de 86-100%, sendo menor em pacientes com infecções disseminadas e com aids, 89% e 86% respectivamente. Apesar disso, a especificidade do EIA neste estudo foi de 96% (GUIMARÃES *et al.*, 2010).

- Pesquisa de antígenos

A detecção do antígeno foi desenvolvida usando anticorpos contra galactomanana de *H. capsulatum* pelo laboratório MiraVista Diagnostico (MVista), em Indianápolis/EUA, através de radioimunoensaio (WHEAT; KOHLER; TEWARI, 1986). Posteriormente, EIA de 1ª geração foi desenvolvido para detecção de antígenos do fungo (DURKIN; CONNOLLY; WHEAT, 1997; WHEAT; WITT III *et al.*, 2007). A segunda geração deste teste reduziu consideravelmente os resultados falso-positivos, devido às reações cruzadas com outros fungos dimórficos patogênicos (WHEAT; WITT III *et al.*, 2007), e a terceira geração, permitiu a quantificação do antígeno (CONNOLLY *et al.*, 2007). Atualmente, este teste tem sido usado para diagnóstico e monitoramento da resposta ao tratamento, particularmente em pacientes com HD/aids (HAGE; KIRSCH *et al.*, 2011). A terceira geração do EIA (MVista) apresenta elevada sensibilidade para detecção de antígenos circulantes na urina (95-100%) e no soro (92-100%) (CONNOLLY *et al.*, 2007; HAGE; RIBES *et al.*, 2011). Nas formas pulmonares o teste pode ser utilizado em amostras de lavado broncoalveolar com até 93% de sensibilidade (HAGE; WHEAT, 2010). Reações cruzadas podem ocorrer com blastomicose, paracoccidiodomicose, penicilinose, coccidiodomicose, aspergilose e esporotricose (ASSI; LAKKIS; WHEAT, 2011; HAGE; RIBES *et al.*, 2011). Apesar disso, este método está validado para o diagnóstico de histoplasmose em pacientes HIV positivos, entretanto, seu uso comercial está restrito somente aos EUA (CONNOLLY *et al.*, 2007; WHEAT, 2009; HAGE; RIBES *et al.*, 2011).

Outros testes usando EIA para a detecção de antígenos de *H. capsulatum* na urina em pacientes imunocomprometidos foram descritos, mas assim como o EIA do MVista, não estão

disponíveis comercialmente (GOMEZ *et al.*, 1999; CLOUD *et al.*, 2007; SCHEEL *et al.*, 2009; ZHANG; GIBSON; DALY, 2013). ELISA de captura para detecção de antígeno de *Histoplasma* na urina foi desenvolvido pelos Centros de Controle e Prevenção de Doenças/EUA (LINDSLEY *et al*., 2007), sendo avaliada em coortes de pacientes HIV positivos, oriundos da Guatemala e Colômbia (SCHEEL *et al*., 2009; CACERES *et al*., 2014). Os resultados demonstraram uma sensibilidade entre 81%-86%, e uma especificidade de 95% em (SCHEEL *et al.*, 2009; CACERES *et al*., 2014). Reações cruzadas foram observadas apenas com paracoccidiodomicose. Atualmente, o mesmo teste está sendo validado para uso na Guiana (ADENIS; AZNAR; COUPPIE, 2014).

Um teste usando anticorpos monoclonais contra galactomanana purificada de *H. capsulatum* através de EIA (ASR-EIA) tem alcançado resultados semelhantes ao EIA do MVista (THEEL *et al.*, 2013; ZHANG; GIBSON; DALY, 2013). Zhang et al. (2015) encontraram uma especificidade do ASR-EIA semelhante ao MVista (98% vs. 100%), entretanto, a sensibilidade foi bem menor (72% vs. 96%) (ZHANG *et al.*, 2015).

- Testes Moleculares

O diagnóstico molecular usando PCR e suas variantes têm resultados rápidos com boa sensibilidade e especificidade em tecidos e fluidos corporais. Entretanto, estes testes não estão disponíveis comercialmente, e em geral, têm sido utilizados somente a nível regional em centros de pesquisa (ADENIS; AZNAR; COUPPIE, 2014).

O alvo molecular mais frequentemente usado para a detecção de DNA de *H. capsulatum* em amostras biológicas é o lócus do gene de uma proteína específica de *H. capsulatum* de 100 kDa – Hcp100, por meio de PCR aninhada (nested PCR) (SCHEEL; GÓMEZ, 2014). O primeiro estudo usando esse lócus para o diagnóstico da histoplasmose foi realizado em amostras de tecidos parafinados e fixados em formalina (TPFF) por Bialek et al. (2002). A sensibilidade é de 100%, e a especificidade varia entre 94-100% (WHEAT; FREIFELD*; et al.*, 2007; ARUNMOZHI BALAJEE *et al.*, 2013). PCR em tempo real também tem sido utilizada para a detecção de Hcp100, com 86-89% de sensibilidade e 100% de especificidade (KOEPSELL; HINRICHS; IWEN, 2012; BUITRAGO *et al.*, 2013). Amplificação isotérmica "loop-mediada" (LAMP) de Hcp100 encontrou uma sensibilidade e

especificidade de 100% em isolados clínicos do fungo, e 67% de sensibilidade na detecção do DNA do fungo em amostras urinárias (SCHEEL *et al.*, 2014).

O lócus da região ITS do rDNA é um marcador universal, que funciona como "código de barras" para identificação de espécies fúngicas ("DNA *barcode*"). Essas regiões são formadas por sequências de 500-800 pb e identificam espécies de todo reino eucarioto (SCHOCH *et al.*, 2012; IRINYI *et al.*, 2015). Caracterizam-se por apresentarem maiores distâncias interespécies do que intraespécies, criando um "*barcoding gap*" (MEYER; PAULAY, 2005; SCHOCH *et al.*, 2012), sendo uma sequência gênica única para uma determinada espécie e constante entre cada espécie (HEBERT *et al.*, 2003; HEBERT; RATNASINGHAM; DEWAARD, 2003; LETOURNEAU *et al.*, 2010). Em *H. capsulatum,* diagnóstico molecular usando esse alvo genético tem sido utilizado para detectar DNA do fungo em TPFF (MUNOZ-CADAVID *et al.*, 2010). PCR em tempo real da região ITS, para detecção de DNA de *H. capsulatum* em amostras clínicas humanas, tem encontrado uma sensibilidade de 70-95%, e especificidade de 96-100% (BUITRAGO *et al.*, 2006; MUNOZ, C. *et al.*, 2010; SIMON *et al.*, 2010; BUITRAGO *et al.*, 2013; GAGO *et al.*, 2014).

Poucos estudos têm utilizado outras regiões genéticas para o diagnóstico molecular da histoplasmose, como PCR convencional do antígeno M (GUEDES *et al.*, 2003), semi-nested PCR do antígeno H (BRACCA *et al.*, 2003), e PCR-RAPD de SCAR (*sequence-characterized amplified region*), uma sequência gênica específica amplificada através dos iniciadores 1281-1283_{220} e 1281-1283_{230} (FRIAS DE LEON *et al.*, 2012).

1.1.3.7 Tratamento

O tratamento da histoplasmose deve sempre ser realizado nos casos grave ou moderado, e naqueles que cursam com disseminação da doença, ou que acometem o sistema nervoso central (Tabelas 2 e 3) (WHEAT; FREIFELD *et al.*, 2007). Os agentes antifúngicos que têm melhor efetividade e que são de escolha para o tratamento são: anfotericina B, nas suas diversas formulações, e o itraconazol. A anfotericina B é indicada para formas disseminadas e pulmonares graves. É usada até a resposta clínica favorável, por meio da melhora dos sintomas, quando então a terapia pode ser continuada com antifúngicos azólicos por via oral (WHEAT; FREIFELD *et al.*, 2007; KAPLAN *et al.*, 2009).

Itraconazol é o antifúngico de escolha para terapia das formas leves ou moderadas, e também para dar continuidade à terapia inicial com anfotericina B. Outros azólicos, como fluconazol, podem ser usados em pacientes com HD sem aids, sendo efetivo em até 70% dos casos (WHEAT; FREIFELD *et al.*, 2007; KAPLAN *et al.*, 2009). Apesar do posaconazol e voriconazol terem demonstrado atividade *in vitro* contra *H. capsulatum, o*s resultados ainda são considerados insuficientes *in vivo* (WHEAT; FREIFELD *et al.*, 2007).

Em pacientes com HD/aids, a terapia deve ser realizada, iniciando com a terapia de indução com anfotericina B ou itraconazol, dependendo da gravidade clínica. Nos casos graves, a terapia de indução deve ser realizada com anfotericina B, por uma a duas semanas ou até a melhora clínica, seguida de itraconazol na dose de 600 mg/dia, dividida em três tomadas por três dias, e posteriormente 400 mg/dia, dividida em duas tomadas por 12 meses. Nos casos leves, inicia-se com itraconazol na dose de 600 mg/dia, dividida em três tomadas por três dias, seguida da dose de 400 mg/dia dividida em duas tomadas por 12 meses (WHEAT, 2009).

Tabela 2: Indicações de tratamento específico em pacientes com histoplasmose.

Indicação definida – eficácia comprovada
• HPA difusa com sintomas graves ou moderados
• HPC
• HD
• Histoplasmose de SNC
Indicação indefinida – eficácia desconhecida
• HPA em pacientes assintomáticos ou com sintomas leves e persistentes por mais de um mês
• Linfadenites ou granuloma mediastinal
• Síndromes inflamatórias
Não recomendada – eficácia desconhecida e ineficaz
• Fibrose mediastinal
• Nódulo pulmonar
• Broncolitíase
• Histoplasmose ocular

Fonte adaptada: WHEAT; FREIFELD *et al.*, 2007, p. 811.

Após a terapia de indução, inicia-se a terapia de supressão ou de manutenção em pacientes com coinfecção HD/aids, devendo ser realizada com itraconazol 200 mg/dia por tempo indeterminado. Entretanto, estudos têm demonstrado que a suspensão da terapia de

manutenção é possível em pacientes aderentes a HAART, e que evoluem com recuperação imunológica dos linfócitos T CD4+ (> 150 céls/mm^3), associado ao uso de itraconazol por 12 meses (GOLDMAN *et al.*, 2004; DAMASCENO *et al.*, 2013). Caso o paciente evolua com redução dos linfócitos T CD4+ abaixo de 150 céls/mm^3, a terapia de supressão deve ser reiniciada (WHEAT, 2009).

Para pacientes de áreas endêmicas, com linfócitos T CD4+ menor do que 150 céls/mm^3, em que a incidência de histoplasmose é maior do que dez casos/100 pacientes-ano ou quando há risco de exposição ocupacional, deve ser realizada a profilaxia primária com itraconazol 200 mg/dia, a qual deverá ser suspensa quando o paciente alcançar a recuperação imunológica sustentada por mais de seis meses em uso de HAART (WHEAT, 2009).

Tabela 3: Recomendações de antifúngicos para tratamento de histoplasmose.

Forma clínica	**Antifúngico**	**Grau de recomendação e força de evidencia**
HPA grave ou moderada	Anfotericina B lipossomal (3-5 mg/Kg/dia) ou Anfotericina B deoxicolato (0,7-1 mg/Kg/dia), por 1-2 semanas, seguido de itraconazol (200 mg duas vezes ao dia), até completar 12 semanas.	A-III
HPA leve	Se sintomas com menos de 4 semanas – terapia não é indicada.	A-III
	Se sintomas com mais de 4 semanas - Itraconazol (200 mg uma ou duas vezes ao dia), por 6-12 semanas.	B-III
HPC	Itraconazol (200 mg uma ou duas vezes ao dia), por até 12 meses.	A-II
HD grave ou moderada	Anfotericina B lipossomal (3 mg/Kg/dia) ou Anfotericina B deoxicolato (0,7-1 mg/Kg/dia), por 1-2 semanas, seguido de itraconazol (200 mg duas vezes ao dia), por até 12 meses.	A-I
HD leve	Itraconazol (200 mg duas vezes ao dia), por até 12 meses.	A-II
Histoplasmose de SNC	Anfotericina B lipossomal (5 mg/Kg/dia) por 4-6 semanas, seguido de itraconazol (200 mg 2-3 vezes ao dia), até completar 12 meses.	B-III

Fonte adaptada: WHEAT; FREIFELD *et al.*, 2007, p. 816-817.

2 JUSTIFICATIVA

Histoplasma capsulatum responsável pela histoplasmose clássica é encontrado em praticamente todas as regiões do mundo, devido ao alto potencial de adaptação do fungo aos diversos ambientes. O aumento da frequência da histoplasmose em zonas endêmicas está diretamente associado com o aumento da população imunodeprimida, especialmente em PVHA, onde o fungo atua como oportunista produzindo formas clínicas disseminadas e muitas vezes fatais. No Brasil, a incidência real da histoplasmose é pouco conhecida uma vez que esta micose não está incluída na lista de enfermidades de notificação compulsória. Com isso, pouco se conhece sobre a epidemiologia desta micose e os perfis genéticos das cepas de *H. capsulatum* circulantes no Brasil, e principalmente no estado do Ceará, onde a histoplasmose é endêmica com altas taxas de mortalidade e de recidiva.

Estudos sobre a variabilidade genética do *H. capsulatum* em diferentes regiões geográficas do país vêm proporcionando um melhor entendimento sobre as fontes de infecção (endógena *versus* exógena), o mecanismo de transmissão da infecção durante surtos da doença, a relação dos genótipos com o estado imunológico do paciente e a forma clínica da doença, além de revelar subtipos de linhagens individualmente.

Acredita-se que micronichos de *H. capsulatum* existam nas diversas regiões do mundo e possam estar atuando como fonte de infecção. Com isso, tem-se identificado diferenças fenotípicas e genotípicas dentro de uma mesma área geográfica, e subtipos de *H. capsulatum* dentro de uma mesma linhagem. Sabe-se que cepas isoladas nos EUA são geneticamente diferentes de cepas da América Latina. Esses achados contribuíram para melhor esclarecimentos sobre as diferenças nas manifestações clínicas da doença, como por exemplo, o encontro de lesões de pele associadas a forma disseminada principalmente em PVHA do Brasil. A diversidade de isolados clínicos de *H. capsulatum* está de acordo com o fato de este fungo residir em muitos tipos de solos e climas, em diferentes áreas geográficas.

É possível que a diversidade genética e fenotípica das amostras de *H. capsulatum* possa explicar o comportamento endêmico da histoplasmose no Ceará. Estudar os genótipos autóctones de *H. capsulatum* nos permitirá conhecer o fluxo das cepas nessa região, além de avaliar a introdução de genótipos exóticos, comparar as linhagens observadas com os perfis genéticos previamente encontrados no Brasil, e realizar possíveis associações com características epidemiológicas, formas clínicas e de desfecho da doença no Ceará.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar o perfil fenotípico e genotípico de isolados clínicos de *H. capsulatum* de pacientes diagnosticados com histoplasmose no Hospital São José de Doenças Infecciosas (HSJ) do Estado do Ceará, e correlacionar com os aspectos clínico-epidemiológicos.

3.2 OBJETIVOS ESPECÍFICOS

- Contribuir e descrever os aspectos da epidemiologia molecular da histoplasmose;
- Identificar as características epidemiológicas e clínicas de pacientes com histoplasmose;
- Avaliar a morfologia e o perfil de exoantígenos dos isolados clínicos de *H. capsulatum;*
- Identificar os tipos de compatibilidade sexual dos isolados clínicos de *H. capsulatum*;
- Classificar taxonomicamente os isolados clínicos de *H. capsulatum*;
- Avaliar a variabilidade genética dos isolados fúngicos;
- Relacionar os dados fenotípicos e genotípicos dos isolados de *H. capsulatum* com as características clínicas e epidemiológicas dos pacientes com histoplasmose.

4 MATERIAS, MÉTODOS E RESULTADOS

As técnicas utilizadas para caracterização fenotípica e genotípica dos isolados clínicos de *H. capsulatum*, assim como a forma de coleta dos dados clínicos e epidemiológicos, e os resultados obtidos durante este estudo, foram descritos nos manuscritos que estão identificados sob a forma de capítulos como se observa a seguir.

Capítulo 1: Could *Histoplasma capsulatum* be related to healthcare-associated infections? (artigo publicado na revista BioMed Research Internacional em Maio de 2015, doi: 10.1155/2015/982419).

Capítulo 2: The use of genetic markers in the molecular epidemiology of histoplasmosis: a systematic review (artigo publicado na revista European Journal Microbiology of Infectious Diseases em Novembro de 2015, vol. 35: 19-27).

Capítulo 3: Correlation between phenotype and mating type genotype of *Histoplasma capsulatum* isolates and clinical manifestations of histoplasmosis patients from Ceará, Brazil (manuscrito em revisão).

Capítulo 4: Mixed infection by *Histoplasma capsulatum* isolates with different mating types in Brazilian AIDS-associated histoplasmosis patients (manuscrito em revisão).

Capítulo 5: Postpartum histoplasmosis in a HIV negative woman: a case report and phylogenetic characterization by ITS region analysis (manuscrito em revisão).

Capítulo 6: A restrict genetic population of *Histoplasma capsulatum* clinical isolates identified in Ceará/Brazil by (GA)n microsatellite typing (manuscrito em revisão).

Capítulo 7: Emerging clinical genotypes of *Histoplasma capsulatum* in Northeast Brazil (manuscrito em elaboração).

4.1 Capítulo 1

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Review Article

Could *Histoplasma capsulatum* Be Related to Healthcare-Associated Infections?

Laura Elena Carreto-Binaghi,[1] Lisandra Serra Damasceno,[2] Nayla de Souza Pitangui,[3] Ana Marisa Fusco-Almeida,[3] Maria José Soares Mendes-Giannini,[3] Rosely Maria Zancopé-Oliveira,[2] and Maria Lucia Taylor[1]

Artigo publicado na revista BioMed Research Internacional em Maio de 2015, doi: 10.1155/2015/982419.

Infecções relacionadas à assistência à saúde (IRAS) referem-se às infecções associadas aos cuidados de saúde, sejam eles intra ou extra-hospitalar. Os principais agentes etiológicos de IRAS são as bactérias (85%) e fungos (13%). Em geral, *Candida spp.* são os fungos mais associados às IRAS, causadas usualmente por fontes endógenas (translocação fúngica). Este artigo de revisão questiona a possibilidade de *Histoplasma capsulatum* comportar-se como um agente etiológico não usual de IRAS. *H. capsulatum* é um patógeno dimórfico, de distribuição mundial, geralmente encontrado em lugares contaminados com fezes de pássaros ou morcegos. Seus propágulos infectantes dispersam-se no ambiente através de aressóis.Além disso, podem formar biofilme e quoro sense, e comportam-se como patógenos oportunistas em hospedeiros imunocomprometidos. Embora, até o momento, nenhuma técnica convencional na rotina laboratorial tenha identificado *H. capsulatum* no ambiente hospitalar, alguns casos de histoplasmose já foram associados a dispositivos invasivos, implantes cirúrgicos, biofilmes no sistema cardiovascular, e em indivíduos pós-transplantes. Acreditamos que mais estudos sobre o tema e a aplicação de técnicas de biologia molecular possam ajudar a esclarecer o papel de *H. capsulatum* como agente etiológico de IRAS.

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Healthcare-associated infections (HAI) are described in diverse settings. The main etiologic agents of HAI are bacteria (85%) and fungi (13%). Some factors increase the risk for HAI, particularly the use of medical devices; patients with severe cuts, wounds, and burns; stays in the intensive care unit, surgery, and hospital reconstruction works. Several fungal HAI are caused by *Candida* spp., usually from an endogenous source; however, cross-transmission via the hands of healthcare workers or contaminated devices can occur. Although other medically important fungi, such as *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, and *Histoplasma capsulatum*, have never been considered nosocomial pathogens, there are some factors that point out the pros and cons for this possibility. Among these fungi, *H. capsulatum* infection has been linked to different medical devices and surgery implants. The filamentous form of *H. capsulatum* may be present in hospital settings, as this fungus adapts to different types of climates and has great dispersion ability. Although conventional pathogen identification techniques have never identified *H. capsulatum* in the hospital environment, molecular biology procedures could be useful in this setting. More research on *H. capsulatum* as a HAI etiologic agent is needed, since it causes a severe and often fatal disease in immunocompromised patients.

1. Introduction

The term healthcare-associated infection (HAI) refers to infections associated with healthcare delivery in any setting (e.g., hospitals, long-term care facilities, ambulatory settings, and home care). This term reflects the inability to determine with certainty where the pathogen is acquired since patients may be colonized or exposed to potential pathogens outside the healthcare setting, before receiving healthcare or during healthcare delivery [1, 2].

In recent years, there has been an overall increase in HAI, which is likely a consequence of the advances in medical and surgical procedures related to specific therapies, in addition to the large number of immunocompromised patients who are hospitalized [3]. It is estimated that every day one out of 25 hospital patients has, at least, one HAI. In 2011, there were 722,000 HAI in the United States' hospitals and about 75,000 hospital patients with HAI died during their hospitalization. More than half of all HAI occurred outside the intensive care unit [4].

HAI commonly occur by direct transmission from individual to individual or through fomites manipulated by healthcare workers, as well as through surfaces and devices contaminated by biofilms (surgical instruments, catheters,

mechanical ventilation systems, and others) [5, 6]. Other mechanisms of transmission are aerial dispersion of opportunistic or environmental microorganisms and endogenous dissemination of commensal or opportunistic pathogens [7–9].

Although the role of the inanimate hospital environment in the spread of HAI has been controversial, nowadays molecular biology methodologies are being used to identify pathogens, measure the quality of environmental and hand hygiene over time, and establish a link between outbreaks and cross-transmission events, according to geographic and temporal variables [8].

Currently, changes in morbidity and mortality patterns due to aging of the world population, treatments with immunosuppressive drugs, and the use of invasive devices (particularly long-term ones) have led to a rise in the need of healthcare facilities for patients who are more susceptible to opportunistic infections [10]. Environmental disturbances associated with construction activities near health institutions pose additional airborne and waterborne disease threats for those patients who are at risk for healthcare-associated fungal infections [2]. Particularly, hospitalized patients could be exposed to infective fungal propagules such as microconidia and small hyphal fragments of *Histoplasma capsulatum* that thrive in bat and bird droppings, deposited in the surrounding hospital recreational areas.

Thus, the aims of this paper were to review the reported cases of *H. capsulatum* infections in healthcare settings, in order to propose the different factors that could be related to healthcare-associated histoplasmosis and discuss the features that could favor the presence of this fungus in the hospital environment.

2. Etiologic Agents of HAI

The etiologic agents of HAI are mainly bacteria (85%) and fungi (13%), in contrast to viruses and parasites that are rarely reported. Some environmental factors have been identified to increase the risk for fungal HAI, particularly the use of medical devices, like central venous and urinary catheters; the presence of severe cuts, wounds, and burns; stays in the intensive care unit, surgery, and hospital reconstruction works [4].

Host factors, such as extremes of age and underlying diseases, human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS), malignancy, and transplants, can increase susceptibility to infection, as well as a variety of medications that alter the normal flora, like antimicrobial agents, gastric acid suppressants, steroids, antirejection drugs, antineoplastic agents, and immunosuppressive drugs [2].

Most HAI associated with fungi are caused by *Candida* spp. These infections usually come from an endogenous source. However, cross-transmission via the hands of healthcare workers or contaminated devices can occur [11]. HAI outbreaks by other yeasts, such as *Malassezia* spp., *Saccharomyces* spp., and *Trichosporon* spp., have also been identified in newborns, patients with hematologic malignancies, and transplant recipients [12–18]. Mechanical ventilation, duration of hospital stay, prolonged use of intravascular catheters, parenteral lipid formulations, and prior exposure to broad spectrum antibiotics (including antifungal therapy) are important predisposing conditions identified in these outbreaks [13, 14, 16–18].

The occurrence of invasive fungal infections (IFIs) depends on several factors like the time of exposure to an infectious agent, the patient's immune status, the pathogen's virulence factors, and the host-pathogen interaction [19]. IFI associated with healthcare is mainly caused by opportunistic fungi, from endogenous or environmental sources, which form biofilms in fomites and abiotic surfaces [9].

Species of filamentous fungi, such as *Aspergillus* spp. [20, 21], *Rhizopus* spp. [22], *Rhizomucor* spp. [22, 23], *Absidia corymbifera* [22, 24], *Fusarium* spp. [25–27], *Paecilomyces* spp. [28, 29], *Curvularia* spp. [30], *Phialemonium* spp. [31–34], and *Scedosporium* spp. [35–37], have been particularly associated with HAI in patients with hematologic diseases. The most common sources reported in the above-mentioned filamentous fungal infections were contamination of medical supplies, like intravenous solutions, contact lens solutions [38, 39], bandages [24], pressure cuffs, and invasive devices (endotracheal tubes) [11, 21–23, 40]. Besides, other species of fungi such as *Aureobasidium* spp. [41], *Trichosporon* spp. [42, 43], *Rhodotorula* spp. [44–46], and *Phaeoacremonium parasiticum* [47] have been implicated in nosocomial pseudooutbreaks through contamination of endoscopes.

A very important opportunistic fungus, *Pneumocystis jirovecii*, has also been associated with HAI by person-to-person airborne transmission [48–58]. Infection by *P. jirovecii* presents as an interstitial pneumonia in immunocompromised hosts, particularly HIV patients; in this group, pneumocystosis is considered an AIDS-definitory condition, when CD4+ T lymphocytes are below 200 cells/μL [59]. Currently, an increase of pneumocystosis in non-HIV patients is being observed, especially in patients with transplants, individuals with autoimmune disorders or malignancies, and those using immunosuppressive treatments, like steroids and immunobiological drugs [50, 55, 60–62]. Molecular biology techniques have detected a high prevalence of colonization (10–55%) in immunocompromised patients and in the general population. Individuals colonized by *P. jirovecii* can be considered reservoirs and therefore contribute to the transmission of this pathogen among immunosuppressed patients in the hospital environment [62].

Other important respiratory pathogens, such as *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, and *H. capsulatum*, have never been associated with infections in the hospital environment; however, *B. dermatitidis* has been found in pseudooutbreaks associated with contaminated bronchoscopes [63].

3. *H. capsulatum* Infection

H. capsulatum is a dimorphic fungus with a mycelial saprobe-geophilic morphotype (infective M-phase), usually found in bat and bird guano, and a yeast morphotype (parasitic and virulent Y-phase) preferentially located within phagocytes. Infection occurs through inhalation of aerosolized M-phase

propagules, mainly microconidia and hyphal fragments, accumulated in confined spaces usually inhabited by bats or birds [64].

There are eight genetic populations of *H. capsulatum* distributed worldwide [65], between the latitudes 54° North [66] and 38° South [67], suggesting a broad geographic dispersion of the pathogen. *H. capsulatum* has been found in ecological niches with special conditions: air and soil temperatures (18–28°C), humidity (>60%), and darkness (fosters sporulation). Particularly, this fungus needs the presence of high concentrations of nitrogen and phosphorus for the M-phase growth, in addition to other micronutrients, which are plentiful in bat or bird guano [64, 68–70]. Besides, this fungus' ubiquitous distribution in nature (soil, treetops, yards, and public parks, among others) makes it feasible to find the M-phase in open spaces, either in rural or urban areas around hospitals [64, 70]. In a large outbreak that occurred in Acapulco, Mexico, the presence of the fungus was revealed in ornamental potted plants, containing organic material known as compost supplemented with bat guano that is used as fertilizer [70].

Histoplasmosis is a systemic mycosis preferentially distributed in endemic areas of the Americas. Most *H. capsulatum* infections are asymptomatic. A low number of individuals develop pneumonia, which is the main clinical form in immunocompetent patients (primary pulmonary histoplasmosis) with distinctive histopathological features, like chronic granulomatous infiltrate [69]. Epidemic outbreaks of histoplasmosis are related to occupational exposure or recreational activities and affect individuals worldwide [66, 71–73]. However, this disease is one of the most common opportunistic infections among HIV/AIDS patients with CD4+ T lymphocytes below 150 cells/μL (known as AIDS-definitory condition), who may develop severe and fatal disseminated histoplasmosis [59]; approximately 30% of these patients die from this infection [74–76]. *H. capsulatum* infections have also been described in patients with transplants [77, 78], invasive devices, and/or surgical implants [79–81].

H. capsulatum shares some features with the etiologic agents of HAI, bacteria or fungi, which support the nosocomial involvement of *H. capsulatum* infection: worldwide distribution (facilitated by flying reservoirs), its ubiquity, production of aerosolized infective propagules that spread the fungus in the environment and favor the infection by the respiratory pathway, development of biofilm and quorum-sensing (QS) events, and opportunistic behavior in immunosuppressed hosts.

4. Biofilm Formation

It is estimated that 95% of the microorganisms found in nature are attached in biofilms [82]. Over 60–65% human infections involve the formation of biofilms by normal commensal flora or nosocomial pathogens [83–89]. A biofilm is a complex structured community of microorganisms, surrounded by an extracellular matrix of polysaccharides, adhering to each other over a surface or interface [82]; sometimes protein-like adhesins of the pathogen are also involved in biofilm formation [90]. Biofilms constitute a potential source of chronic, recurrent infections and cross-contamination events [7, 89]. Microorganisms in biofilms are protected from the host's immune system and may be 1,000-fold more resistant to antibiotics than planktonic cells [91], due to poor penetration of drugs, low growth rate, and development of the microorganism's resistant phenotypes within biofilms [84, 92].

Fungal biofilms have been found not only in wild soil and water, but also in urban environments, like piping systems, water reservoirs, and constructions, and in healthcare equipments [8, 85, 93–96]. Among the fungal biofilms found on these surfaces, the medically important fungi, such as *Candida* spp., *Aspergillus* spp., *Cryptococcus* spp., *Rhodotorula* spp., *Penicillium* spp., *Sporothrix* spp., *Acremonium* spp., and *Paecilomyces* spp., must be highlighted [8, 90, 93–98].

In medical devices, *Candida* spp. is the most common fungi associated with biofilm formation, usually with endovascular and urinary catheter-related infections in intensive care units, resulting in invasive candidiasis with high mortality [99–101]. The distribution of *Candida* species is variable and in recent years non-*albicans Candida* species have been frequently found in patients with hemodialysis catheter-related candidemia [102].

The presence of biofilms has also been described in ventriculoperitoneal (VP) shunts in patients with *Candida* spp., *Cryptococcus neoformans*, and *Coccidioides immitis* meningoencephalitis. These biofilms were associated with recurrent peritonitis and meningitis [88, 103]. Various fungi have been able to form biofilms on abiotic surfaces in experimental models, such as *A. fumigatus* [104], *M. pachydermatis* [105], *Blastoschizomyces capitatus* [106], *Candida* spp. [107], *Pneumocystis* spp. [108], *Rhodotorula* spp. [109, 110], *C. neoformans* [111], *S. cerevisiae* [112], *Fusarium* spp. [113], *T. asahii* [114], and zygomycetes [115].

Epidemiological surveillance definitions of HAI include surgical site infections associated with surgical implants or medical indwelling devices, when they occur within 30–90 days after the surgical procedure [4]. Clinically, *H. capsulatum* infections have been identified in individuals with invasive devices or surgical implants, and some authors have described endovascular histoplasmosis in patients with vascular prosthetic or synthetic implants [80, 81, 116–119]. Usually, the diagnosis is made by isolation of the fungus in vegetation or over synthetic materials. In addition, histopathological observation has revealed fibrin, large aggregates of yeast cells, mild chronic inflammatory cell infiltrates (predominantly macrophages) [80, 116, 118], and *H. capsulatum* hyphae (M-phase) in a few cases [117]. Furthermore, *H. capsulatum* endocarditis has also been described in native heart valves [118–120]. The aforementioned factors suggest the ability of *H. capsulatum* Y-phase to form biofilms in vivo (human solid organs and medical devices). Recently, it was described that *H. capsulatum* is able to form biofilms on abiotic surfaces [121]. Besides, *H. capsulatum* yeasts have been found clustered in the cells of bats' spleen, lung, and liver and in the lamina propria of intestine villi [122].

There are some reports of *H. capsulatum* peritonitis associated with infected catheters in patients with end-stage renal disease under continuous ambulatory peritoneal dialysis [123–128]. All of these peritoneal histoplasmosis'

cases occurred in residents from an endemic area, in a period longer than 90 days, in contrast with the epidemiological definition of HAI. Thus, continuous exposure to the fungus' infective M-phase propagules appears to be an important risk factor, since no other epidemiological feature could be associated with these cases.

Veeravagu et al. [129] reported a case of *H. capsulatum* meningitis associated with a VP shunt that was diagnosed two days after surgery. It is noteworthy that the patient did not come from an endemic area. Furthermore, *H. capsulatum* was isolated from the VP shunt tip and the surgical instruments, so this could be considered a nosocomial histoplasmosis.

Currently, it is unknown if *H. capsulatum* is able to form biofilms in its filamentous form, which could contaminate hospital environments, medical devices, and supplies, facilitating the direct inoculation of the infective form through cross-contamination. However, it is not a farfetched idea, because biofilms have been described in filamentous fungi, such as *Aspergillus* spp. [104] and zygomycetes [115].

5. Quorum Sensing (QS)

QS is a mechanism of microbial communication dependent on cell density that can regulate several behaviors in bacteria, such as secretion of virulence factors, biofilm formation, survival, and bioluminescence. Fungal QS systems were first described in the pathogenic fungus *C. albicans*, with important signaling molecules, called farnesol and tyrosol (alcohols derived from aromatic amino acids), which control fungal growth, morphogenesis, and biofilm formation, inducing detrimental effects on host cells and other microbes. The concentration of these alcohols increases proportionally to the microbial population and, after reaching a critical threshold, a regulatory response is triggered leading to the coordinated expression or repression of QS-dependent target genes in the entire microbial population [130].

QS activities have also been described in other fungi, such as *H. capsulatum* [131], *Ceratocystis ulmi* [132], and *Neurospora crassa* [133]; however, the molecules responsible for such activities have not yet been purified. In *H. capsulatum*, regulation of α-(1,3)-glucan synthesis in the Y-phase cell wall has been shown to occur in response to cell density [134].

Albuquerque and Casadevall [130] proposed that fungal QS molecules are not only a product of fungal catabolism, but they should have some characteristics: to accumulate in the extracellular environment during fungal growth at a concentration proportional to the population cell density restricted to a specific stage of growth, to induce a coordinated response in the entire population once a threshold concentration is reached, and to reproduce the QS phenotype when added exogenously to the fungal culture. More research about these molecules is needed to elucidate the QS mechanisms in each fungus model, involving different pathogenic events, including biofilm formation.

6. *H. capsulatum* Infection in Drug-Induced Immunocompromised Individuals

IFIs related to immunosuppression caused by drugs in patients with transplant occur because cellular immunity is modified, usually within the first six months posttransplant. During this period, the IFI acquired an opportunistic nature and emerged as HAI [135–137]. After six months posttransplant, patients usually remain stable and continue receiving immunosuppressive drugs at low doses. Thus, they are susceptible to common infections acquired in the community [79, 135–137].

H. capsulatum infections have been identified in solid organ transplant (SOT) recipients [138, 139]. However, a low frequency of histoplasmosis related to HAI has been observed in the first six months posttransplant. Freifeld et al. [138] identified nine cases of pulmonary histoplasmosis in SOT recipients in a period of 30 months, but only four patients developed the disease in the first six months posttransplant. In a 10-year cohort study, Cuellar-Rodriguez et al. [140] found only three cases of histoplasmosis in SOT recipients in the first six months posttransplant; however, eleven cases were identified after the first year posttransplant.

Other authors evaluated the incidence of IFIs in SOT recipients, in a 5-year cohort study [141, 142]. Of the 1,208 cases of IFI, histoplasmosis was diagnosed in 48 patients, where 18 cases (37.5%) occurred in the first six months posttransplant, particularly in kidney, liver, and kidney-pancreas transplants [141]. In general, these patients were receiving immunosuppressive drugs, like tacrolimus, sirolimus, mycophenolate mofetil, and steroids [138, 140, 142].

A more recent study about IFIs identified an increase in the number of histoplasmosis cases in SOT recipients [142]. Among the 70 cases of IFIs reported, 52 (80%) were diagnosed as histoplasmosis, in a 5-year period. The median time from transplant to the diagnosis of this fungal disease was one year. Five SOT recipients developed histoplasmosis within 30 days of transplant; two patients acquired the infection from their donated organs, and three patients developed pulmonary histoplasmosis irrespectively of the transplanted organs [142]. In rare cases, histoplasmosis has also been diagnosed in patients treated with immunobiological molecules, like different monoclonal antibodies [143, 144].

7. Fungal Respiratory Infections in the Hospital Environment

Inadvertent exposure to opportunistic environmental and airborne pathogens can result in infections with significant morbidity and mortality [9]. Fungal infections can range from mild to life-threatening; they vary among mild skin rashes, fungal pneumonia, meningitis, and IFIs. In the hospital, the most common fungal HAI are caused by *Candida* spp. and *Aspergillus* spp. [19].

Airborne infections in susceptible hosts may result from exposure to environmental microorganisms that are ubiquitous in nature, growing in soil, water, dust, or organic matter [2, 9]. Spores or hyphal fragments of fungi usually lie scattered in the environment, especially near decomposing organic matter. *Aspergillus fumigatus* is the species most often associated with pulmonary IFIs [3]. Infection occurs after inhalation of conidia stirred up from construction or renovation works in the hospital. The main risk factor for this HAI is the concentration of *Aspergillus* conidia in the

air [2, 145–149], and the most susceptible individuals are hematopoietic stem cell transplant recipients, neutropenic patients, and those with hematologic malignancies [136, 145, 150–153].

Infections due to *C. neoformans*, *H. capsulatum*, or *C. immitis* can occur in healthcare settings if the nearby ground is disturbed and a malfunction of the facility's air-intake components allows these pathogens to enter the hospital ventilation system [9]. Several outbreaks of histoplasmosis have been associated with disruption of the environment [67, 72, 154]. *H. capsulatum* contaminated environments related to bat and bird colonies living in abandoned buildings and on treetops could disperse the fungus around the hospital. Under this statement, the dispersion of *H. capsulatum* infective propagules could represent a potential risk factor for hospital-acquired histoplasmosis, especially in individuals hospitalized in units lacking adequate air quality control. *H. capsulatum* has never been identified in air quality studies from hospital settings [152]. This could be explained by the difficulties in this fungus' isolation, including prolonged culture growth in laboratory conditions, special nutritional needs, and culture inhibition by the presence of other fast-growing fungi [64, 68].

8. Molecular Biology as a Diagnostic Tool in HAI

Hospital-acquired pneumonia represents one of the most difficult treatment challenges in infectious diseases. Many studies suggest that the timely administration of appropriate pathogen-directed therapy could be lifesaving. However, results of bacterial cultures and antimicrobial susceptibility testing can take 48 hours or longer, but some fungi may not even be able to grow in the first week after culturing.

Nowadays, physicians rely on clinical and epidemiological factors to choose an initial empiric therapy for HAI. A number of rapid molecular tests have been developed to identify pathogens and the bases for most molecular assays are polymerase chain reaction and nucleic-acid-sequence-based amplification. These methodologies offer the promise of dramatically improving the ability to identify pathogens in respiratory tract specimens with high sensitivity and specificity. Data from such applications can also be electronically integrated into shared molecular databases, where clinicians and epidemiologists could ascertain local, regional, national, and international trends [155].

Molecular identification of fungi in hospitals has been scarcely described [156–158]. Lo Passo et al. [156] reported transmission of *Trichosporon asahii* by an endoscopic procedure, when isolated from an esophageal ulcer. *T. asahii* isolates were genotyped by restriction fragment length polymorphism and random amplification of polymorphic DNA, confirming the endoscopic device as the source of transmission.

Notwithstanding, there are some undefined issues regarding the use of these molecular biology tools.

(i) Molecular assays have been used mainly for bacteria and viruses, leaving aside the importance of other microorganisms, such as pathogenic fungi; however, there are specific markers for almost every fungal pathogen, which have recently improved the molecular diagnostic bundle for HAI [157].

(ii) The significance of finding a pathogen's DNA in respiratory tract specimens, in the absence of a positive culture, will show different airway ecology from what it is known, and it exposes the inability to distinguish between infecting and colonizing organisms [155].

(iii) The complexities of the pulmonary microbiome and its metagenomic diversity represent a great challenge with many unanswered questions remaining [155].

(iv) New procedures combining molecular biology techniques and environmental sampling of air have revealed some fungal pathogens living in the hospital surroundings [158], which may be relevant for the acquisition of respiratory HAI.

9. Conclusions

H. capsulatum infection associated with healthcare has been linked to medical devices and surgical implants. The M-phase of *H. capsulatum* may be present in hospital settings, as this fungus adapts to different types of climates and has great dispersion ability. Although conventional pathogen identification techniques have never identified *H. capsulatum* in the hospital environment, histoplasmosis HAI cases have been reported in the last decades. Molecular biology procedures could be useful in this fungus' identification in the air of hospitals and in the diagnosis of this mycosis. More research is needed about *H. capsulatum* involvement in HAI, since it causes a severe and often fatal disease in immunocompromised individuals.

Conflict of Interests

The authors declare that there is no conflict of interests among them and with any financial organization regarding the material discussed in the present paper.

Authors' Contribution

Maria Lucia Taylor, Laura Elena Carreto-Binaghi, and Lisandra Serra Damasceno contributed equally to the design of this study. Laura Elena Carreto-Binaghi and Lisandra Serra Damasceno contributed equally to draft the paper. Nayla de Souza Pitangui, Ana Marisa Fusco-Almeida, and Maria José Soares Mendes-Giannini contributed with their critical opinion to improve the paper. Rosely Maria Zancopé-Oliveira and Maria Lucia Taylor were supervisors of this study. All of the authors read and approved the final version of the paper. Laura Elena Carreto-Binaghi and Lisandra Serra Damasceno contributed equally to the development of the review.

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4.2 Capítulo 2

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REVIEW

The use of genetic markers in the molecular epidemiology of histoplasmosis: a systematic review

L. S. Damasceno[1] · T. M. J. S. Leitão[2] · M. L. Taylor[3] · M. M. Muniz[1] · R. M. Zancopé-Oliveira[1]

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Histoplasmose é uma micose sistêmica causada por *Histoplasma capsulatum*, um fungo dimórfico, que causa infecções tanto no homem como em outros animais. No ambiente, *H. capsulatum* cresce sob a forma filamentosa, mas em parasitismo e em condições especiais, sob a forma leveduriforme. Técnicas moleculares são importantes métodos para conduzir investigações epidemiológicas como na detecção do fungo, identificação das fontes de infecção e determinação de genótipos associados a diferentes manifestações clínicas da doença. Neste estudo, foi realizada uma revisão sistemática através das bases de dados PubMed e Web of Cience, para melhorar o entendimento sobre a epidemiologia molecular da histoplasmose. O estudo foi restrito a artigos em Inglês e Espanhol, sendo incluída uma combinação específica de palavras chaves: tipagem molecular [ou] diversidade genética [ou] polimorfismo [e] *H. capsulatum*; epidemiologia molecular [e] histoplasmose; epidemiologia molecular [e] *Histoplasma*. Além disso, foram usados termos específicos como histoplasmose [e] surtos. Os principais métodos usados para tipagem molecular foram: RAPD, Microssatélites, sequenciamento da região ITS, e MLST. Diferentes perfis genéticos foram identificados, sendo agrupados de acordo com a fonte do isolamento, origem geográfica e manifestações clínicas.

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REVIEW

The use of genetic markers in the molecular epidemiology of histoplasmosis: a systematic review

L. S. Damasceno[1] · T. M. J. S. Leitão[2] · M. L. Taylor[3] · M. M. Muniz[1] · R. M. Zancopé-Oliveira[1]

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Abstract Histoplasmosis is a systemic mycosis caused by *Histoplasma capsulatum*, a dimorphic fungal pathogen that can infect both humans and animals. This disease has worldwide distribution and affects mainly immunocompromised individuals. In the environment, *H. capsulatum* grows as mold but undergoes a morphologic transition to the yeast morphotype under special conditions. Molecular techniques are important tools to conduct epidemiologic investigations for fungal detection, identification of infection sources, and determination of different fungal genotypes associated to a particular disease symptom. In this study, we performed a systematic review in the PubMed database to improve the understanding about the molecular epidemiology of histoplasmosis. This search was restricted to English and Spanish articles. We included a combination of specific keywords: molecular typing [OR] genetic diversity [OR] polymorphism [AND] *H. capsulatum*; molecular epidemiology [AND] histoplasmosis; and molecular epidemiology [AND] *Histoplasma*. In addition, we used the specific terms: histoplasmosis [AND] outbreaks. Non-English or non-Spanish articles, dead links, and duplicate results were excluded from the review. The results reached show that the main methods used for molecular typing of *H. capsulatum* were: restriction fragment length polymorphism, random amplified polymorphic DNA, microsatellites polymorphism, sequencing of internal transcribed spacers region, and multilocus sequence typing. Different genetic profiles were identified among *H. capsulatum* isolates, which can be grouped according to their source, geographical origin, and clinical manifestations.

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Introduction

Histoplasmosis is a systemic mycosis caused by the dimorphic fungus *Histoplasma capsulatum*. This disease has worldwide distribution, and areas of high endemicity are located in the Americas, especially in the USA, Mexico, Brazil, and Guiana Shield [1].

The target of this fungal infection is the lung. However, less than 10 % of immunocompetent individuals in endemic areas develop disease symptoms. Most infections course with mild symptoms, characterized by non-specific respiratory distress [1, 2]. Infection depends on the individual's exposure to the pathogen in particular environments (inoculum size inhaled and time of exposure), the strain virulence, and the host immune response [3]. Immunocompromised patients, especially individuals with human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS), can develop a severe and fatal disease, due to fungal dissemination to many organs [1, 4].

H. capsulatum grows as mold in its habitat, but undergoes morphotype transition to intracellular yeasts during parasitism in mammals. It is a haploid organism and has a heterothallic mating system [5, 6]. To date, three biological varieties are still considered for this fungus: *H. capsulatum* var. *capsulatum*;

H. capsulatum var. *duboisii*; and *H. capsulatum* var. *farciminosum* [7].

Structural, chemical, pathogenic, and genetic differences are observed among *H. capsulatum* strains from diverse regions of the world [8, 9]. Methodologies based on phenotypic characteristics of *H. capsulatum* were initially applied for typing this fungus [10]. However, there are serious restrictions with the use of phenotype studies due to a very low degree of differentiation for this fungus, as well as difficulties in the reproducibility of phenotype analyses, constituting a limitation for reliable epidemiological analysis [11].

In infectious diseases epidemiology, molecular techniques are important tools to conduct investigations regarding detection and strain typing procedures, identification of exposure sources, and determination of different genotypes associated to disease pathogenesis [12]. The main molecular methodologies for the identification and genetic typing of infectious fungi are based on DNA fingerprinting methods [13].

In this study, we reviewed previously published data about the use of molecular typing in the epidemiology of histoplasmosis. We summarize the main molecular methods used for typing this fungus and highlight the association of genetic profiles with the clinical manifestations, infection process of the disease, and outbreaks. The results of this literature search are described in the following sections of this work.

Materials and methods

Search strategy and selection criteria

We searched the PubMed and Web of Science databases, restricting to English and Spanish publications. There were no limitations on the study type or date. The search terms included a combination of specific keywords: molecular typing [OR] genetic diversity [OR] polymorphism [AND] *H. capsulatum*; molecular epidemiology [AND] histoplasmosis; and molecular epidemiology [AND] *Histoplasma*. In addition, we used the specific terms histoplasmosis [AND] outbreaks to identify all the articles that applied molecular typing in investigating histoplasmosis outbreaks. In addition, we searched other relevant studies provided in the reference lists of key studies. Non-English or non-Spanish articles, dead links, and duplicate results were excluded from the manuscript review. The search was performed between February to November 2014, and the last access was on November 14, 2014.

Quality assessment

Abstracts were analyzed by two reviewers and were included for full-text review if they were definitively related with the proposed topic in this article. Therefore, we excluded all papers that addressed any other disease and different microorganisms, or those not reporting on the projected idea in this systematic review. Discrepancies between reviewers were solved by discussion and consensus. The present paper is in compliance with the PRISMA checklist [14] for systematic reviews.

Results and discussion

Identification of studies

The initial search identified 304 studies, of which 139 researches were related with a combination of specific keywords, and 165 additional studies were identified by searching using specific terms. After assessment of titles and abstracts, 112 studies were selected for full-text assessments, and, subsequently, 68 studies were excluded. Four studies were excluded since they did not fulfil the inclusion criteria, and 64 articles were excluded because they were duplicate studies. Therefore, seven additional studies were included based on the analysis of reference lists of key studies. Thus, 51 studies were considered for this systematic review (Fig. 1).

Molecular typing of *H. capsulatum*

A large number of DNA-based techniques have been used for the genetic characterization of *H. capsulatum* [15]. These techniques differ according to the accessed target and methodologies applied (Table 1). The first studies about *H. capsulatum* genome diversity were done in the 1980s and performed using restriction fragment length polymorphism (RFLP), which identified intra-species differences among fungi [52, 53, 58]. This tool consists of a total genomic DNA digestion with restriction endonucleases, followed by Southern blot and hybridization of the fragments with labeled probes [54]. The main regions of *H. capsulatum* isolates analyzed using this method were mtDNA, rDNA, and the nuclear *yps-3* locus (gene expressed in *H. capsulatum* yeast phase that encodes a specific protein).

RFLP analyses of mtDNA and rDNA from *H. capsulatum* grouped the fungal strains studied into four classes, on the basis of source and geographic origin. Class 1 was represented by the Downs strain (USA); class 2 comprised strains from North America (G217 [A and B] and G222) and Africa; class 3 included Central American strains (G184 [A and B] and G186 [A and B] from Panama); and class 4 included only one environmental isolate (FSL1 from the USA) [53]. Analyses of the *yps-3* locus identified two new classes (RFLP classes 5 and 6 obtained from AIDS patients from Latin America and the USA). Based on differences within mtDNA, class 5 was subtyped into four new subclasses: class 5a, isolates from Panama; 5b and 5c, isolates of American immigrants from Puerto Rico; and 5d, obtained from an American patient who had recently traveled to Central America [16].

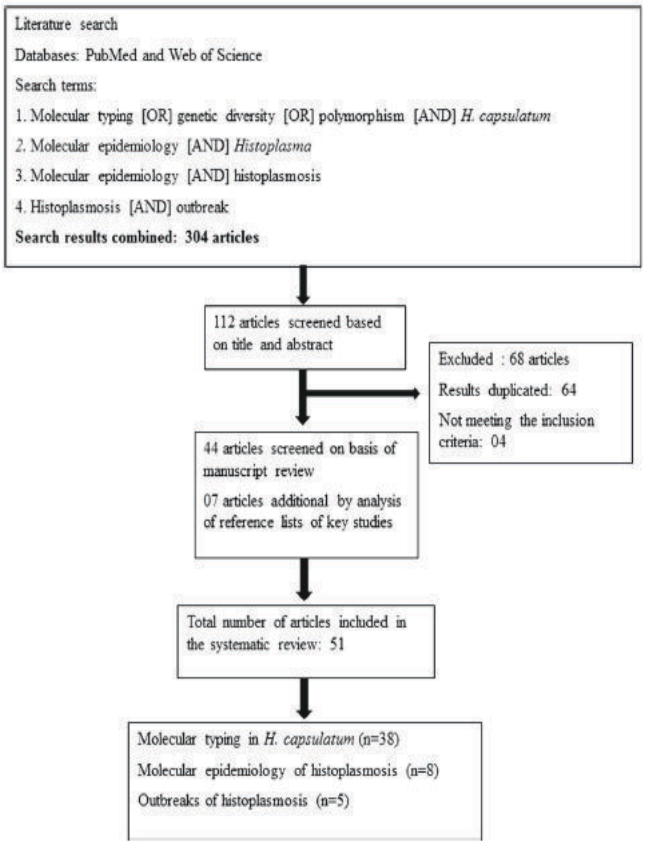

Fig. 1 Systematic review process used in the study

A molecular method frequently used to distinguish *H. capsulatum* strains is random amplified polymorphic DNA (RAPD) analysis. This tool is based on the polymerase chain reaction (PCR) amplification of DNA segments using short primers with arbitrary sequences [17]. RAPD-PCR analyses have revealed even higher polymorphisms among *H. capsulatum* strains than RFLP [18, 19]. Some studies by RAPD-PCR analyses also identified a relationship between RAPD profiles and geographic origin, revealing an important genetic difference between *H. capsulatum* isolates from the USA and Latin America [20–23].

In Rio de Janeiro, Brazil, environmental, animal, and clinical *H. capsulatum* isolates (including patients with and without HIV/AIDS) were analyzed by RAPD-PCR, showing 80–

Table 1 Molecular methods used in *Histoplasma capsulatum* typing

Molecular methods	No. of articles	Target	References
RAPD[a]	20	DNA	[16–35]
MLST[b]	9	*arf*, *ole1*, *tub1*, *H-anti* nuclear genes	[26, 27, 33, 36–41]
ITS-PCR[c]	6	ITS1-5.8S-ITS2	[36, 42–46]
Microsatellite	6	C/A(T)n, (CA)n, (GA)n, (AT)n, (CT)n	[40, 47–51]
RFLP[d]	4	mDNA, rDNA, *yps3* locus	[36, 52–54]
PFGE[e]	2	Chromosomes	[55, 56]
PRP8 intein sequences	1	*prp8* gene	[57]

[a] Random amplified polymorphic DNA
[b] Multilocus sequence typing
[c] rDNA internal transcribed spacer (ITS1-5.8S-ITS2)
[d] Restriction fragment length polymorphism
[e] Pulsed-field gel electrophoresis

100 % genetic similarity, and, despite this similarity, the isolates were grouped into four to six different genotypes. The RAPD profile of Brazilian isolates was different from that of the USA (Downs, G222B, and FLS1) and Panama strains (G186B), included as references in this study, in which the genetic similarity was less than 70 % with each of the primers tested [20]. However, *H. capsulatum* isolates from different sources of Brazil in studies more recently developed showed that there were no genotypes restricted to different regions from Brazil, since *H. capsulatum* isolates from diverse geographical regions were grouped in a same genetic population or clade [24, 42].

The amplification of a non-coding region of the rDNA internal transcribed spacer (ITS1-5.8S-ITS2) by PCR has been widely used for examining inter-species polymorphisms in closely related human fungal pathogens. A high genetic diversity among *H. capsulatum* isolates has been found within the same geographic region [43]. However, some studies report an association between the polymorphic profile of the ITS1-5.8S-ITS2 region and the geographic origin and environmental source of *H. capsulatum* isolates [25, 36, 44].

In the ITS1-5.8S-ITS2 region, PCR was performed in combination with RFLP analysis in Brazil to distinguish *H. capsulatum* isolates from source and diverse states of this country. The results revealed that *H. capsulatum* isolates from the state of Rio de Janeiro grouped into three major clusters, which were genetically distinct from the isolates from other Brazilian states, as well from isolates from other Latin American countries or other continents. The high genetic similarity among Rio de Janeiro isolates suggests that only one genetic population is present in the microenvironment of Rio de Janeiro. However, this study also highlighted that there is not a sole genotype for other regions of Brazil, because high genetic diversity was found among *H. capsulatum* isolates supplied by other Brazilian states [47].

Microsatellite markers are applied widely for the study of the structure of the genetic population of some eukaryotic species. Microsatellites are regions of the genome having short repeats (two to four nucleotides) and are found in tandem arrays ranging from 10 to 20 or more repeats [48]. The original analyses using microsatellites in *H. capsulatum* were performed by Carter et al. [49, 50]. These authors developed three multiallelic markers (microsatellite loci: *L638REP*-C/A(T)n, *L610.2CA*-(CA)n, and *HSP-TC*-(TC)n or (GA)n), and studies utilizing them were able to distinguish isolates at the individual level and were polymorphic in both North American and South American populations [50, 51].

McEwen et al. analyzed four microsatellites in *H. capsulatum* isolates: (CA)n and (GA)n of Carter et al. [50, 51] and two other microsatellites found by querying the GenBank database, ATPase-AT (AT)n and ATPase-CT (CT)n. The results showed that these microsatellites could distinguish *H. capsulatum* from diverse geographic origins. However, (AT)n and (CT)n failed to amplify *H. capsulatum* isolates from the USA and (CT)n for isolates from Australia [48]. In 2012, a study was conducted using the sequence polymorphism of a 240-nucleotide fragment, which included the (GA)n length microsatellite and flanking regions within the *HSP60* gene. The results demonstrated that *H. capsulatum* isolates recovered from migratory bats (captured randomly in Mexico, Brazil, and Argentina) are distributed according to the bat migration routes in the Americas and, hence, this molecular approach could be used as biogeographical markers [59].

Pulsed-field gel electrophoresis (PFGE) or electrophoresis karyotyping is a molecular method applied for the separation of intact chromosomes of small size [55]. Steele et al., using PFGE, observed differences in band size and migration among three reference strains: Downs, seven chromosomes; G186B, four chromosomes; and G217B, three chromosomes [56]. Canteros et al. performed PFGE analysis of *H. capsulatum* clinical isolates from different countries and identified high chromosome-length polymorphism. Moreover, these authors observed variations in the chromosomal banding profiles of the human isolates, which were composed of five to seven bands (ranging from 1.3 to 10 Mbp in size). However, there was no association among electrokaryotype and geographical or clinical source [37].

Multilocus sequence typing (MLST) is another molecular technique applied to studying the genetic diversity of *H. capsulatum* [38, 39]. Based on MLST examination, using the partial DNA sequences of four protein-coding nuclear genes (*ole 1*-delta-9 fatty acid desaturase, *tub1*-alpha-tubulin, *arf*-ADP ribosylation factor, and *H-anti*-H antigen precursor), *H. capsulatum* can be distinguished in eight phylogeographic clades: North America class 1 (NAm1), North America class 2 (NAm2), Latin America group A (LAmA), Latin America group B (LAmB), Australian, Netherlands/Indonesia, Eurasian, and African. Seven clades are considered phylogenetic species, and the only exception is the Eurasian clade, which is grouped within the LAmA clade. *H. capsulatum* var. *capsulatum* was identified in all phylogenetic clades; *H. capsulatum* var. *duboisii* was only found in the African clade; and *H. capsulatum* var. *farciminosum* was found in the African, NAm2, and Eurasian clades. In addition, *H. capsulatum* isolates from Latin America were the most phylogenetically diverse [39]. Other MLST analyses of *H. capsulatum* isolates revealed new clades and lineages (sole genetic population) in different geographic regions of the world [26, 40, 47, 60].

More recently, Theodoro et al. performed a PRP8 intein sequences analysis (*prp8* gene that encodes for the highly conserved Prp8 nuclear protein) [57]. These authors showed that there are clearly different clades to those found previously by Kasuga et al. [39]. Therefore, the same authors observed LAmB isolates inside the LAmA clade and suggested that

there is an eventual hybridization or admixture between populations with overlapping geographical areas, leading to the introgression of a gene from one species to other. Another possible hypothesis is the presence of shared ancestral polymorphism [57].

The polymorphic profiles of *H. capsulatum* referred in this review characterize the high genetic diversity of this pathogen around the world. Several methods can be used to interpret *H. capsulatum* strain typing (Fig. 2). However, to evaluate the basic features of fungal populations, a series of simple, well-characterized, independent, and stable polymorphic loci is necessary. Molecular assays such as electrophoretic karyotyping and fingerprinting methods cannot provide this type of data, although these methods are useful for distinguishing clones populations [61]. On the other hand, MLST has great potential as a tool to interpret the fine-scale population genetic structure of microbes. In addition, MLST can be used to increase the discrimination among the possible causes of emerging diseases, to recognize fungal species, and to determine if the fungi are purely clonal or if they also recombine in nature [62].

Use of genetic markers in the epidemiology of histoplasmosis

Molecular typing is one of the most important methodologies used to clarify the pathogenesis of diseases, as well as to assist in specific treatment, to identify multidrug-resistant pathogens, and to elucidate the infection mechanism and exposure sources of these diseases [45, 63–65]. Epidemiological studies have shown differences in manifestations and clinical outcomes among patients with histoplasmosis from the USA and South American countries. High mortality and relapse rates, as well as the presence of mucocutaneous lesions, are more frequent in patients with histoplasmosis from Brazil, Colombia, and Argentina, especially among AIDS patients [1, 4].

Molecular studies have been helpful in the elucidation of these queries. Some authors have shown genetic differences between *H. capsulatum* strains from North America and South America, which were related to the high frequency of skin lesions. RFLP analysis of the nuclear gene *yps-3* demonstrated that classes 5 and 6, originally from Latin America, were the predominant genotype associated with this clinical manifestation [18].

Durkin et al. studied the clinical differences between *H. capsulatum* isolates from Latin America (RFLP classes 5 and 6) and North America (RFLP class 2) after intra-tracheal infection of mice with this fungus' yeast cells. The animals infected with *H. capsulatum* RFLP class 2 developed a chronic disease and had 100 % survival, whereas those infected with Latin American isolates had an increased number of deaths, with a survival rate of only 20 % at 21 days post-infection [41].

In Rio Grande do Sul, in southern Brazil, *H. capsulatum* isolates obtained by skin culture from eight patients were analyzed through the ITS1-5.8S-ITS2 region sequencing. Two different *H. capsulatum* genetic profiles were identified, which were similar to isolates from Colombian and Argentinean patients, although they were dissimilar to the USA isolates. This study supports differences in pathogenic characteristics among Latin American and USA *H. capsulatum* isolates [27].

In China, a study described the presence of an Australian MLST genotype in a *H. capsulatum* isolate from an indigenous patient with chronic disseminated histoplasmosis. Moreover, this patient received treatment for six months and no evidence of relapse was noted after one year of clinical follow-up [28]. This was the only study found in our analysis that associated MLST genotype with histoplasmosis outcome and clinical follow-up.

Molecular techniques have also been important to characterize the infection process of this mycosis. Reactivation and mixed *H. capsulatum* infection have been identified in few cases by molecular tools. Poonwan et al. identified the

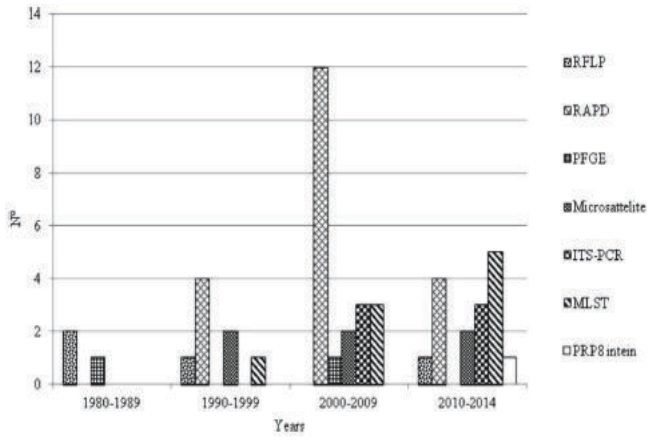

Fig. 2 Articles published about molecular typing in *Histoplasma capsulatum* between the years 1980 and 2014

presence of mixed *H. capsulatum* infection in an HIV/AIDS patient, after identifying two distinct genetic profiles by RAPD-PCR analysis, obtained from two different clinical sources (bronchoalveolar lavage fluid and blood) [22]. In 2001, RAPD-PCR analysis of four samples of *H. capsulatum* isolated at different times from two patients showed that each patient harbored the same genetic population of this fungus at different times, suggesting the reactivation of previous histoplasmosis clinical cases [24]. However, the pathophysiological consequences of these findings were not discussed in the original article.

Based on the studies reviewed, molecular techniques have been useful to find genetic differences among *H. capsulatum* strains from Latin America and North America, which are associated with the morbidity and mortality of this systemic mycosis. Despite that fact that few molecular studies are related to the epidemiology of histoplasmosis, they have been helpful in the identification and association of fungal genetic profiles with the presence of manifestations and clinical outcomes of histoplasmosis. The more frequent mucocutaneous lesions in Latin American patients have been associated with a specific genetic profile found in this region [27]. In experimental models, a higher mortality due to *H. capsulatum* has been related to Latin American isolates characterized by RFLP as classes 5 and 6 when compared with North American strains [41].

Use of molecular studies in histoplasmosis outbreaks

RAPD-PCR analysis and sequencing of two nuclear genes (*H-anti* and *ole*) were performed to investigate the source of an outbreak of histoplasmosis in Acapulco, Guerrero, Mexico. DNA sequences of *H. capsulatum* isolates obtained from a soil sample of a planter (organic fertilizer) collected in a hotel in Acapulco were analyzed and compared with clinical isolates of histoplasmosis patients from Guerrero, and other *H. capsulatum* isolates from Colombia, Argentina, and Guatemala. Although DNA banding profiles of the fungal samples isolated from the organic fertilizer were found to be different when compared with the other isolates included in this study, the sequence analyses of *H-anti* and *ole* genes of all *H. capsulatum* isolates studied (from human and organic fertilizers) revealed high similarity for these two genes tested (92–99 %). Thus, it was possible to confirm the infectious source of the outbreak [29].

An investigation of a histoplasmosis outbreak was carried out in Mexico among six individuals that developed severe histoplasmosis, after conducting excavation activities. Two fungal isolates were recovered and analyzed by RAPD-PCR, which confirmed the same polymorphic profile indistinguishable from the E-53 *H. capsulatum* reference strain, which is highly virulent and prevalent in Mexico [30].

In Argentina, an outbreak of acute pulmonary histoplasmosis (APH) occurred in six cadets of the Argentinean Air Force, after cleaning an abandoned hangar. Although the diagnosis was reached through the clinical manifestations, serologic tests, and skin tests with histoplasmin, the outbreak source was identified through collection of local soil and subsequent inoculation in hamsters. Moreover, by RAPD-PCR, it was possible to identify *H. capsulatum* with 96 % similarity to other strains of Argentinean patients previously characterized by the same analysis [46].

Another outbreak of APH occurred in a non-endemic area, Neuquén region (Patagonia, Argentina), in road workers who stirred soil. It was possible to isolate and evaluate the genetic profile of *H. capsulatum* from the index patient of this outbreak by fungal identification in blood and lung biopsy cultures. RAPD-PCR analysis revealed that the *H. capsulatum* sample isolated from the index patient had a restricted and autochthonous genotype compared to that from the geographic region. Low similarity was observed in this *H. capsulatum* isolate when comparing to other strains recovered from Argentinean patients (60 %) and USA strains (62–68 %). Therefore, it was suggested that the strain identified in this study has been introduced in this region by dispersion mechanisms, which adapted to an adverse environment for its development [31].

In summary, molecular techniques have been an important instrument to identify possible sources of infection in outbreaks of histoplasmosis [45, 64, 65]. In addition, autochthonous *H. capsulatum* genotypes in regions with unfavorable climate for fungal development have been identified by molecular techniques [31].

Molecular studies of histoplasmosis in mammals

Histoplasmosis can also occur in different mammal species [24, 32, 33, 66]. Bats are the most important mammals that have developed a very close relationship during a period of millions of years with the fungus *H. capsulatum* and, consequently, they are the main dispersers of this fungus in nature [59]. A tight relatedness (87–100 % similarity) between clinical and bat *H. capsulatum* isolates was demonstrated in a study performed using RAPD-PCR analysis with *H. capsulatum* isolates recovered from migratory and non-migratory bats from three states of the Mexican Republic. In Argentina, Canteros et al. found results similarity between bat isolates and those from patients living in Buenos Aires [67]. These studies demonstrated an important correlation between the source of infection and development of histoplasmosis [66].

In an epidemiologic study of histoplasmosis performed in the state of Morelos, Mexico, the RAPD-PCR polymorphic profiles of a human clinical isolate was similar to a bat isolate [67]. Chávez-Tapia et al. performed RAPD-PCR analysis in

Tadarida brasiliensis (migratory bats). They identified a distinct polymorphic profile between human and bat *H. capsulatum* isolates from Mexico, and they suggested that *T. brasiliensis* was infected with the fungus outside Mexico [68]. Besides, *H. capsulatum* samples isolated from the spleen, liver, and adrenal gland from two captive maras (*Dolichotis patagonum*) of the same colony were analyzed by RAPD-PCR and the DNA band profiles found were highly similar [33]. Both studies support the fact that different hosts could share the same source of infection. It is very important to ascertain the polymorphic profiles of *H. capsulatum* isolated from bats and humans, because this has helped to understand the distribution of histoplasmosis in certain endemic regions [66, 67]. These statements are consistent with the fungal ability to adapt to different types of soils and climates, and its capacity to infect multiple animal species.

In Japan, cases of disseminated and cutaneous histoplasmosis in dogs were identified. Phylogenetic analysis of *H. capsulatum* isolates from these dogs, using the ITS1-5.8S-ITS2 region, showed more than 99 % identity with human isolates of this country. In addition, *H. capsulatum* var. *farciminosum* was responsible for all these cases of Japanese histoplasmosis, in both humans and dogs [32]. More recently, *H. capsulatum* var. *farciminosum* was identified from a wild badger (*Meles meles*) with a severe nodular dermatitis by the ITS1-5.8S-ITS2 region sequence. This fungal isolate was analyzed by MLST, which demonstrated that the fungus in our case belonged to the Eurasian clade [33]. These two studies demonstrated that *H. capsulatum* var. *farciminosum* cause infections in diverse host species, and it causes not only cutaneous but also systemic infections.

Summary

The findings of this systematic review indicate that molecular typing could be critical to elucidate the origin, course, and pathogenesis of histoplasmosis infection, depending on the *H. capsulatum* strain involved. However, further studies on genotyping are required in order to better understand the full range of clinical differences in histoplasmosis among patients from Latin America and North America, as well as other continents, in order to promote the correct treatment and follow-up for these patients.

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4.3 Capítulo 3

Correlation between phenotype and mating type genotype of *Histoplasma capsulatum* isolates with epidemiological aspects and clinical manifestations of histoplasmosis patients from Ceará, Brazil

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Histoplasma capsulatum é um fungo dimórfico, que causa desde infecções assintomáticas à doença grave e fatal. O objetivo deste estudo foi descrever as características clínico-epidemiológicas de pacientes com histoplasmose, e identificar os aspectos morfológicos, perfil de exoantígenos e tipo de compatibilidade sexual ou *mating type* de isolados de *H. capsulatum* recuperados destes indivíduos. Os pacientes incluídos neste estudo foram recrutados do Hospital São José, localizado no Ceará/Brasil, no período de 2011- 2014. Os dados clínico-epidemiológicos foram obtidos através da revisão dos prontuários médicos. Todos os isolados fúngicos foram procedentes de culturas de diferentes amostras biológicas, e identificados através de métodos fenotípicos convencionais e detecção de exoantígenos, no Instituto Nacional de Infectologia Evandro Chagas da Fundação Oswaldo Cruz. PCR do lócus *MAT1* foi realizada para identificação dos tipos de compatibilidade sexual de cada isolado. Durante o período do estudo, um total de 43 hospitalizações ocorreu em 40 pacientes, sendo recuperados 51 isolados fúngicos. A maioria das colônias apresentou pigmentação branco e branco-bege (70,6%), e tinham textura cotonosa (82%). A presença de todas as estruturas de esporulação nas colônias filamentosas foi observada em 74,5% dos isolados. Conversão dimórfica ocorreu em 98% das cepas. Ambos os antígenos H and M foram identificados em 54,9% dos isolados através de WB. Dentre os 51 isolados fúngicos estudados, 53% eram *MAT1-2* e 47%, *MAT1-1*. Colônias pulverulentas foram associadas com a presença de manifestações hemorrágicas nos pacientes, e colônias de pigmentação branco e branco-bege foram relacionadas à insuficiência renal aguda. Óbito foi associado com a presença de isolados que continham todas as estruturas de esporulação. Os aspectos fenotípicos parecem estar associados com as manifestações clínicas e desfecho da histoplasmose.

Correlation between phenotype and mating type genotype of *Histoplasma capsulatum* isolates with epidemiological aspects and clinical manifestations of histoplasmosis patients from Ceará, Brazil

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Abstract

Histoplasma capsulatum is a dimorphic fungus responsible for asymptomatic infections to severe diseases. The aim of this study was to describe the epidemiological and clinical aspects, and to indentify the mycological characteristics, exoantigens profile and mating types of *H. capsulatum* clinical isolates recovered from histoplasmosis patients. The patients included in this study were recruited at the São José Hospital from Ceará/Brazil between 2011 to 2014 period. Epidemiological and clinical data were obtained by review of medical records. The fungal isolates obtained from distinct anatomical sites were identified by conventional phenotypical methods and exoantigen detection, in the Evandro Chagas National Institute of Infectious Diseases (INI) at Oswaldo Cruz Foundation. Moreover, PCR to identify mating type of isolates was also performed. Fifty-one fungal isolates obtained from 40 patients were identified. White and white-beige pigments were observed in 70.6% of isolates and 82% of strains had cottony appearance. The presence of all characteristic structures of mycelia-form was observed in 74.5% of fungal isolates. Dimorphism occurred in 98% of strains. Both H and M antigens was observed in 54.9% of fungal isolates by western blot in the exoantigen test. Among the 51 isolates studied, 53% were MAT1-2 and 47% were MAT1-1. Powdery colonies were associated with hemorrhagic manifestation, and colonies with pallid pigmentation were related with acute renal failure. Colonies that contained macroconidia were associated with death. The presence of some mycological characteristics appears to be associated with clinical aspects as well as severity and disease outcome.

Key words: histoplasmosis, *H. capsulatum*, mycological characteristic, exoantigen, mating type.

Introduction

Histoplasmosis is a systemic mycosis that usually occurs in individuals with immunosuppression or in persons that acquired a high parasitic burden by inhalation of infectious propagules. This fungus is found in diverse microniches around the world. In the Americas, cases of histoplasmosis are observed from North of Canada until Patagonia in Argentina [1, 2]. In Ceará, northeastern of Brazil, histoplasmosis is an endemic mycosis and occurs predominantly in AIDS-patients [3, 4].

The pathogen *Histoplasma capsulatum* is a dimorphic fungus that can cause since asymptomatic infections to severe diseases, after inhalation of microconidia or hyphae fragment. Usually, the infection resolves within 1 to 2 weeks, but in some cases, acute respiratory histoplasmosis or disseminated disease can develop [5]. This fungus is found as a mycelial (M) form in the environment and in vitro at 25-28ºC temperature. The M-stage is constituted for hyaline, thin, septate and branched hyphae, microconidia and tuberculate macroconidia [6,7]. The yeast (Y) phase is found in parasitism or by cultures in rich medium at 34-37ºC. Microscopically, the yeast-like form is seen as small oval structures with thin-walled, 2-5µm, which reproduced by a single bud from the pointed end [5, 8].

In countries with low resourses, the diagnosis of histoplasmosis is based on the identification of *H. capsulatum* by morphological and physiological assays [9, 10]. The conventional identification of *H. capsulatum* is linked to the M-Y conversion in order to establish the dimorphic nature of fungus. However, immunological test, such as exoantigen test, can also identify *H. capsulatum* by M and H antigens detection, specific molecules localized in the fungal wall, which are associated with pathogen virulence [11, 12]. This test is useful as a definitive and rapid identification of *H. capsulatum* and other dimorphic fungus [13-15].

Histoplasma capsulatum is a heterothallic ascomycete that has an anamorphic or asexual stage with two types of sexual compatibility, (+) major and (-) minor, represented at the mating locus (*MAT1*). This locus has a bipolar system which contains two idiomorphs, *MAT1-1* and *MAT1-2* that define the mating types, respectively [16]. The teleomorphic (sexual) stage known as *Ajellomyces capsulatus,* results from crossover of *MAT1-1* and *MAT1- 2* idiomorphs (17-19). The species *H. capsulatum* and *A. capsulatus* constitute the same holomorphic organism. The stage of sexual compatibility of fungus or mating type is a phenotypic characteristic involved in the pathogenesis of some mycoses as cryptoccocosis and aspergillosis [20-23]. However, the role of mating type in the histoplasmosis pathogenesis is not clearly defined. Despite this, the intra-species morphological differentiation and the

association of the phenotypic characteristics of this pathogen with clinical features have not been entirely determined.

The aim of this study was to describe the epidemiological and clinical features of histoplasmosis patients diagnosed in Ceará, Northeastern of Brazil, and identify the mycological characteristics, exoantigen profiles and mating types of *H. capsulatum* clinical isolates. Moreover, it was investigated the association between the phenotypical characteristics and clinical manifestations.

Material and Methods

Patients

Patients included in this study were recruited in São José Hospital from Fortaleza/Ceará, between 2011 to 2014 period. This hospital is a single reference unit to hospitalization of individual with infectious diseases at Ceará State. All patients had histoplasmosis diagnosis confirmation based on isolation of *H. capsulatum* in biological samples cultures. Epidemiological and clinical data were obtained by review of medical records. It was investigated variables involving its respective parameters such as; epidemiological (sex, age, origin, occupational risk for histoplasmosis, drug user and co-infection with tuberculosis), clinical (fever, weight loss, cough, dyspnea, hepatomegaly, diarrhea, asthenia, splenomegaly, vomiting, abdominal pain, headache, hemorrhagic manifestation, skin lesion, adenomegaly and acute renal failure - ARF), and laboratory variables (HIV serological test and CD4+ T lymphocyte count).

Isolates

Fungal isolates obtained from distinct anatomical sites were identified by conventional phenotypic methods and exoantigen detection, as described below, in the Mycology Laboratory of INI/FIOCRUZ.

Mycological Characteristics

Fungal isolates were maintained for 21 days in the M-phase by culture at 25ºC in Potato Dextrose Agar (PDA – Difco, Detroit, MI, USA). The macromorphology features of *H. capsulatum* M-phase such as texture and pigmentation of colonies were described. The micromorphology characteristic of *H. capsulatum* M-phase was observed in 10 fields by optical microscopy, with a magnification of 40X, after staining by Lactophenol Cotton Blue (Fluka Analyted, France). Dimorphism was demonstrated by mycelial- to yeast-phase transitions induced by a temperature shift from 25 to 37°C in the ML-Gema medium [24] for 7 to 14 days.

Production of Exoantigens

The fragments of 2-4 cm^2 of *H. capsulatum* M-phase were grown for 14 days in 16 × 125 mm test tube of Potato Dextrose Agar slants (PDA) and transferred (one slant per flask) to 1 Erlenmeyer flask containing 25 mL of Brain Heart Infusion Broth (Difco, Detroit, MI, USA) and incubated at 25°C on a rotatory flask shaker at 150 rpm for 7 days (New Brunswick Scientific, Edison, NJ). Thimerosal (concentration of 1: 5,000) was added in the seventh day, and the flasks were re-incubated overnight at 25°C. Cultures were centrifuged at 1,050 x g for 10 minutes, and the supernatants were filtered through 0.45µm porosity membranes (Nalgene Co., Rochester, NY). The pooled filtrate was concentrated to 50 X in the Amicon Minicon Macrosolute B 15 (Amicon Corp., Lexington, Mass) [13]. To evaluate the exoantigen profiles of each isolate it was performed immunoidentification tests as double immunodifusion and western blot.

Double Immunodiffusion (ID) Test

The ID test was performed as previously described [25]. This assay is based on the interaction between antigens released into fungal cultures and homologous antibody, which was obtained from serum of histoplasmosis patient (stored at 4ºC at Mycology Laboratory - INI/Fiocruz). If the antibodies recognize the antigens, they will form an immune complex as a precipitate into the agarose gel showing a thin white line, which is a visual signature of antigen recognition. Before use, the serum of histoplasmosis patient was analyzed by ID so as to verify the presence of specifics antibodies M and H. Histoplasmin antigen was produced in the Mycology Laboratory - INI/Fiocruz and used as control.

Western Blot (WB)

This assay was based on Pizzini et al. (1999) protocol with some modifications [26]. A total of 25µl of exoantigen filtrate solution from each fungal isolate was first dissociated at 100°C for 5 min in 0.125MTris-HCl buffer (pH 6.8) containing 2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, and 0.025% bromophenol blue. Then sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted with 10% polyacrylamide resolving gel and 4% polyacrylamide stacking gel. The voltage for electrophoresis performance were 20 mA constant current for stacking gel and at 35 mA for protein separation. The second step was electrotransfer of gel contents onto nitrocellulose membranes at 0,2 µm in a Mini Trans Blot cell (Bio-Rad) containing transfer buffer (25 mM Tris-HCl, 192 mM glycine, and methanol (20% [vol/vol]; pH 8.3) and operated at 400 mA for 1 h. Nitrocellulose washing procedure of membranes were performed using 20 mM Tris-HCl–1,500 mM NaCl–0.2%–NaN3–2% Tween 20 (pH 7.5) (Tris-buffered saline with Tween 20

(TBST) buffer) during 5 minutes for 3 times. Free membrane binding sites were blocked by incubation for 60 min in 5% (wt/vol) nonfat dry milk in TBST buffer.

Membranes were incubated for 60 min at room temperature with serum specimen from histoplasmosis patient (stored at 4ºC at Mycology Laboratory - INI/Fiocruz), diluted 1/100 in TBST containing 5% nonfat milk. After that, it was performed washing in TBST during 5 minutes for 3 times; and finally, incubated for 1 h with alkaline phosphatase-conjugated goat anti-human IgG antibody (Jackson ImmunoResearch, EUA) diluted in TBST (1:3.000). Following incubation, blot membranes were washed and incubated with substrate solution consisting of 5-bromo-4-chloro-3- indolylphosphate (BCIP; 15 mg/ml in dimethylformamide [DMF]) and nitroblue tetrazolium (NBT; 30 mg/ml in 70 % aqueous DMF). Substrate stock solutions were diluted 1:100 before use in Tris/NaCl buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 50 mM MgCl2). After color development blot membranes were rinsed exhaustively in deionized water.

Mating type genotype

Genomic DNA was extracted from *H. capsulatum* Y-phase by chloroform/isoamyl (24:1) alcohol method as previously described [27]. The *MAT1* locus of the *H. capsulatum* isolates was identified by (PCR, using specific primer pairs for idiomorphs *MAT1-1* (MAT1-1S: 5' CGTGGTTAGTTACGGAGGCA-3' and MAT1-1AS: 5'-TGAGGATGCGAGTGATGGGA-3'), which generated an amplicon of 412 base pairs (bp); and *MAT1-2* (MAT1-2S 5'-ACACAGTAGCCCAACCTCTC-3' and MAT1-2AS 5'-TCGACAATCCCATCCAATACCG-3'), which generated an amplicon of 533 bp according to previously described conditions with minor modifications [28]. The PCR was performed in a 25-µl reaction mixture, containing 200µM each deoxynucleoside triphosphate (dNTP) (Applied Biosystems Inc., Foster City, CA, USA), 1.5 mM $MgCl_2$, 50 ng/µl of each primer, 1.5 U *Taq* DNA polymerase (New England BioLabs Inc., MA, USA), 1 X *Taq* commercial buffer, and 75 ng (25 ng/µl) of each DNA sample. The G-217B from USA (*MAT1-1*) and G-186A from Panama (*MAT1-2*) references strains were used as positive controls.

PCR assays were performed in a Thermal iCycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) programmed as follows: (a) 3 min at 95°C; (b) 35 cycles, consisting of 30 s at 95°C, 30 s at 58°C, and 1 min 30 s at 72°C; and (c) 10 min at 72°C. Amplicons were resolved by 1.5% agarose gel electrophoresis. The 100-bp DNA ladder was used as a molecular marker. The amplicons were purified using a QIAquick PCR purification Kit (Qiagen AG, Basel, Switzerland). Automated sequencing was done using the Sequencing Platform at Fundação Oswaldo Cruz, PDTIS/FIOCRUZ, Brazil [29] and at the High-Throughput Genomics Center

(University of Washington, Seattle, WA, USA). The nucleotide sequences obtained were edited and aligned with G-217B (*MAT1-1*) and G-186A (*MAT1-2*) references strains using Clustal-W [30] in MEGA 6.0 software [31]. The consensus sequence was compared with reference strain (G-217B – EF433757.1; G-186A – EF 433756.1) retrieved from GenBank database for homologous nucleotide sequences with the BLASTn algorithm (Basic Local Alignment Search Tool). All the nucleotide sequences obtained in this study were deposited in GenBank (Table 2).

Statistical analysis

Epi-Info, version 7.1.5 (Centers for Disease Control and Prevention, Atlanta, GA, USA), was used for data entry. The statistical analysis was carried out using the software program STATA 11.2 (StataCorp LP, College Station, TX, USA). A descriptive analysis of data was performed and median and interquartile range (IQR) were used as measures of central tendency. The univariate analysis were performed to identify the association between the different phenotypic characteristics, using the Chi-squared test or Fisher exact test, if any value in the cells of the contingency table was less than five, where it was applied a significance level of 5% ($\alpha = 0.05$).

The relationship between mycological characteristics, exoantigen profiles and mating types with clinical manifestation were evaluated. The presence of skin lesion, hemorrhagic manifestations, dyspnea, ARF, and death were considered dependent variables. The relative risk (RR) was considered as measures of association, with their respective confidence intervals (95% CI). Individuals who had more than a fungal isolate in the same hospitalization, with different phenotypical characteristics were not considered in the statistical analysis. The study was approved in the Research Ethical Committee of INI/FIOCRUZ (No. 19342513.2.0000.5262).

Results

Patients

A total 43 hospitalizations occurred in 40 patients. Three patients had recurrence of histoplasmosis in the period of study. The median age of patients was 31 years (IQR: 28-39). Twelve patients had risk occupation related to microniches of *H. capsulatum* such as, civil construction, agriculture and gardening, and all of them have had disseminated histoplasmosis. Co-infection with tuberculosis was identified in 15% of patients. Fever, weight loss, cough, dyspnea, and hepatomegaly were the main symptoms. Just one individual had negative HIV test result. This patient was in the early postpartum period. Other epidemiological and clinical aspects are described in the Table 1. In AIDS-patients the CD4+

T lymphocyte count was obtained from only 19 patients. Eighty percent of these patients had the CD4+ T lymphocyte count lower than 150 cells/mm^3 (range: 3-303 cells/mm^3; median: 40 cells/mm^3; IQR: 19-134 cells/mm^3). All patients received Amphotericin B deoxicholate (1mg/Kg/day) until the clinical improved, following by itraconazole (400mg/day). Death occurred in 33% of patients. Skin lesion (p=0.003) and ARF (p=0.010) were risk factor associated with death.

Isolates

A total of 51 *H. capsulatum* clinical isolates were isolated from 40 patients from different biological specimens such as buffy coat (n=30), blood (n=14), bone marrow (n=6) and tracheo-bronchial aspirate (n=1) by methods applied to the mycological identification. Six patients had two or more clinical isolates during the same hospitalization (Table 2).

Morphological characteristics of M-phase

Five types of pigmentations were observed (Fig. 1): albino colonies (31.4% -16/51), albino-beige (39.2% - 20/51), beige (7.8% - 4/51), beige-brow (19.6% - 10/51), and brow (2% - 1/51). The albino and albino-beige colonies were reclassified in the "pallid" pigmentation group (70.6%); and beige, beige-brown and brown colonies were grouped in the "dark" pigmentation group (29.4%). Regarding texture of colonies, 82.4% (42/51) exhibited cottony aspect, and 17.6% (9/51) presented powdery appearance in the macroscopy.

A total of 34 (81%) cottony colonies had pallid pigmentation (16 – albino and 18 – albino-beige), and 8 (19%) cottony colonies had dark pigmentation (4 – beige and 4 – beige-brow). Of 9 powdery colonies, 7 (78%) isolates had dark pigmentation (6 – beige-brow and 1 – brow), and 2 (22%) colonies had white-beige pigment (p = 0.000).

The presence of all structures of M-phase (hyphae, microconidia and tuberculate macroconidia) was identified in 38 (74.5%) *H. capsulatum* isolates (Fig. 1). Nevertheless, thirteen colonies (25.5%) presented just hyphae and microconidia in the microscopy examination.

Twenty-five (65.8%) of colonies with macroconidia had pallid pigmentation (9 – albino and 16 – albino-beige), and 13 (34.2%) isolates had dark pigmentation (3 – beige and 10 – beige-brown). In colonies without macroconidia, 11 isolates had pallid pigmentation (7 – albino and 4 – albino-beige), and 2 colonies had dark pigmentation (1 – beige and 1 – brown) (p = 0.033). In addition, tuberculate macroconidia densely crowded were observed in beige-brown colonies by simple optical microscopic. There was no association between texture and presence of tuberculate macroconidia, as the majority of cottony (30/42 – 71.4%) and powdery colonies (8/9 – 89%) had tuberculate macroconidia (p=0.417).

A total of six patients had more than one *H. capsulatum* isolate identified in the same hospitalization (patients 1, 2, 17, 19, 22 and 28), and presenting different morphological characteristics (Table 2). The patients 1, 2, 19, 22 and 28 had isolates with different pigmentations. Only the patient 17 had colonies with the same pigment in all the three isolates. Cottony appearance was observed in all isolates of patients 1, 2, 17 and 22; the patient 28 presented isolates with powdery texture, and the patient 19 had isolates with cottony and powdery textures. Concerning to micromorphologic aspects, it was identified the presence of tuberculate macroconidia in all isolates from patients 2, 19, 22 and 28; two patients (1 and 17) had fungal isolates with different structures sporulation.

Dimorphism

Fifty fungal isolates achieved the M-Y conversion in the first culture after 7 to 14 days of growth in ML-Gema medium [24]. Forty-five *H. capsulatum* isolates realized the M-Y conversion in the first subcultive, and five fungal isolates the dimorphism occurred after 3 or 4 subcultures. Only the CE0613 isolate has not converted from M to Y phase. This fungal isolate was submitted to four subcultures at 37ºC. All Y-phase colonies presented creamy and moist texture into the macroscopy, and showed oval structures, 2-5µm of size (Fig. 1) by microscopy examination.

Exoantigen Profile

In ID test, 18% (9/51) of *H. capsulatum* isolates showed bands precipitins. Eight fungal isolates had M single band (CE0211, CE0311, CE1211, CE1012, CE0513, CE0214, CE0314, and CE1714) and only one strain had H and M bands precipitins (CE0611). However, all fungal isolates were identified by WB (Table 2). Both H and M antigens were identified in 29 (56.8%) isolates; 18 (35.3%) had single M-band [94 kDa], and 4 (7.9%) isolates just presented H antigen [115kDa] (Fig. 2). There was no association between isolates that produced the both H and M antigens with some specific texture (p=0.268) or pigmentation (p=0.167) of colonies, as well as the presence or absence of macrococonidia (p=0.106).

Mating type genotype

The *MAT1-2* idiomorph was identified in 53% of fungal isolates, and *MAT1-1* in 47% of strains (Fig. 3). *MAT1-1* isolates presented a high genetic similarity (98-99%) with *Ajellomyces capsulatus* (teleomorph of *H. capsulatum*) G-217B strain (EF433757.1), and *MAT1-2* (96-98%) isolates with *A. capsulatus* G-186A strain (EF 433756.1), by BLASTn analysis. The accession numbers of nucleotide sequence are described in the Table 3.

There was no statistical association with the mating type among three parameter; texture (p=1.000), presence or absence of macroconidia (p=0.211), or both H and M antigens

production (p=1.000). However, *MAT1-2* was observed in 11/16 (68.8%) colonies with dark pigmentation, and *MAT1-1* in 20/36 (55.5%) isolates with pallid pigmentation (p=0.030). Two patients (2 and 28) had mixed infection with different mating types in the same hospitalization.

Relationship between phenotypic characteristics, mating type genotype, and epidemiological feature or clinical manifestation

There was no association between sex, origin, tuberculosis, risk activities or drug user group and phenotype or mating type genotype. Patients with powdery colonies (5/7 – 71.4%) had higher risk to develope hemorrhagic manifestations than individuals with cottony colonies (5/35 – 16.7%) (RR=8.0; CI 95% 1.82-35.07). Individuals who had colonies with pallid pigmentation (5/7 – 71.4%) were more susceptible to ARF than patients with dark pigmentation (0/12 – 0%) (RR=1.57; CI 95% 1.21-2.03). Death occurred principally in patients who had colonies with macroconidia (13/30 – 43%) than in isolates without macroconidia (1/11 – 9%) (RR=1.47; CI 95% 1.06-2.03). There was no association between fungi isolates that presented both H and M antigens and mating type and the presence of clinical manifestation (Table 4).

Discussion

This study is the most complete and large research about three important issues: (i) morphological characteristics, (ii) exoantigen profiles and (iii) mating type of *H. capsulatum* isolates performed up to now in Brazil. Pigmentation diversity in M-phase, exoantigen detection by WB and different mating type profile were the main results found out in isolates from Ceara. In addition, specifics phenotypes were associated with several clinical manifestations and outcome of histoplasmosis patients.

In the last years, histoplasmosis has been the most frequent systemic mycosis in Brazil, especially in Ceará (Northeastern of Brazil) [3,4]. Similar another studies realized in endemic regions of Latin America, disseminated histoplasmosis occurred mainly in AIDS-patients, with a prominent frequency of hemorrhagic manifestations, skin lesions, ARF and death [32, 33].

The first study about morphology of *H. capsulatum* found only two pigmentation type, albino (or type A) and brown (or type B) colonies, and rarely colonies types intermediate between albino and brown pigmentation, in isolates cultured in Dextrose Sabouraud Agar [34]. Other authors have identified only albino pigment colonies cultured in Mycobiotic Agar [35]. Contrarily, in this research, we found five pigmentations; however, this observation was performed based on a slight modification i.e, the use of PDA instead of the medium

previously propose. The pigmentation of colonies can be influenced by composition of culture media, number of successive subcultives and age of cultures, and origin of fungi isolate [36-38]. Older cultures usually are albino, and isolates from environmental have frequently brown pigmentation [36, 37]. Moreover, phenolic compounds as L-DOPA when additionated in culture medium can lead to melanization of fungal colonies due to oxidative polymerization of these compounds [38]. However, it is known that *H. capsulatum* conidia possess the enzymatic processes necessary to synthesize precursors required for the formation of melanin, wich can observed by pigmentation of colonies in culture mediums with absence of phenolic compounds as PDA [38].

Few studies have described the texture of colonies in the M-phase. These studies have observed that the majority of isolates have cottony aspect [34, 35], similar situation was found out in this study where we observed a low frequency of powdery appearance colonies.

The lack of sporulation capacity, characterized by absence of macroconidia in cultures, occurs mainly in *H. capsulatum* isolates from old cultures after successive *in vitro* subculturing, in until 25% of strain [36]. In this study, the fungal isolates were submitted only one subculture for morphological characteristics descriptions, and it was young cultures, lead us to come to the conclusion that the absence of macroconidia could be a specific and unique condition of these strains.

After analysis of correlation between pigmentation and sporulation capacity, we found out similar data such as observed for Berline et al. (1968). These authors identified that the presence of macroconidias were related with brown colonies, and, absence of macroconidia with albino colonies [34]. However, the association between texture and pigmentation was firstly explored in our study, where there was the statistical association between dark pigmentation and powdery appearance colonies.

Herein, we have shown for the very first time the association between phenotype and symptom of histoplasmosis, and highlighting the strong correlation among pallid colonies and ARF, powdery colonies and mucosal hemorrhage, and presence of macroconidia and death. Regarding strain virulence, a study suggested that brown colonies could be more pathogenic than albino colonies, due its high rates of M-Y conversion [39]. However, experimental studies have not shown difference in the virulence between white and brown strains [40,41].

The dimorphism is a complex process strain-dependent associated with physical, chemistry and biological conditions. The presence of oldest culture shows difficulties to M-Y conversion as well as, white colonies can convert later than brown colonies [34]. Moreover, other saprophytic fungi such as *Chrysosporium* and *Sepedonium* have similar sporulation

structures to *H. capsulatum* [7,8]. In our study a high rate M-Y conversion was observed, similar to other researchers [35, 42], and this event occurred regardless of pigmentation appearance. In just one isolate (CE0613) has not been seen this process. To better characterize this isolate, we submitted the M-phase to subcultered (2 times) at 34ºC (data not shown), because some *H. capsulatum* strains had the M-Y conversion in low temperatures as 34ºC as Downs strain (temperature-sensitive), since they can suffer cellular degeneration in high temperatures [43]. However, there was not the conversion M-Y of fungus. The immunoidentification assay of CE0613 by WB identified the band M, specific antigen of *H. capsulatum*, which confirmed the nature of fungus. In addition, we obtained the partial amplification of ITS1-5.8S-ITS2 region of rDNA by PCR to confirm the fungal specie (data not shown) based in the conditions previously described [44].

The immunoassays are techniques used for faster and accurate identification of fungal isolates [13, 15], especially in those strains identification with atypical morphology or isolates without characteristics sporulation structures [14, 45, 46]. In addition, this technique reduces the potential for exposure to biohazardous fungi by eliminating the need for the in vitro conversion or cultural manipulations used in animal inoculations. Rapid exoantigen tests eliminate costs arising from time-consuming and laborious morphologic, physiologic, and cultural studies.

In previous studies, ID has shown a high sensitivity to detect *H. capsulatum* exoantigens [13, 14] in contrast with our results. In several studies fungal immunoidentification the ID test was used and generally it was detected both H and M antigens in isolate from different source [13, 14, 35, 42]. Nevertheless, our study have shown different results because we have found just M antigen in the majority of positive results in exoantigen test by ID. The influence of some factors on the accuracy of exoantigens production were reported as growth time of colonies (> 3 days of incubation in a rotatory shaker), inoculum quantifity and size (2-4cm^2), and efficiency in the exoantigen concentration methods of (50x) [13, 14, 47, 48]. However, in our experiment such factors have not interfered in the exoantigen production, since all recommended working conditions were followed.

Although the ID test have demonstrated poor results in the *H. capsulatum* immunoidentification, the WB showed better sensitivity and specific for identification of all isolates included in this study. Another study obtained similar results in 12 *H. capsulatum* isolates, where 100% of fungal isolates were identificated by WB, contrarily to ID, which identified only 33% *H. capsulatum* isolates [49]. WB is an immunoassay more accurate than ID, due its capacity to detect low amounts of antigenic protein using primary antibodies,

which binds specifically to antigens coating the nitrocellulose membrane. Moreover, in the final step enzyme-conjugated antibodies incubation and substrate for this enzyme is then added so as to amplifying a color development [50].

In addition, it is noteworthy that this was the first study that better characterized the M and H bands profile in *H. capsulatum* isolates detected by WB. Both M and H or single M antigens in fungal isolates were the main profiles observed. Antibody to H and M antigens are observed frequently in individuals with severe forms of histoplasmosis [51]. On the other hand, antibody to single M antigen are more frequently in individual that developed pulmonary acute infection [51]. In this research, all fungal isolates were obtained from patients with disseminated form of mycosis, what would explain a high frequency of both M and H antigens identified in the population. However, it has not been demonstrated statistical association between exoantigen profiles and clinical manifestations of histoplasmosis in our study. However, the increasing of sample size will lead us a much more powerful and accurate analysis of this issue.

Until now, few studies have investigated the frequency of mating type in *H. capsulatum* clinical isolates. In 2007, a molecular study identified *MAT1-1* idiomorph in 02 clinical strains from USA (G-217B – strain from patient with unusual histoplasmosis; and UH1 – isolate obtained from a transplant patient with disseminated histoplasmosis), and *MAT1-2* idiomorph in clinical strain from Panama (G-186AR) and clinical isolate from HIV-patient with disseminated histoplasmosis (VA1) [16]. More recently, *H. capsulatum* isolates from diverse sources from Mexico and Brazil were studied their *MAT1* locus. All human clinical isolates (3 of each country) were obtained from patients with disseminated histoplasmosis. In Mexico, the *MAT1-2* idiomorph was identified in two isolates from HIV-negative individuals, and only one fungal isolate was *MAT1-1* idiomorph identified in HIV-positive patient. However, in Brazilian patients, all from Southeast of Brazil, only *MAT1-1* idiomorph was found, which two individuals were HIV-positive [28].

Hence, we analyzed a major casuistic of *H. capsulatum* clinical isolates from humans by *MAT1* locus analysis. Contrarily, to a previous study realized with Brazilian isolates [28], *MAT1-2* idiomorph was firstly described and had a slight predominance among all fungal isolates from Ceará, Northeast of Brazil. Moreover, we identified two patients with mixed infection with different mating type, an inedited event in individuals with histoplasmosis. This suggests that mating type could be associated with geographic origin of *H. capsulatum* isolates. Despite this, more studies are necessary to clarify better the distribution of mating type in Brazil.

Sexual compatibility performed by conventional methods *in vitro* identified a predominance of *MAT1-2* idiomorph in clinical isolates from acute pulmonary histoplasmosis outbreak and in immunocompromised individuals from USA [52, 53]. The *MAT1-2* idiomorph was also considered to be the more virulent than *MAT1-1* idiomorph, due to a higher M-Y conversion rate [52]. On the other than, virulence assays *in vivo* have not sustained this hypothesis, because both mating types of *H. capsulatum* are able to perform dimorphic conversion and had capacity to cause infection in the host [54]. In this research, the M-Y conversion also occurred independently of mating type profile of fungal isolates.

Despite, it was not possible to establish any association among the sexual compatibility type with clinical severe manifestations, mycological characteristics and/or exoantigen profiles. Nevertheless, it is necessary more studies in vivo and in vitro in order to clarify the impact of mating type in the fungal virulence and consequently, in the pathogenesis of disease.

In summary, the phenotypical characterizations are still important methodologies to identifying *H. capsulatum.* However, it is time-consuming, and several times presenting low sensitivity. Immunoidentification test, such as WB, is faster and more specific method for identification of *H. capsulatum* isolates than ID test, and should be used (WB) in daily laboratory practical procedures. Even though the morphological aspects and conversion process are influenced by external factors (such as medium of culture and temperature of incubation), the results observed herein are strongly suggest that some phenotypical characteristics such as pigmentation, texture and sporulation structures of colonies, could be associated with clinical manifestation of histoplasmosis, as well as severity and disease outcome.

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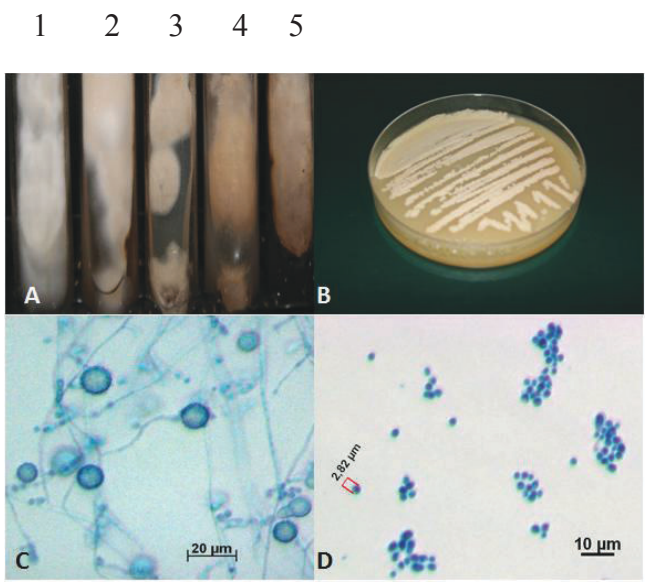

Figure 1: Morphology of *H. capsulatum*. (A) Macromorphology of M – phase, where it's possible to identify five different pigmentations (1 – albino; 2 – albino-beige; 3 – beige; 4 – beige-brown; 5 – brown); (B) Macromorphology of Y – phase, which present cream color and moist colonies; (C) and (D) Micromorphology of M and Y-phase stained by cotton blue lactophenol, respectively.

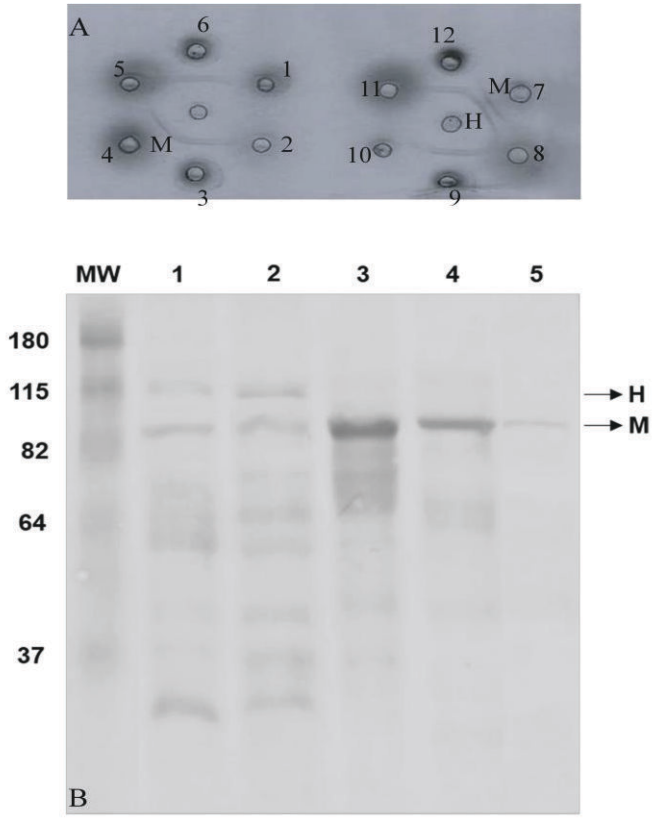

Figure 2: Representative exoantigen profile. (A) Immunodiffusion test with specific H and M lines precipitins. Center well with serum of histoplasmosis patient, wells 3, 6, 9 and 12 with histoplasmina reference; wells 1 (CE0511), 2 (CE0711), 4 (CE1012), 5 (CE1312), 7 (CE0611), 8 (CE2111), 10 (CE0212) and 11 (CE1511) with crude exoantigens. (B) Western Blot assay with specific H and M antigens. Lanes 1 – CE0311, 2 – CE0411, 3 – CE1611, 4 – CE0812, 5 – CE1012, MW – Molecular weight.

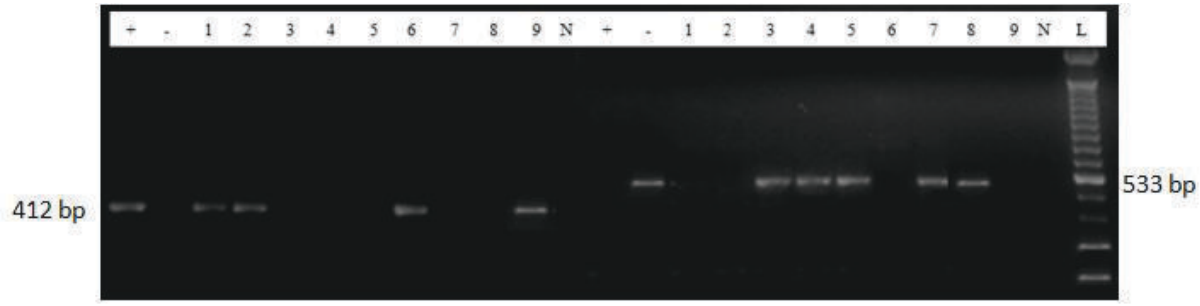

Figure 3: Representative mating type PCR: (+) G217-B reference strain of MAT1-1 idiomorphic, (-) G-186AR reference strain of MAT1-2, (1) CE0311, (2) CE0411, (3) CE0211, (4) CE1911, (5) CE 0511, (6) CE 0611, (7) CE 1111, (8) CE 1211, (9) CE 0112, (N) negative control, (L) 100bp DNA Ladder.

Table 1: Epidemiological and clinical characteristics of patients with histoplasmosis from Ceará/Brazil, 2011-2014.

Epidemiologic and clinical characteristics	Frequency (%)
Sex (n=40)	
Man	31 (77.5%)
Woman	9 (22.5%)
Origin (n=40)	
Fortaleza	23 (57.5%)
Other cities of Ceará	17 (42.5%)
HIV/AIDS (n=40)	
Yes	39 (97.5%)
No	1 (2.5%)
Drug user (n=40)	
Yes	6 (15.0%)
No	34 (85.0%)
Co-infection with tuberculosis (n=40)	
Yes	6 (15.0%)
No	34 (85.0%)
Signal/symptoms (n=43)*	
Fever	43 (100%)
Weight loss	33 (77%)
Cough	27 (63%)
Dyspnea	27 (63%)
Hepatomegaly	26 (60%)
Diarrhea	25 (58%)
asthenia	22 (51%)
Splenomegaly	20 (46%)
vomiting	18 (42%)
Abdominal pain	12 (28%)
Headache	11 (25%)
Hemorrhagic manifestation	10 (23%)
Skin lesion	9 (21%)
Adenomegaly	8 (19%)
Acute renal failure	6 (14%)

*The signal/symptoms were obtained from 43 hospitalizations of histopasmosis patients.

Table 2: Morphological data and exoantigen profile of *H. capsulatum* isolates from Ceará/Brazil, 2011-2014.

Patient	Fungal isolate	Source of sample	Date of culture	Pigmentation	Texture	Macro[a]	First conversion	Exoantigen by WB[b]
1	CE 0211	Buffy coat	03/01/2011	Albino-beige	Cottony	Yes	Yes	H/M
1	CE 1911	Blood	02/02/2011	Albino	Cottony	No	Yes	H/M
2	CE 0311	Buffy coat	20/01/2011	Albino	Cottony	Yes	Yes	H/M
2	CE 0411	Bone marrow	20/01/2011	Albino-beige	Cottony	Yes	Yes	H/M
2	CE 0511	Buffy coat	21/01/2011	Beige	Cottony	Yes	Yes	M
3	CE 0611	Blood	29/12/2010	Albino-beige	Cottony	Yes	Yes	H/M
4	CE 0711	Buffy coat	18/01/2011	Beige	Cottony	Yes	Yes	M
5	CE 1111	Bone marrow	17/12/2010	Beige	Cottony	Yes	Yes	H/M
6	CE 1211	Buffy coat	24/01/2011	Albino-beige	Cottony	No	Yes	H/M
7	CE 1511	Buffy coat	27/12/2010	Beige-brown	Cottony	Yes	Yes	H
8	CE 1611	Buffy coat	05/01/2011	Beige-brown	Cottony	Yes	Yes	M
9	CE 2111	Blood	10/02/2011	Beige	Cottony	Yes	Yes	H
10	CE 0112	Blood	14/02/2012	Brown	Powdery	No	Yes	H
11	CE 0212	Buffy coat	10/04/2012	Albino-beige	Cottony	Yes	Yes	H/M
12	CE 0812	Buffy coat	05/07/2012	Albino-beige	Cottony	Yes	Yes	M
13	CE 1012	Buffy coat	21/08/2012	Beige-brown	Cottony	Yes	Yes	H/M
14	CE 1312	Buffy coat	16/11/2012	Albino-beige	Cottony	Yes	No	H/M
15	CE 0913	Blood	29/05/2013	Albino-beige	Cottony	Yes	Yes	H/M
16	CE 0213	Buffy coat	11/04/2013	Albino-beige	Powdery	Yes	Yes	M
17	CE 0313	Buffy coat	16/04/2013	Albino	Cottony	Yes	No	H/M
17	CE 0713	Bone marrow	15/04/2013	Albino	Cottony	Yes	Yes	M
17	CE 1013	Buffy coat	17/04/2013	Albino	Cottony	No	No	H/M
17*	CE 2713	Buffy coat	13/09/2013	Beige-brown	Powdery	Yes	Yes	M
18	CE 0413	Blood	20/05/2013	Albino	Cottony	Yes	Yes	H/M
19	CE 0513	Buffy coat	08/05/2013	Albino-beige	Cottony	Yes	Yes	M
19	CE 0914	Blood	14/05/2013	Beige-brown	Powdery	Yes	Yes	M
19*	CE 0814	Buffy coat	11/06/2013	Albino-beige	Cottony	No	Yes	H/M
20	CE 0613	Buffy coat	11/04/2013	Albino	Cottony	Yes	---	M

21	CE 0813	Bone marrow	25/02/2013	Albino	Cottony	Yes	Yes	H
22	CE 1113	Bone marrow	09/04/2013	Albino-beige	Cottony	Yes	Yes	M
22	CE 1513	Buffy coat	11/04/2013	Albino	Cottony	Yes	Yes	M
23	CE 1213	Buffy coat	05/06/2013	Beige-brown	Powdery	Yes	Yes	H/M
24	CE 1313	Buffy coat	11/06/2013	Beige-brown	Powdery	Yes	Yes	M
25	CE 0414	Buffy coat	14/06/2013	Albino	Cottony	Yes	No	M
26	CE 1414	Blood	10/05/2013	Albino	Cottony	No	No	H/M
27	CE 1713	Buffy coat	18/01/2013	Beige-brown	Powdery	Yes	Yes	H/M
28	CE 2513	Buffy coat	27/08/2013	Beige-brown	Powdery	Yes	Yes	H/M
28	CE 2813	Buffy coat	18/09/2013	Albino-beige	Powdery	Yes	Yes	M
29	CE 3013	Buffy coat	20/04/2013	Albino-beige	Cottony	Yes	Yes	H/M
30	CE 0214	Blood	30/10/2013	Albino	Cottony	No	Yes	H/M
31	CE 0314	Buffy coat	15/10/2012	Albino	Cottony	Yes	Yes	M
32	CE 0514	Buffy coat	15/07/2013	Albino-beige	Cottony	No	Yes	H/M
33	CE 0614	Blood	31/05/2013	Albino-beige	Cottony	No	Yes	H/M
33*	CE 1014	Bone marrow	11/10/2013	Albino	Cottony	No	Yes	M
34	CE 0714	Buffy coat	05/06/2013	Albino-beige	Cottony	No	Yes	H/M
35	CE 1114	Blood	20/09/2013	Albino	Cottony	No	Yes	H/M
36	CE 1214	Blood	30/10/2013	Albino-beige	Cottony	Yes	Yes	H/M
37	CE 1714	Blood	04/02/2014	Beige-brown	Cottony	Yes	Yes	H/M
38	CE 2214	Buffy coat	19/02/2014	Albino	Cottony	No	Yes	H/M
39	CE 2514	Tracheo-bronchial aspirate	23/01/2014	Albino-beige	Cottony	Yes	Yes	M
40	CE 2614	Blood	01/02/2014	Albino-beige	Cottony	Yes	Yes	H/M

* These patients were re-hospitalized due to new histoplasmosis episode; (a) Presence of macroconidia; (b) Western Blot.

Table 3: Mating type genotype and accession number of each sequence of *H. capsulatum* isolates from Ceará/Brazil, 2011-2014.

Fungal isolate	Mating type genotype	GenBank
CE 0211	MAT 1-2	KX685597
CE 1911	MAT 1-2	KX685617
CE 0311	MAT 1-1	KX058315
CE 0411	MAT 1-1	KX058314
CE 0511	MAT 1-2	KX058317
CE 0611	MAT 1-1	KX685628
CE 0711	MAT 1-1	KX685631
CE 1111	MAT 1-2	KX685607
CE 1211	MAT 1-2	KX685608
CE 1511	MAT 1-2	KX685613
CE 1611	MAT 1-2	KX685614
CE 2111	MAT 1-2	KX685618
CE 0112	MAT 1-1	KX685622
CE 0212	MAT 1-1	KX685623
CE 0812	MAT 1-1	KX685633
CE 1012	MAT 1-1	KX685636
CE 1312	MAT 1-2	KX685610
CE 0913	MAT 1-1	KX685634
CE 0213	MAT 1-1	KX685624
CE 0313	MAT 1-2	KX685599
CE 0713	MAT 1-2	KX685602
CE 1013	MAT 1-2	KX685605
CE 2713	MAT 1-2	KX685621
CE 0413	MAT 1-1	KX685626
CE 0513	MAT 1-1	KX685627
CE 0914	MAT 1-1	KX685635
CE 0814	MAT 1-2	KX685604
CE 0613	MAT 1-1	KX685629
CE 0813	MAT 1-2	KX685603
CE 1113	MAT 1-1	KX685637
CE 1513	MAT 1-1	KX685640
CE 1213	MAT 1-2	KX685609
CE 1313	MAT 1-2	KX685611
CE 0414	MAT 1-2	KX685600
CE 1414	MAT 1-2	KX685612
CE 1713	MAT 1-2	KX685615
CE 2513	MAT 1-2	KX058316
CE 2813	MAT 1-1	KX058313
CE 3013	MAT 1-1	KX685642
CE 0214	MAT 1-2	KX685598
CE 0314	MAT 1-1	KX685625
CE 0514	MAT 1-2	KX685601
CE 0614	MAT 1-1	KX685630
CE 1014	MAT 1-2	KX685606
CE 0714	MAT 1-1	KX685632
CE 1114	MAT 1-1	KX685638
CE 1214	MAT 1-1	KX685639
CE 1714	MAT 1-2	KX685616
CE 2214	MAT 1-2	KX685619
CE 2514	MAT 1-2	KX685620
CE 2614	MAT 1-1	KX685641

Table 4: Correlation between clinical manifestations and phenotypical characteristics of *H. capsulatum* isolates from Ceará/Brazil, 2011-2014.

	Clinical manifestations									
Phenotypical characteristics	Skin lesion	RR (95% IC)	Hemorrhagic manifestation	RR (95% IC)	Dyspnea	RR (95% IC)	ARF	RR (95% IC)	Death	RR (95% IC)
Texture[a]										
Powdery (n=7)	2	1.46 (0.33-6.34)	5	**8.0 (1.82-35.07)**	4	0.82 (0.21-3.20)	2	2.4 (0.59-9.67)	4	2.66 (0.68-10.31)
Cotton (n=35)	7		5		22		4		10	
Pigmentation[b]										
Pallid (n=26)	7	1.38 (0.94-2.01)	6	0.96 (0.57-1.63)	19	1.58 (0.90-2.77)	5	**1.57 (1.21-2.03)**	11	1.41 (0.94-2.09)
Dark (n=12)	1		3		5		0		2	
Macroconidio[c]										
Presence (n=30)	7	1.08 (0.71-1.63)	9	0.9 (0.96-1.82)	18	0.77 (0.55-1.09)	5	1.16 (0.77-1.76)	13	**1.47 (1.06-2.03)**
Absence (n=11)	2		1		9		1		1	
Exoantigens profile by WB[d]										
M and H (n=24)	3	0.42 (0.11-1.54)	6	1.41 (0.41-4.88)	16	1.25 (0.74-2.13)	2	0.47 (0.88-2.52)	6	0.60 (0.24-1.48)
M or H (n=17)	5		3		9		3		7	
Mating type[e]										
MAT1-1 (n=19)	5	1.92 (0.52-7.03)	6	2.31 (0.66-8.02)	12	0.99 (0.62-1.58)	2	0.77 (0.14-4.14)	7	1.35 (0.54-3.32)
MAT1-2 (n=22)	3		3		14		3		6	

These patients were not considered in the statistical analyses: (a) One patient had colonies with different texture; (b) Five patients had isolates with different pigmentation; (c) Two patients had colonies with and without macroconidia; (d) Two patients had isolates with different exoantigen profile; (e) Two patients had isolates with different mating type. RR – relative risk; IC – confidence interval; ARF – acute renal failure.

4.4 Capítulo 4

Mixed infection in Brazilian AIDS-associated histoplasmosis patients produced by *Histoplasma capsulatum* isolates with different mating types

Running title: Mixed infection by *H. capsulatum*

Manuscrito em revisão para submissão na revista Journal of Clinical Microbiology.

Histoplasma capsulatum é um microorganismo eucarioto e hetrotálico, o qual é encontrado no ambiente como micélio haploide associado com + (MAT1-1) ou – (MAT1-2) tipos de compatibilidade sexual ou *mating type*. *Ajellomyces capsulatus* é o estágio sexual ou teleomorfo de *H. capsulatum,* e resulta do pareamento dos tipos de compatibilidade sexual. Ambos os fungos representam o mesmo organismo holomorfo. Neste estudo, foram relatados dois casos de infecção mista causadas por diferentes tipos de compatibilidade sexual de *H. capsulatum* em pacientes com aids. Além disso, foram avaliados os aspectos morfológicos e de conversão dimórfica. Foram realizados também, estudos moleculares (mating type e MLST) e análise filogenética nos cinco isolados obtidos dos dois pacientes do estudo. No paciente 1, três isolados foram obtidos: CE0311 (1 º dia – creme leucocitário), CE0411 (1º dia – medula óssea) e CE0511 (2 º dia – creme leucocitário); e no paciente 2, os isolados obtidos foram: CE2813 (3º dia) e CE2513 (12º dia), ambos provenientes de culturas do creme leucocitário. A textura e pigmentação das colônias foram bastante diversas. *MAT1-1* idiomorfo foi observado nos seguintes isolados: CE0311, CE0411 e CE2813; e *MAT1-2* idiomorfo , nos isolados CE0511 e CE2513. Todos os isolados foram agrupados no clado LAm A, entretanto, foi possível destacar dois subgrupos diferentes, baseado no alto valor de *bootstrap*. Os isolados CE0311 e CE0411 formaram um único subgrupo, e os demais isolados deste estudo agruparam-se com o isolado H151 (São Paulo). Este artigo relata pela primeira vez no mundo, infecção mista causada por diferentes tipos de compatibilidade sexual de *H. capsulatum*. Mais estudos são necessários para avaliar as consequências desse tipo de infecção, que é incomum, na patogenia da histoplasmose.

Mixed infection by *Histoplasma capsulatum* isolates with different mating types in Brazilian AIDS-associated histoplasmosis patients

Mixed infection by *H. capsulatum*

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ABSTRACT

Mixed infection by *Histoplasma capsulatum* isolates with different mating types, in AIDS-patients are described in this study. Morphological, mating type-specific PCR assay and multilocus sequencing type analysis of *H. capsulatum* isolates recovered from two Brazilian AIDS-patients were performed. Five *H. capsulatum* isolates were recovered at different times from the two patients. Three isolates were obtained from bone marrow (day 1 – CE0411) and buffy coat cultures (day 1 – CE0311; day 2 – CE0511) of patient 1, and two isolates were isolated from buffy coat cultures (day 3 – CE2813; day 12 – CE2513) of patient 2. The mycelial colonies depicted different textures and pigmentation features. Dimorphic conversion to yeast-phase in ML-Gema medium was reached in all isolates. MAT1-1 idiomorph was identified in CE0311, CE0411 and CE2813 isolates; and MAT1-2 idiomorph was found in CE0511 and CE2513 isolates. These *H. capsulatum* isolates were grouped within LAm A clade, highlighting that CE0311 and CE0411 isolates formed a subgroup supported by a high bootstrap value. The CE0511, CE2513, and CE2813 isolates clustered together with a Brazilian H151 isolate. This research reports, for the first time in the world, mixed infections caused by *H. capsulatum* isolates with different mating types.

Key words. Mixed infection; *Histoplasma capsulatum;* mating types; multilocus sequence typing; histoplasmosis.

INTRODUCTION

Histoplasma capsulatum is a dimorphic fungus found in the form of mold in the environment and *in vitro* at 25 - 28ºC. The yeast form is observed during parasitism conditions or in cultures at 34 - 37ºC in enriched media (1). *Histoplasma* infection can occur in individuals that are exposed to fungal micro-niches rich in bat guano or bird droppings (1). Severe forms of histoplasmosis usually occur in individuals with immunosuppression, such as AIDS-patients (2).

The asexual stage or anamorph (*H. capsulatum*) is an eukaryotic and heterothallic microorganism found in the environment as haploid mycelium associated with + or - mating types (3). *Ajellomyces capsulatus* is the sexual stage or teleomorph of *H. capsulatum,* as a result of the sexual compatibility + and – of *H. capsulatum* isolates (4). Both fungal stages represent the same holomorph (3-5).

H. capsulatum has a bipolar mating system that expresses transcription factors encoded at the *MAT1* locus (6, 7). In this fungus this locus presents two idiomorphs, MAT1-1 and MAT1-2*,* which define the + and – mating types, respectively (6). In some fungi, the mating type is associated with the virulence of the pathogen (8, 9). In addition, mixed infection with different mating types has been rarely described in pathogenic fungi (9).

A high genetic diversity of *H. capsulatum* has been reported worldwide (10-13). In 2003, the performance of multilocus sequencing type (MLST) by partial amplification of four nuclear protein coding genes, ADP-ribosylation factor (*arf*), H antigen precursor (*H-anti*), delta-9 fatty acid desaturase (*ole1*), and alpha-tubulin (*tub1*), indicated the presence of eight phylogenetic clades among 149 *H. capsulatum* isolates from 25 countries (10). The results of theses analyses revealed the presence of NAm 1 and NAm 2 clades in North America; LAm A and LAm B clades in Latin America; Africa clades restrict to Africa; Euroasian clades constituted from fungal isolates from Egypt, India, China, Thailand, and England; Netherlands clade; and, Australian clade (10). In addition, diverse new lineages have also been identified in different geographic regions of the world (10, 11). A recent study performed with a broad number of fungal isolates (n=234) and more robust phylogenetic analyses identified five new phylogenetic clades, with a great admixture among *H. capsulatum* isolates from Latin America (13). The LAm A clade was regrouped in LAm A1, LAm A2, and LAm B clade was reclassified in LAm B1 and LAm B2 (13). In addition, two new phylogenetic clades (RJ from Southeast Brazil and BAC-1 from Mexico), and four monophyletic clusters in Brazil (BR1-4) were identified.

Here, two cases of mixed infection with different mating types of *H. capsulatum* in AIDS-patients are reported. In addition, the five *H. capsulatum* isolates obtained from these histoplasmosis-AIDS cases were evaluated by morphological criteria, mating type determination, and phylogenetic classification by MLST analysis.

MATERIALS AND METHODS

Patients

Clinical data of two patients with histoplasmosis and AIDS from Ceará, Brazil, were retrospectively reviewed from their medical records. These patients were selected because they presented more than one *H. capsulatum* isolate in different clinical samples, during their period of hospitalization and treatment. The study was approved by the Research Ethics Committee at the Instituto Nacional de Infectologia Evandro Chagas/FIOCRUZ (No. 19342513.2.0000.5262), Rio de Janeiro, Brazil.

Fungal Isolation and Phenotypic Characterization

Fungal isolates (buffy coat from whole blood and bone marrow aspirate) from patients were cultured on Potato Dextrose Agar **(**Difco, Detroit, MI, USA) at 25ºC during 21 days. Macromorfology of the filamentous fungal cultures were visually examined and recorded. Their micromorphology was observed in 10 different fields by optical microscopy at a 40X magnification of the *H. capsulatum* colonies, stained with Lactophenol Cotton Blue (Fluka Analyted, France). Dimorphism was demonstrated by conversion to the yeast-like form on MLGema-agar medium (14), for 7 to 14 days at 37ºC.

Mating type determination

DNA was isolated as previously reported (15). The *MAT1* locus of the *H. capsulatum* isolates was identified by polymerase chain reaction (PCR) using specific pair of primers for MAT1-1 and MAT1-2 idiomorphs based in a previous protocol with minor modifications (16). Briefly, the PCR was performed in a 25 µl reaction mixture, containing 200µM each deoxynucleoside triphosphate (dNTP) (Applied Biosystems Inc., Foster City, CA, USA), 1.5 mM $MgCl_2$, 50 ng/µl of each primer, 1.5 U *Taq* DNA polymerase (New England BioLabs Inc., MA, USA), 1 X *Taq* commercial buffer, and 75 ng (25 ng/µl) of each DNA template. The G-217B from USA (*MAT1-1*) and G-186AR from Panama (*MAT1-2*) references strains were used as controls.

PCR assays were performed in a Thermal iCycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) programmed as follows: (a) 3 min at 95°C; (b) 35 cycles, consisting of 30 s at 95°C, 30 s at 58°C, and 1 min 30 s at 72°C; and (c) 10 min at 72°C. Amplicons were resolved by 1.5% agarose gel electrophoresis. The 100-bp DNA ladder was used as a molecular marker.

Amplicons were sequenced at the High-Throughput Genomics Center (University of Washington, Seattle, WA, USA). The sequences obtained were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov). They were edited and aligned for BLASTn analysis (17), using as reference the sequence of the strains G-217B from USA (MAT1-1, GenBank accession number EF433757) and G-186AR from Panama (MAT1-2, GenBank accession number EF433756).

Phylogenetic relationship among the studied isolates

Genetic reconstruction analysis was performed by MLST, using PCR amplification of partial DNA sequences of four nuclear genes (*arf, H-anti, ole1,* and *tub1*) according to the protocol described by Kasuga et al. (10) with some modifications. The PCR was performed with 25 µl reaction mixture, containing 200µM each deoxynucleoside triphosphate (dNTP) (Applied Biosystems Inc., Foster City, CA, USA), 2.0 mM $MgCl_2$, 50 ng/µl of each primer, 1.0 U *Taq* DNA polymerase (New England BioLabs Inc., MA, USA), 1 X *Taq* commercial buffer, and 20 ng (10 ng/µl) of each DNA template. The G-217B from USA reference strains was used as a control. PCR assays were performed in a Thermal iCycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) programmed as follows: (a) 3 min at 95°C; (b) 32 cycles, consisting of 15 sec at 94°C, 30 sec at 65ºC in the first cycle, which was subsequently reduced by 0.7ºC/cycle for next 12 cycles, and 1 min at 72ºC. The remaining 20 cycles, the annealing temperature was continued at 56ºC; (c) a final extension cycle of 5 min at 72ºC (touchdown PCR) (18).

Generated amplicons were also sequenced at the High-Throughput Genomics Center (University of Washington) and the sequences were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov). The resulting sequences were analyzed by BLASTn (17), using the sequences of the G-217B strain as reference (GenBank accession numbers: L25117.1, U20346.1, X85962.1, and M28358.1).

To identify the relationships of the five studied isolates with other *H. capsulatum* isolates previously characterized by the four aforementioned nuclear genes, a combined matrix using partial sequences of these genes was generated and edited manually by MESQUITE ver. 2.75 (19). This matrix was constructed with 260 sequences, considering four genes per isolate, out of 65 *H. capsulatum* analyzed isolates: 57 isolates from TreeBASE (http://treebase.org, study ID S1063) reported by Kasuga et al. (10); three from GenBank with their respective accession numbers for arf, H-anti, ole1, and tub1 genes (EH-383I isolate- AF495619, AF495620, AF495621, and AF495622; and EH-375 isolate- AF495607, AF495608, AF495609, and AF495610; and the G-217B strain, see the aforementioned GenBank accession numbers); and

the five *H. capsulatum* clinical isolates reported in this study. Details of the 65 *H. capsulatum* isolates can be seen in Tables 1 and 2.

The generated combined matrix containing 1539-nt was analyzed through two methods: 1) Maximum likelihood (ML) in RaxMLGUI ver. 1.31 (20) through the General Time Reversible substitution model gamma distribution; and 2) Bayesian inference (BI) by MrBayes ver. 3.2 (21) with a final run using four chains for a total of 100,000,000 generations and sampling trees every 10,000 generations. The substitution models considered in BI for each partition were K80 (H-anti), K80+G with four categories (arf and tub1), and K80+I (ole1). The substitution models for ML and BI methods were selected according to Akaike Information Criterion and Bayesian information criterion tests, implemented in Jmodeltest ver. 2.1.4 for ML and BI (22), respectively.

Bootstrap values (bt) for ML analysis were based on 1000 heuristic search replicates, using Tree-Bisection-Reconnection. For the BI analysis, the maximum clade credibility tree was selected with a posterior probability (pp) limit of 0.95, using TreeAnotator ver. 1.8.2, implemented in BEAST - Bayesian Evolutionary Analysis Sampling Trees (23, 24). An unrooted tree was constructed using the combined matrix.

RESULTS

Patient 1

A 22-year-old male AIDS-patient was admitted in an infectious diseases hospital with fever, diarrhea, asthenia, and a weight loss of 6 kg. He was a craftsman and lived in an urban area of Baturité/Ceará, Brazil. Physical examination revealed cervical adenomegaly and hepatosplenomegaly. Pulmonary and cardiac auscultation as well as vital signs were normal. He used irregularly antiretroviral drugs (estavudine, lamivudine, and efavirenz). Laboratory evaluations revealed hemoglobin level of 9.3g/dL, white blood cells count 1200/mm^3 (neutrophils = 80%; lymphocytes = 10.8%; monocytes = 4.2%; eosinophils = 5%), and platelets count 65,000/mm^3. Renal function was normal. Level of lactate dehydrogenase (LDH) was high (2,086 U/L). Aspartate aminotransferase (AST) level was of 274 U/L, alanine aminotransferase (ALT) level was of 67 U/L, and alkaline phosphate (AP) was of 184 U/L. The patient had CD4+ lymphocytes count of 273 cells/mm^3 and plasma HIV-RNA of 127,240 copies/mL. *H. capsulatum* yeast-like was visualized by Giemsa staining of buffy coat smear. Therapy with amphotericin B (1mg/kg per day) was administered and the patient was discharged after 35 days of hospitalization. In the clinical follow-up, the antifungal therapy was maintained with amphotericin B once a week for 6 month.

Patient 2

A 52-year-old male AIDS-patient was admitted in an infectious diseases hospital with fever, abdominal pain, cough, dyspnea, hematochezia, and weight loss. He had a history of an oral mucosa lesion for the last 2 months. He was engaged in farming activities and lived in a rural area of Maracanaú/Ceará. His vital signs were as follow: temperature 37.2ºC; pulse rate 106/min, respiratory frequency 30/min, and blood pressure 70/30mmHg. A physical examination revealed pallor, oral ulcer with partial destruction of uvula, and oral candidiasis. Cardiac auscultation revealed systolic murmur, and chest auscultation detected crackles in the base of the left lung. The abdominal examination revealed hepatosplenomegaly. He used irregularly antiretroviral drugs (zydovudine, lamivudine, atazanavir, and ritonavir). Laboratory evaluations revealed hemoglobin level of 4.3 g/dL, white blood cells count 3860/mm^3 (bands = 1%; neutrophils = 64%; lymphocytes = 24%; monocytes = 8%; eosinophils = 2%; basophiles = 1%), and platelets count 34,000/mm^3. Renal function was normal. A high level of LDH was observed (1,402 U/L). Hepatic function was altered (AST = 97 U/L, ALT = 32 U/L, AP = 908 U/L, GGT = 197 U/L). The patient had CD4+ lymphocytes count of 80 cells/mm^3. A chest radiograph showed diffuse reticulonodular pulmonary infiltrate. Empiric therapy with amphotericin B (1 mg/kg per day) was started on day 1. However, the patient presented respiratory and renal failure. He died after 18 days of hospitalization.

Fungal cultures and morphological identification

H. capsulatum were isolated from patient's clinical samples during their hospitalization and treatment. Three *H. capsulatum* isolates were obtained from patient 1, one from bone marrow (day 1 – CE0411) and two from buffy coat (day 1 – CE0311; day 2 – CE0511). Two *H. capsulatum* isolates were recovered from buffy coat of patient 2 at day 3 (CE2813) and day 12 (CE2513) of hospitalization. The *H. capsulatum* mycelial cultures of patient 1 presented cottony texture macromorphology, whereas mycelial cultures of patient 2 were powdery. Different pigmentations were observed in the fungal cultures of both patients. These morphological characteristics are recorded on Table 2. In regard to micromorphology, all fungal isolates had hyaline, septated and branched thin hyphae, microconidia and tuberculate macroconidia. Dimorphic conversion occurred in all of *H. capsulatum* isolates, with typical budding yeast cells. Fig. 1 shows a representative micromorphology of the five fungal isolates recovered in this study.

Mating types of *H. capsulatum* isolates recovered from the AIDS-patients

The *MAT1-1* idiomorph was identified in *H. capsulatum* isolates CE0311 and CE0411 (patient 1) as well as in CE2813 (patient 2) isolates; whereas the *MAT1-2* idiomorph was found in CE0511 (patient 1) and CE2513 (patient 2) isolates. The sequences of the *MAT1* locus for these five fungal isolates are available in the GenBank (accession numbers in Table 3) and they were compatible with *A. capsulatus* through BLASTn analysis (15). The CE0311 and CE0411 isolates showed 99% similarity and CE2813 isolate showed 100% similarity with the sequence of the G-217B reference strain (*MAT1-1*), whereas CE0511 and CE2513 isolates showed 97% similarity with the sequence of the G-186AR reference strain (*MAT1-2*).

Phylogenetic analyses of the *H. capsulatum* isolates

These partial sequences obtained from the five *H. capsulatum* isolates were deposited in the GenBank (accession numbers in Table 3).

The phylogenetic trees for the four genes analyzed by either ML or BI methods presented similar topologies. A BI phylogenetic tree was constructed to support both ML and BI data, where bt and pp values were represented in each tree node (Fig. 2). The five *H. capsulatum* isolates from the AIDS-associated histoplasmosis patients were grouped in the LAm A clade together with other LAm A isolates included in the present study (Table 1, Fig. 2). The CE0311 and CE0411 isolates from patient 1 share the same branch with the H146 isolate from Brazil, supported by bt = 74% (ML) and pp = 1.0 (BI) values. On the other hand, CE0511 (patient 1), CE2513 and CE2813 (patient 2) isolates were clustered together and share a branch with Brazilian H151 and H149 isolates (bt = 56% in ML, pp = 1.0 in BI) (Fig. 2). In regard to the sequences of other isolates used to develop the MLST analyses, Fig. 2 shows that they clustered according to Kasuga et al. (10) criterion, representing the different clades reported in Table 1.

DISCUSSION

This study reports for the first time in the world, two cases of mixed infection caused by *H. capsulatum* isolates with different mating types, highlighting the genetic diversity among the five fungal isolates recovered from clinical samples. The *H. capsulatum* isolates associated with mixed infection were obtained from the Ceará State, which is an endemic area of histoplasmosis in Northeast Brazil. This mycosis usually occurs in AIDS-patients, and a high mortality rate has been recorded (30-40%) for them (2, 25).

Mixed infections caused by microorganisms with different genetic profiles have been described mainly with pathogenic bacteria (26, 27). Fungal mixed infection with different mating types of the same species was described in individuals colonized or infected by *Aspergillus fumigatus* (9). However, the clinical consequences of this event are still unknown.

It is suggested that the mechanisms driving mixed infections include microevolution of pre-existing clones, simultaneous co-infection by recently acquired strains, and superinfection by a new strain different from pre-existing clones (28, 29). Here, mixed infection caused by different isolates was supported by the finding of different *H. capsulatum* mating type's idiomophs from the same patient, at different time points of the histoplasmosis treatment.

Some studies have also shown that the mating system of *Cryptococcus neoformans* and *A. fumigatus* is associated with virulence of these pathogens as well as the level of infections (8, 9, 30, 31). *MAT1*-1 has been associated with invasive aspergillosis (9, 31), and in *C. neoformans* infections, the α-mating type has been described as more virulent than the a-mating type (8, 30). However, more recently, experimental studies revealed that there is not association between mating type and virulence of *C. neoformans* and *A. fumigatus* (32, 33).

The sexual reproduction by meiosis between strains with different mating types can generate genetic variability, which is very important for lineage survival. In addition, this process can lead to the formation of hypervirulent strains, as well as strains with the ability to evade the host's immune response and with more resistance to antifungal drugs (7, 28).

Few studies have investigated the mating type of *H. capsulatum* isolates and its impact on the virulence of the pathogen (6, 16, 34). Conventional methods, such as *in vitro* cross mating between isolates of *H. capsulatum,* identified a predominance of *MAT1-2* in clinical isolates from USA patients with acute pulmonary histoplasmosis (34). In 2007, a molecular study identified *MAT1-1* in strains from USA isolated from a patient with unusual histoplasmosis (G-217B) and UH1 isolate obtained from a transplant patient with disseminated histoplasmosis. *MAT1-2* was related to the strain G-186AR from Panama and the strains VA1 and T-3-1 from USA (6).

More recently, several environmental and clinical *H. capsulatum* isolates from Mexico and Brazil were characterized concerning their *MAT1* locus by PCR (16). Six out of 28 studied fungal isolates were obtained from patients with disseminated histoplasmosis, where three isolates came from HIV-patients (two from Brazil and one from Mexico). The *MAT1-1* was found in all (11 environmental and three clinical) isolates studied from Brazil, whereas the *MAT1-2* was predominantly identified in most of the Mexican *H. capsulatum* isolates, including two clinical isolates. Interestingly, *MAT1-1* idiomorph was also identified in four isolates from Mexico, where one of these was isolated from an HIV-patient (16). As a new data, it was reported here two *MAT1-2 H. capsulatum* isolate from Brazil. Undoubtedly, more studies are necessary to characterize the distribution and the impact of mating types in *H. capsulatum* isolates from different regions of the Americas.

Among the morphological characteristics recorded for the studied *H. capsulatum* isolates, pigmentation was the sole divergent factor. It is well known that pigmentation of fungal isolates depends on the culture medium, age of strain, and melanin production. Although, the melanization of *H. capsulatum* protects the fungus against antifungal drugs, like amphotericin B (35), experimental studies have not found any difference in virulence between albino (non-melanized) and brown (melanized) *H. capsulatum* strains (36).

All the *H. capsulatum* isolates of this study were identified within the former LAm A clade by MLST analyses, which also revealed genetic diversities among the fungal isolates of each patient. According with the new phylogenetic clusters proposed by Teixeira et al. (13), the isolates CE0311 and CE0411 would be classified within the BR4 clade together with H146; and CE0511, CE2513, and CE2813 within the BR2 clade together H151. Thus, isolates from patient 1, CE0311 and CE0411, grouped in a different cluster from that of isolate CE0511; whereas isolate CE2513 from patient 2 showed a high genetic similarity with the CE0511 isolate from patient 1, supported by bt and pp values of ML and BI trees, respectively (Fig. 2). Based on these genetic findings, and considering the differences in the *MAT1* locus described in the *H. capsulatum* isolates of the same patient, it is possible to assume that these fungal mixed infections could be explained by simultaneous co-infection with a new isolate that diverged and co-exists in the same area, or by superinfection of a latent *Histoplasma* infection. Previous phylogenetic studies with different molecular markers have demonstrated that there is a high genetic diversity among *H. capsulatum* isolates from diverse regions, as well as in the same region (10-13, 37-39). Therefore, co-infection and superinfection are events that can occur in mixed infection by pathogens and cannot be discarded for *H. capsulatum* infections. Despite of this, it is necessary more studies that aimed evaluating the consequences of mixed infections with *H. capsulatum* harboring different genetic and morphologic characteristics in the pathogenesis of histoplasmosis.

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POTENTIAL CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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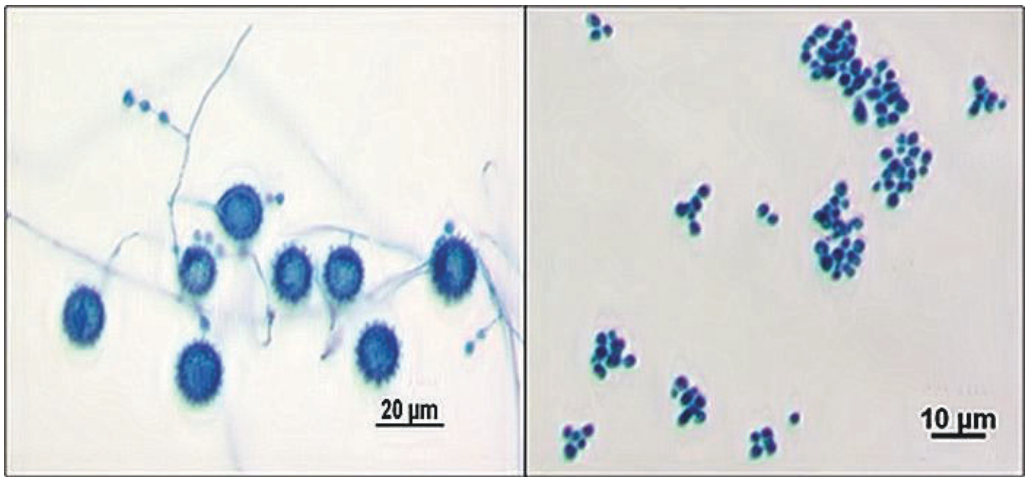

FIG 1 Representative micromorphology of *H. capsulatum* mycelium and yeast phases of CE0411 isolate from an AIDS-patient studied. Both fungal phases were stained by Lactophenol Cotton Blue. Magnification 40X.

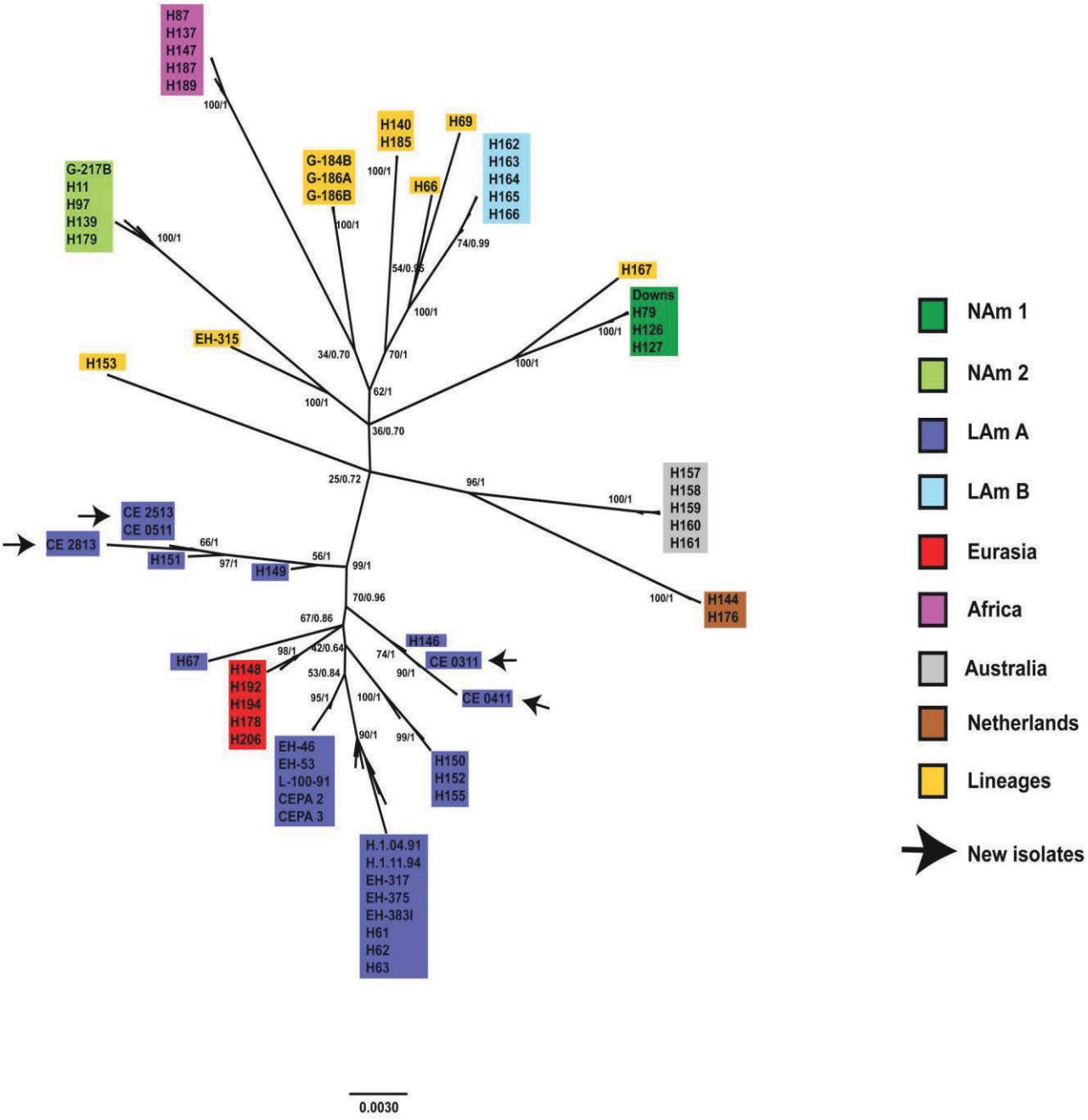

FIG 2 Unrooted phylogenetic tree of *H. capsulatum* isolates. The tree was constructed with a concatenated matrix of 1539-nt using four gene fragments (*arf*, *H-anti*, *ole1*, and *tub1*). It was generated by BI and is representative of both ML and BI analyses. The values of bt/pp are indicated on their corresponding tree nodes.

TABLE 1 Major data of the *H. capsulatum* strains/isolates, which sequences were acquired from databases for the MLST analysis of the present study.

Strain/ Isolate	Phylogenetic clade	Source	Origin	Year of isolation
Downs (H9)*	NAm 1	Human	USA	1968
H79*	NAm 1	Skunk	USA	1967 or before
H126*	NAm 1	Human/HIV+	USA	1987
H127*	NAm 1	Human/HIV+	USA	1987
G-217B (H8)**	NAm 2	Human	USA	1973 or before
H11*	NAm 2	Human	USA	1993 or before
H97*	NAm 2	Human	USA	1995 or before
H139*	NAm 2	Soil	USA	1975
H179*	NAm 2	Human	USA	Not known
H146*	LAm A	Human	Brazil	1979
H149*	LAm A	Human/HIV+	Brazil	1996
H150*	LAm A	Human	Brazil	1996
H151*	LAm A	Human/HIV+	Brazil	1997
H152*	LAm A	Human/HIV+	Brazil	1997
H155*	LAm A	Human/HIV+	Brazil	1998
H67*	LAm A	Human	Colombia	1993
H61*	LAm A	Human	Colombia	1993
H62*	LAm A	Human	Colombia	1993
H63*	LAm A	Human	Colombia	1989
EH-46*	LAm A	Human	Mexico	1979
EH-53*	LAm A	Human	Mexico	1977
EH-317*	LAm A	Human/HIV+	Mexico	1992
L-100-91 (EH-333)*	LAm A	Black bird excreta	Guatemala	1991
EH-383I**	LAm A	Bat	Mexico	1997
CEPA 2 (EH-362)*	LAm A	Black bird excreta	Guatemala	1996
CEPA 3 (EH-363)*	LAm A	Human	Guatemala	1996
H.1.04.91 (EH-304)*	LAm A	Human	Guatemala	1991
H.1.11.94 (EH-332)*	LAm A	Human	Guatemala	1994
EH-375**	LAm A	Bat	Mexico	1997
H162 *	LAm B	Human/HIV+	Argentina	1998-1999
H163*	LAm B	Human/HIV+	Argentina	1998-1999
H164*	LAm B	Human/HIV+	Argentina	1998-1999
H165*	LAm B	Human/HIV+	Argentina	1998-1999
H166*	LAm B	Human/HIV+	Argentina	1998-1999
H148*	Eurasia	Horse	Not known	1935
H178*	Eurasia	Human	China	Not known
H192*	Eurasia	Human	India	Not known
H194*	Eurasia	Horse	Egypt	Not known
H206*	Eurasia	Human	Thailand	1994
H87*	Africa	Human	Guinea-Liberian border	1970
H137*	Africa	Human	Zaire	1962
H147*	Africa	Human	Senegal	1957
H187*	Africa	Bat guano	Nigeria	1991
H189*	Africa	Not known	Not known	Not known
H157*	Australia	Human	Australia	1970s
H158*	Australia	Soil/bat guano	Australia	1984

H159*	Australia	Human	Australia	1984
H160*	Australia	Human/HIV+	Australia	1988
H161*	Australia	Human/HIV+	Australia	1990
H144*	Netherlands	Human	Netherlands	1965
H176*	Netherlands	Human	Netherlands	1969
H66*	Lineage	Human	Colombia	1986
H69*	Lineage	Human	Colombia	1991
H153*	Linage	Human	Brazil	1997
G-186B (H83)*	Lineage	Human	Panama	1967 or before
G-186A (H82)*	Lineage	Human	Panama	1967 or before
G-184B (H81)*	Lineage	Human	Panama	1967 or before
H140*	Lineage	Owl monkey	USA/Peru	1997
H185*	Lineage	Owl monkey	USA/Peru	1999
EH-315*	Lineage	Bat	Mexico	1994

Sequences of all *H. capsulatum* strains/isolates of this table were obtained from two databases, most from *TreeBase and some from **GenBank; Numbers in parenthesis are acronyms.

TABLE 2 Characteristics of the *H. capsulatum* isolates associated with the mixed infection.

Characteristics of fungal	**Patient 1**			**Patient 2**	
isolates	**CE0311**	**CE0411**	**CE0511**	**CE2813**	**CE2513**
Source	Buffy coat	Bone marrow	Buffy coat	Buffy coat	Buffy coat
Day of hospitalization	day 1	day 1	day 2	day 3	day 12
Pigmentation of colonies	Albino	Albino-beige	Beige	Beige-brown	Albino-beige
Texture of colonies	Cottony	Cottony	Cottony	Powdery	Powdery
Mating type	MAT1-1	MAT1-1	MAT1-2	MAT1-1	MAT1-2

TABLE 3 GenBank accession numbers of the sequences of each gene used to characterize the five *H. capsulatum* isolates of the present study.

Fungal isolate	*MAT1*	*Arf*	*H-anti*	*ole1*	*tub1*
CE 0311	KX058315	KX058302	KX058322	KX058307	KX058312
CE0411	KX058314	KX058301	KX058321	KX058306	KX058311
CE0511	KX058317	KX058300	KX058320	KX058305	KX058310
CE2813	KX058313	KX058298	KX058318	KX058303	KX058309
CE2513	KX058316	KX058299	KX058319	KX058304	KX058308

4.5 Capítulo 5

POSTPARTUM HISTOPLASMOSIS IN A HIV NEGATIVE WOMAN: A CASE REPORT AND PHYLOGENETIC CHARACTERIZATION BY ITS REGION ANALYSIS

Manuscrito em revisão para submissão na revista Clinical Microbiology and Immunology.

Histoplasmose é uma micose sistêmica, que pode causar infecções desde assintomáticas ou auto-limitadas, a formas graves e disseminada, dependendo do número de propágulos inalados, da virulência da cepa, e da resposta celular imune do hospedeiro. No Ceará, esta micose é endêmica, ocorrendo principalmente em pacientes com aids. Durante a gestação, algumas micoses têm sido descritas, devido à supressão da imunidade celular, bem como durante a recuperação da resposta celular imune durante o período pós-parto. Este trabalho descreve o primeiro caso de histoplasmose disseminada no período pós-parto, em uma mulher HIV negativa. Além disso, foi realizada análise filogenética da região ITS do isolado fúngico. O caso ocorreu em uma mulher de 24 anos de idade, no 10º dia após parto vaginal, que apresentava dispneia, febre, hipotensão, taquicardia, crepitações nos 2/3 inferiores do hemitórax esquerdo, e hepatomegalia dolorosa. Leveduras compatíveis com *H. capsulatum* foram visualizadas no sangue periférico, com subsequente crescimento do patógeno em cultivo de creme leucocitário. Análise filogenética demonstrou que o isolado fúngico deste estudo foi semelhante a outras cepas de *H. capsulatum* da América Latina. Em conclusão, embora casos graves de histoplasmose ocorram principalmente em pacientes com aids, no Ceará, a equipe de saúde deve estar alerta para a ocorrência de histoplasmose em indivíduos com imunossupressão transitória ou naqueles que estão em período de recuperação imunológica, como ocorrem em mulheres no pós-parto. É possível haver um micronicho específico de *Histoplasma* nesta região do Brasil, já que o perfil filogenético do isolado fúngico deste estudo teve uma alta similaridade genética principalmente com outras cepas oriundas dessa mesma região.

POSTPARTUM HISTOPLASMOSIS IN A HIV NEGATIVE WOMAN: A CASE REPORT AND PHYLOGENETIC CHARACTERIZATION BY ITS REGION ANALYSIS

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ABSTRACT

Histoplasmosis is a systemic mycosis, which can range from asymptomatic or self-limited presentation to a severe and disseminated form, depending on the number of infective propagules inhaled, the strain virulence, and the cellular immune response of host. In Ceará, this mycosis in endemic, and occur mainly in AIDS-patients with severe immunossupression. Systemic mycoses have been described during pregnancy, due to suppression of cell-mediated immunity, as well as during the recovery of cellular immune response in the postpartum period; however, this report describes a first case of postpartum disseminated histoplasmosis in HIV-negative woman. In addition, we performed a phylogenetic analysis by ITS1-5.8S-ITS2 region of *Histoplasma* isolate. A 24-year-old woman, in the day 10 after vaginal delivery presented dyspnea, fever, hypotension, tachycardia, fine crackles in the lower 2/3 of the left hemithorax, and painful hepatomegaly. *H. capsulatum* yeast-like features was seen in peripheral blood with subsequent growth in buffy coat culture. Phylogenetic analysis demonstrates that the fungal isolate was similar to others *H. capsulatum* isolates identified in HIV-patients from Ceará and Latin America. In conclusion, although severe cases of histoplasmosis occur mainly in AIDS-patients in the Ceará State, the health team has to be alert to the occurrence of histoplasmosis in individuals with transitory immunessupression or in those that are in the period of immune recovery as postpartum woman. *Histoplasma* isolate had the same phylogenetic profile identified for HIV patient from this area, indicating a possible specifics microniche in this endemic area.

Key words: histoplasmosis, postpartum, *Histoplasma capsulatum,* ITS1-5.8SITS2 region analysis.

INTRODUCTION

Histoplasmosis is a systemic mycosis caused for *Histoplasma capsulatum,* a dimorphic fungus that is ubiquitous and isolated in diverse geographic regions with different climates [1, 2]. The fungal is found in soil rich with bat guano or bird dropping, and the infection occur by inhalation of microconidia or hyphae fragments [3]. In the Americas, cases of histoplasmosis have been found since Canada to Patagonia (Argentina) [4, 5].

It is usually asymptomatic or self-limited, however, severe and disseminated infections can occur depending on the number of infective propagules inhaled, the strain virulence, and the cellular immune response of host [3]. The risk factors associated to disseminated histoplasmosis are AIDS-patient, the extremes of age, use of immunosuppressive drugs, hematologic malignancies, solid organ transplantation, pregnancy and immune reconstitution syndrome (IRS) [3, 6-8].

A few numbers of cases of disseminated histoplasmosis have been described during pregnancy, especially in the second and third trimesters, in women with *Diabetes Mellitus* or HIV-positive [8, 9], but not in the post-partum period.

During the pregnancy a complex immunomodulation is conducted by hormones, cytokines, and peripheral blood cellules that results in decreased killer natural cells and citotoxic T lymphocytes level, which are associated with fetal protection. At or next delivery and in immediate postpartum period occurs a recovered Th1 type response [10-13]. Reversal changes during the postpartum period may facilitate the emergence of overt clinical manifestations of quiescent or latent infections [13].

The aims this report is to describe the first case disseminated histoplasmosis in the postpartum period in a HIV-negative woman, from endemic area of the Northeast of Brazil. In addition, we performed molecular analysis of *H. capsulatum* isolated in this case.

CASE REPORT

A 24-year-old woman, in the day 10 after vaginal delivery, was admitted to the Hospital São José of Infectious Diseases (HSJ) in Fortaleza, Ceará. On arrive at the emergency department of HSJ, she was pale, with dyspnea (30 breaths per minute), fever (39 °C), hypotension (blood pressure 100x60 mmHg) and tachycardia (113 beats per minute). She had no lymphadenopathy or oral lesions but complained of cough since two months before delivery. Her heart rhythm was normal and the pulmonary exam revealed fine crackles in the lower 2/3 of the left hemithorax. The abdomen was distended, flaccid with painful hepatomegaly.

The hemoglobin level was 10g/dL, with normal white blood cells (WBC) and platelets count. The renal function was normal, but the lactate dehydrogenase level was high (2,125U/L). Aspartate aminotransferase level was altered (67U/L) and, alanine aminotransferase level was normal (28U/L). Arterial blood gas measurement with 21% fraction of inspired oxygen was as follows: pH=7.48, $PO_{2=}$73.7mmHg, $PCO_{2=}$23.7mmHg, $HCO_{3=}$17.3mmol/L.

The chest X-ray revealed diffuse reticulonodular infiltrate (figure 1). Abdominal ultrasound showed hepatosplenomegaly and thick intrauterine liquid. HIV serology test was negative.

Therapy with antituberculosis drugs, oseltamivir, levofloxacin and dexamethasone were started, but fever and dyspnea persisted with the advent of erythematous macular pruritic skin lesions distributed in the trunk and limbs, compatible with pharmacodermia. The skin biopsy was performed. Anti-tuberculosis drugs were stopped. In the seventh day of hospitalization, the patient evaluated with respiratory and heart failure with refractory septic shock (WBC count= 24,890/mm^3). She was transferred to ICU and submitted to mechanical ventilation. Vasoactive drugs were iniciated as well as ethambutol, moxifloxacin, linezolid, and piperacillin with tazobactam.

Buffy coat and respiratory secretion cultures were performed. In the day 12 of hospitalization, yeast-like structures suggestive of *H. capsulatum* were viewed in the peripheral blood, lately isolated in buffy coat culture (figure 2). Culture and sputum smear for acid fast bacilli were negative. Therapy with deoxycholate amphotericin B was started. The skin biopsy showed nonspecific chronic dermatitis with absence of microorganisms. Other laboratories analyses as: C4 and CH50 complement, rheumatoid factor, anti-neutrophil cytoplasm, antinuclear factor and cryoglobulins were all negatives. A later epidemiologic link to a reform in the patient's home was obtained. After antifungal therapy, the patient developed slow and gradual improvement. She was discharged after 58 days of hospitalization in use of itraconazol 400mg/day, which was maintained for a year with full clinical recovery.

MOLECULAR ASPECTS

DNA of *H. capsulatum* yeast cells was extracted as previously described [14]. The internal transcribe spacer (ITS1-5.8S-ITS2) region of rDNA was amplified by polymerase chain reaction (PCR) using the sense primer ITS5 and the antisense primer ITS4 as previously described [14]. Amplicon was purified using a QIAquick PCR purification Kit (Qiagen AG, Basel, Switzerland). Automated sequencing was done using the Sequencing Platform at Fundação Oswaldo Cruz, PDTIS/FIOCRUZ – Brazil, with the same primers used for PCR amplication [15]. The nucleotide sequence obtained was edited and aligned by Clustal-W [16]

in MEGA 6.0 software [17], using as reference the sequence of the H2 strain from USA (AF322377.1), available on GenBank database. In addition, the consensus sequence of CE1714 isolate was deposited in the GenBank database (KX756764). The sequence of CE1714 isolate was analyzed by BLASTn [18] which revealed that this isolate showed 100% of similarity with *Ajellomyces capsullatus* (anamorph form of *H. capsulatum)* CEMM 05-2-037 strain from Ceará.

To access the relationship among the CE1714 isolate and others *H. capsulatum* isolates from different regions, phylogenetic analysis was performed using 39 isolates retrieved from GenBank database (table 1). The phylogenetic analysis were conducted by maximum likelihood (ML) in Phyml ver. 3.1 [19] and Neighbor Joining (NJ) in MEGA 6.0 [17]. According to results of Bayesian Information (BI) Criterion test [20] implemented in Jmodeltest ver. 2.1.6, Kimura 81 model gamma distribution was the model selected [21]. Bootstrap values (bt) analyses were based on 1000 heuristic search replicates, by estimating alpha of gamma parameter with 4 categories and empiric nucleotide frequency. Nucleotide sequence of *Paracoccidiodes brasiliensis* (AF322389.1) and *Blastomyces dermatitidis* (AF322389.1) strain were used as outgroups (figure 3). The results of this analysis showed that CE1714 isolate has a high genetic similarity with other isolates from Latin America, Mexico and Asia. Moreover, it was possible to identify 3 specific subgroups (bt > 70%) in both analyses: subgroup I (HST1 and HST32 – USA), subgroup II (HST2 and HST31 – USA), and subgroup III (HST3, HST71 and HST8 – Southeast of Brazil).

DISCUSION

This reporter describes the first case of disseminated histoplasmosis in postpartum period, in a HIV-negative woman. Despite this, the phylogenetic analysis demonstrates that the fungal isolate is similar to others *H. capsulatum* clinical isolates identified in patients from Ceará and Latin America.

Disseminated form of histoplasmosis has been described during pregnancy, mainly in HIV-positive women [8, 13]. However, atypical cases of histoplasmosis can also occur during pregnancy such as ocular and meningoencephalites forms [22, 23]. It is well known that during pregnancy there is a state of relative immunosuppression, characterized by decrease of Th1 type response, leading to a Th2 type response that promote fetal antigens tolerance [10, 24, 25]. In addition, local immunoreactivity at the maternal fetal interface also shifts towards Th2 response [10]. In this scenario, the exposure to fungal pathogen could induce more frequently the appearance of disseminated fungal infections [8].

On the other hand, at or near delivery, and in the postpartum period occurs the recovery of Th1 type inflammatory responses, by a broad immune activation, which is identified by elevation of regulatory T cells levels and cytokines in these periods [12, 26]. Some studies have suggested a significantly higher regulatory T cells level (included lymphocytes T CD4+ and CD8+) at delivery, and a decreasing in the shortly postpartum period [27, 28]. These changes can vary conform the delivery type, as well as maternal atopic status, exposure to pets, and number of prior births [12].

Although, the components of immune response during the postpartum period were a complex and controversial event, an intense and exacerbated inflammatory response recovery is associated with the emergence of IRS [10, 13]. Usually, IRS occurs due to a high microorganisms or antigen burden in an unfavorable anatomic location [28]. The diagnosis of IRS is based in the unmasking of occult asymptomatic infection, or the paradoxical worsening of clinical symptoms of an infection in course without other explanation and despite appropriate antimicrobial therapy [29].

Thus, the changes in immune response at or near delivery, and shortly postpartum period can probably trigger the exacerbation of many infections. In the specific case, we do not know if disseminated histoplasmosis occurred due to a transitory immunssupression or to the immune recovery during the postpartum period. IRS-associated histoplasmosis has been observed in AIDS-patients after the highly active antiretroviral therapy initiation [7, 30]. Other infections such as tuberculosis, leprae, cryptococosis, coccidioidomycosis and viral hepatitis have also been associated with IRS in women in the postpartum period [31-36].

Difficulties to differentiate histoplasmosis from tuberculosis have already been observed for other authors [37, 38]. Usually, the initiation of empirical treatment to these two infectious diseases is performed mainly in individuals with severe immunossupression [38], due to in Brazil tuberculosis has a high prevalence (52 cases per 100,000 habitants in 2014) in the general population [39]. However, the prevalence of histoplasmosis is unknown, because this pathology is not of compulsory notification. It is important to highlight the lack of availability of rapid and specific methods, as antigen detection, for the diagnosis of disseminated histoplasmosis in many endemic areas such as Brazil, where the histoplasmosis diagnosis is performed by identification and fungus isolation in cultures of biologic samples. However, this methodology can lead a time up to 4 weeks for fungal identification, and thus, lead to a delay in diagnosis and treatment of this systemic mycosis [40].

In Ceará, histoplasmosis commonly is diagnosed in AIDS-patients with high rates of mortality and relapse [41, 42]. In this case, the patient referred environmental exposure by

remodeling of her house, next to part and in postpartum period. *Histoplasma* microniches are frequently observed in abandoned houses or in civil construction areas. These environmental generally are contaminated with bird dropping or bat guano, and they represent source of *Histoplasma* infection in urban areas [3, 43]. Frequently, acute pulmonary histoplasmosis outbreaks were described in individual's exposure to these places [44, 45].

By ITS region analysis was not possible to identify a specific cluster of *H. capsulatum* from Ceará. Another study performed with *H. capsulatum* isolates from various geographic regions of Brazil also not evidenced one specific genotypic profile for different regions of Brazil based in analysis of this molecular marker [14]. Despite this, some authors have demonstrated the association between *H. capsulatum* clusters and geographic origin [46-48]. In addition, in this study we observed that CE1714 isolate was clearly different of USA strains, but similar genetically to fugal isolates from Latin America, Mexico and Asia. Goldani et al. showed also that fungal isolates obtained of skin lesion of histoplasmosis patients from Rio Grande do Sul (Brazil) had a high genetic similarity with other isolates from Colombia, Argentina and Asia, but they were also different of *H. capsulatum* strain from North America [49].

The ITS1-5.8S-ITS2 region is an excellent DNA-barcode to identified diverse fungal species [50]. However, this genetic marker usually had not evidenced a good intra-specific discrimination between *H. capsulatum* isolates from distinct area [51]. Although, *H. capsulatum* has showed an adaptation capacity to various and different environmental and the migratory flux of bat and individuals with *Histoplasma* infection greatly contribute for genetic diversity of fungal in the world [51, 52]. Thus, to characterize better if there are specifics fungal populations in this area, we believed to be necessary to use another stable molecular marker well characterized, such as microsatellite sequences.

In conclusion, although severe cases of histoplasmosis occur mainly in AIDS-patients in the Ceará State, the health team has to be alert to the occurrence of histoplasmosis in individuals with transitory immunssupression or in those that are in the period of immune recovery as postpartum woman. The evidence of different genetic profile between Latin America strain, including the fungal isolate of this case, and North America strain demonstrate that it's possible there is specifics microniches of *Histoplasma* in this endemic region. Other molecular markers with higher discriminatory power can be helpful to elucidate this issue.

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Table 1. *H. capsulatum* strains and isolates used for ITS region analysis.

Strain/Isolate	Name	Source	Origin	GenBank
HST1	H2	Human/HIV+	USA	AF322377.1
HST2	Downs	Human	USA	AF322378.1
HST3	ES62	Human	ES-Brazil	GU320947.1
HST4	MS53	Human	MS-Brazil	GU320981.1
HST5	GO764	Human	GO-Brazil	GU320955.1
HST6	157CS	Human	RS-Brazil	GU320938.1
HST7	3237	Human	RJ-Brazil	GU320942.1
HST8	9291	Human	RJ-Brazil	GU320940.1
HST9	SP2414	Human	SP-Brazil	GU320951.1
HST10	CE1714	Human	CE-Brazil	KX756764
HST11	CEMM 05-2-072	Human/HIV+	CE-Brazil	JX051637
HST12	CEMM 05-2-039	Human/HIV+	CE-Brazil	JX051639
HST13	CEMM 05-1-098	Human/HIV+	CE-Brazil	JXO51642
HST14	CEMM 05-1-070	Human/HIV+	CE-Brazil	JXO51644
HST15	CEMM 05-1-096	Human/HIV+	CE-Brazil	JXO51643
HST16	CEMM 05-2-001	Human/HIV+	CE-Brazil	JXO51647
HST17	CEMM 05-2-034	Human/HIV+	CE-Brazil	JXO51641
HST18	CEMM 05-2-037	Human/HIV+	CE-Brazil	JXO51634
HST19	CEMM 05-2-002	Human/HIV+	CE-Brazil	JXO51638
HST20	JIEF	Human	CE-Brazil	GU320956.1
HST21	HP12	Human/HIV+	Thailand	AB055240.2
HST22	HP177	Human	China	AB055237.2
HST23	HC28	Human/HIV+	Argentina	KC693532
HST24	HC1	Human/HIV+	Argentina	KC693507
HST25	HC38	Human/HIV+	Argentina	KC693540
HST26	HC47	Human/HIV+	Argentina	KC693548
HST27	H71	Human	Colombia	AF322384.1
HST28	H70	Human/HIV+	Colombia	AF322383.1
HST29	H68	Human	Colombia	AF322382.1
HST30	H62	Human	Colombia	AF322379.1
HST31	IFM41329	Human	USA	AB055228.2
HST32	IFM41659	Human	USA	AB055230.2
HST33	H147	Human	Indonesia	AB055235.2
HST34	HP3	Human/HIV+	Thailand	AB055238.2
HST35	H143		South Africa	AB055246.2
HST36	H147	Human	Senegal	AB055247.2
HST37	H90	Horse	Egypt	AF322387.1
HST38	H95	Horse	Egypt	AB055249.1
HST39	EH383		Mexico	KP132275.1
HST40	EH374		Mexico	KP132271.1
Paracoccidioidis brasiliensis	Outgroup	----	----	AF322389.1
Blastomyces dermatitidis	Outgroup	----	----	AF322388.1

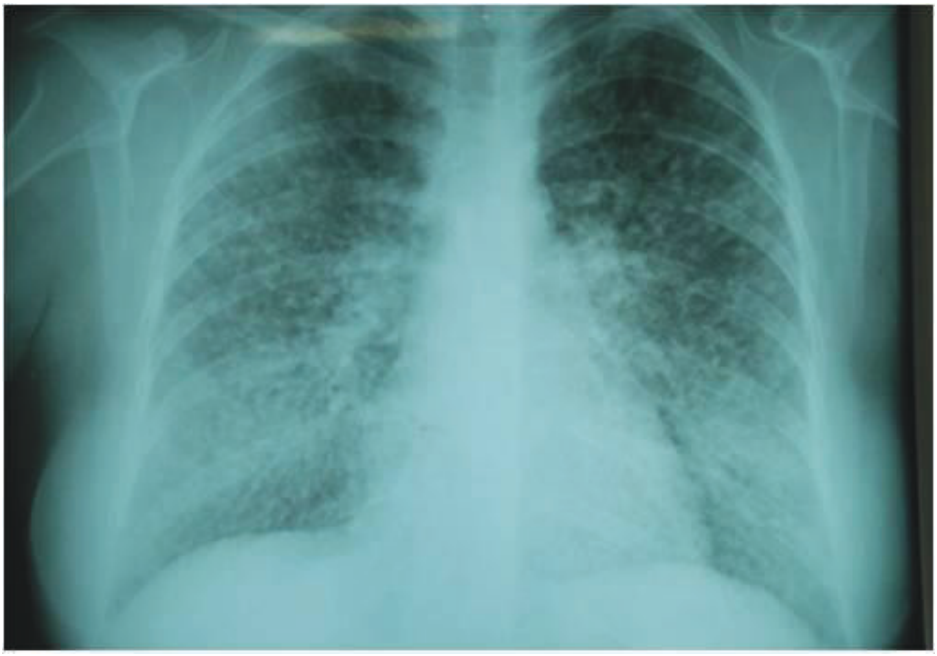

Figure 1: Chest X-ray shown a difuse reticulonodular infiltrate

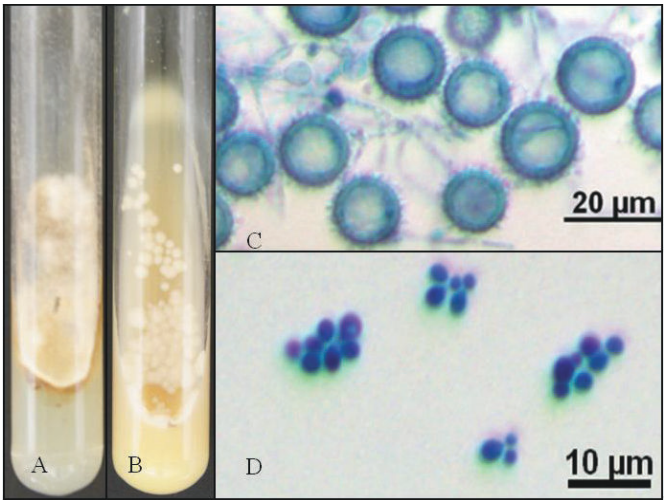

Figure 2: Representative morphology of CE1714 isolate. (A) and (B) Buffy coat culture of *H. capsulatum* mycelium and yeast phase in agar dextrose potate and ML-Gema medium, respectively; (C) and (D) Micromorphology of *H. capsulatum* mycelium and yeast phase stain by cotton blue lactophenol, respectively. These characteristics were observed by optical microscopy at 40X magnification.

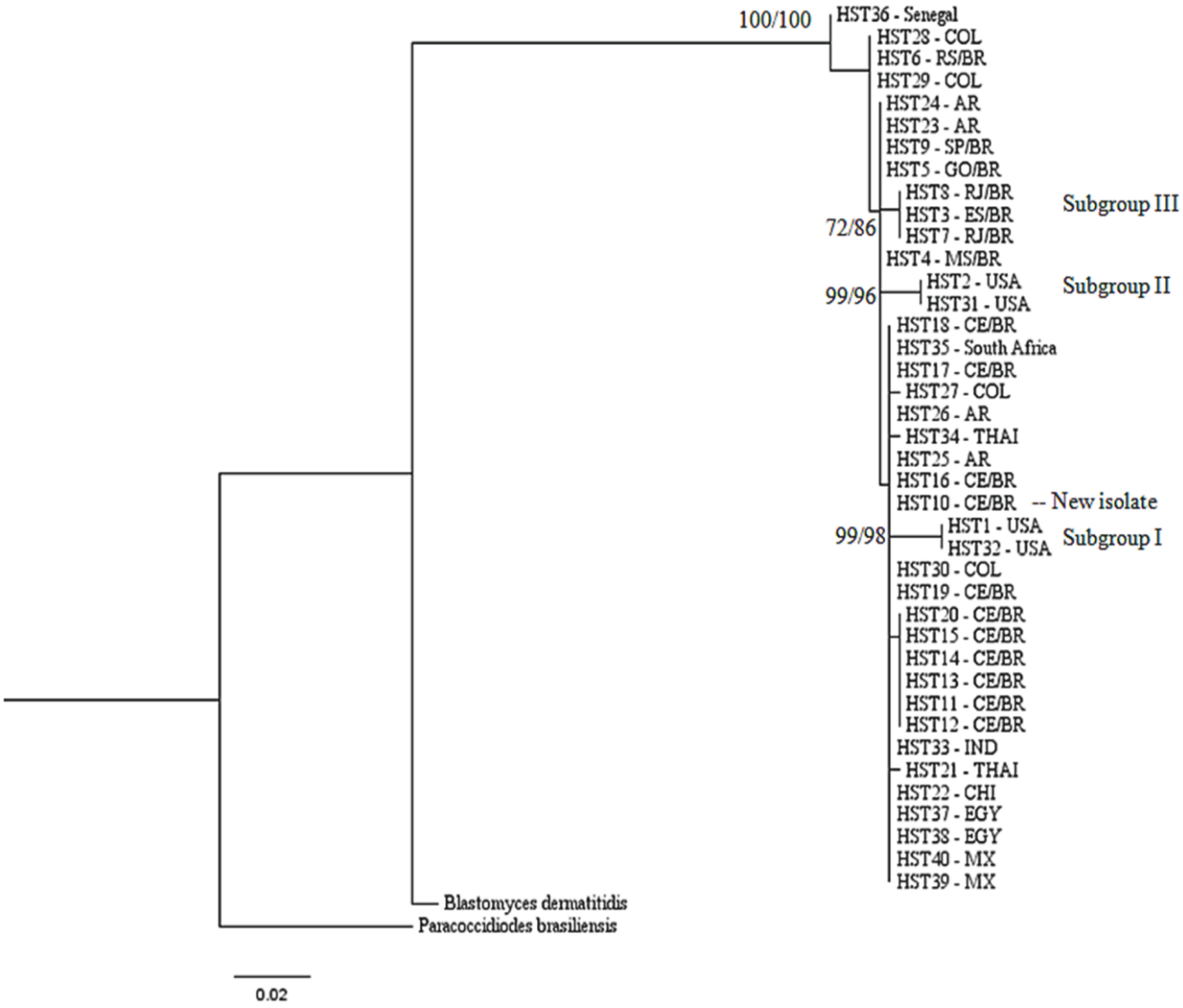

Figure 3: Phylogenetic tree of *H. capsulatum* isolates. The tree was performed using ITS1-5.8S-ITS2 region with 39 fungal sequence retrieved by GenBank and the new sequence of CE1714 isolate. *P. brasiliensis* (AF322389.1) and *B. dermatitidis* (AF322389.1) were considered outgroup. It was generated by BI and is representative of both ML and NJ analyses. The values of bt/bt are indicated on their corresponding tree nodes, respectively.
AR=Argentina; BR=Brazil; CE=Ceará; CHI=China; COL=Colombia; EGY=Egypt; ES=Espírito Santo; GO=Goiás; RJ=Rio de Janeiro; IND=Indonesia; SP=São Paulo; THAI=Thailand.

4.6 Capítulo 6

A restrict genetic population of *Histoplasma capsulatum* clinical isolates identified in Ceará/Brazil by (GA)n microsatellite typing

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Manuscrito em revisão para submissão na revista Genetics, Infection and Evolution

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Histoplasma capsulatum é um fungo dimórfico que apresenta uma ampla diversidade genética entre isolados de diversas áreas geográficas. No nordeste do Brasil, o Estado do Ceará tem se destacado por descrever uma frequência elevada de casos de histoplasmose disseminada (HD), principalmente em pacientes com aids. Neste estudo, isolados fúngicos recuperados de diversas amostras biológicas de pacientes com HD, foram tipados através de amplificação e sequenciamento do microsatélite (GA)n. Cinquenta e um isolados clínicos de *H. capsulatum* obtidos de 40 pacientes foram avaliados. Análise filogenética identificou uma população fúngica genética restrita ao Ceará/Brasil, diferentemente dos outros isolados do estudo, procedentes do México, Argentina, São Paulo/Brasil e dos EUA (cepa de referência). Além disso, 11 haplótipos foram identificados na população fúngica do estudo, sendo quatro pertencentes ao grupo de isolados do Ceará. Vale ressaltar, a identificação de dois haplótipos inéditos nesta população. Portanto, isolados de *H. capsulatum* procedentes desta região do Brasil constituem um grupo restrito, havendo alta variabilidade genética nesta população.

A restrict genetic population of *Histoplasma capsulatum* clinical isolates identified in Ceará/Brazil by (GA)n microsatellite typing

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Abstract

Histoplasma capsulatum is a dimorphic fungus that presents a broad genetic diversity among isolates from diverse geographic areas. In northeastern of Brazil, Ceará State has highlighted due to large frequence of cases of disseminated histoplasmosis (DH) mainly in AIDS-patients. In the present study, fungal isolates recovered from various biologic samples of DH-patients were typed by (GA)n microsatellite polymerase chain reaction. Fifty-one clinical isolates of *H. capsulatum* from 40 individuals were evaluated. The phylogenetic analysis of fungal isolates identified one restrict genetic population in Ceará/Brazil different of strains from Mexico, Argentina, Sao Paulo/Brazil, and G-217B reference strain from US. A total of 11 differents haplotypes were observed in the overall fungal population of this study. Four distinct haplotypes were presented in the fungal isolates from Ceará. It also highlithed 2 newsinedit haplotypes (GA)n sequence lenght in that population. *H. capsulatum* isolates constituted a confine cluster in this region of Brazil, however, there are some genetic variability in the same population.

Key words: (GA)n microsatellite, genetic diversity, *Histoplasma capsulatum*, histoplasmosis.

Introduction

Histoplasmosis is the most frequent systemic mycosis in the Americas. Asymptomatic or sub-clinical infections are the most common clinical form. However, severe and disseminated diseases usually occur in immunossuprimed individuals (Wheat, et al., 2016). In northeastern of Brazil, Ceará State has described a great number of cases of disseminated histoplasmosis mainly in AIDS-patients (Brilhante, et al., 2012; Dasmasceno, et al., 2013). *Histoplasma capsulatum* is a dimorphic fungus that presents a high genetic diversity among isolates from diverse geographic areas. Up to date, 17 different phylogenetic species of *H. capsulatum* were identified by multilocus sequence typing of 4 genes nuclear (*arf*, *H-anti*, *ole1*, *tub1*), wich are distributed worldwide (Kasuga, et al., 2003; Teixeira et al., 2016). In addition, others molecular techniques have also described a large genetic variability of this pathogen from a same geographic area (Damasceno, Leitao, Taylor, Muniz, & Zancope-Oliveira, 2016). The HSP-TC microsatellite, which contains the (GA)n repetitive sequence linked to HSP 60 (heat shok protein-60) have been used to separate *H. capsulatum* isolates from different regions as North America and Latin America (Carter, et al., 2001; Taylor, et al., 2012). In the present study, multiple isolates recovered from various biologic samples of histoplasmosis patients were identified by amplification of ITS1-5.8S-ITS2 region and typed by (GA)n microsatellite marker polymerase chain reaction (PCR).

Material and Methods

Fungal isolates and Patients

Fifty-one clinical isolates of *H. capsulatum* were obtained from 40 patients with histoplamosis disseminated hospitalized at Hospital São José de Doenças Infecciosas (HSJ). This hospital is a healthcare institution reference in infectious diseases in Ceará State. The laboratorial data (as type of biologic sample, year of isolation, and HIV status) were obtained by review medical registers. This study was approved by Research Ethical Committee at Fundação Oswaldo Cruz/FIOCRUZ (No 19342513.2.0000.5262).

DNA extraction

All fungal isolates were primarily identified according to standard morphologic criteria in culture in Potato Dextrose Agar at 25ºC and their ability to grow at 37 °C in ML-Gema (Fressatti, Dias-Siqueira, Svidzinski, Herrero, & Kemmelmeier, 1992). Genomic DNA of *H. capsulatum* yeast-phase was extracted as described previously (Muniz, Morais, Meyer, Nosanchuk, & Zancope-Oliveira, 2010).

ITS1-5.8S-ITS2 (ITS) region PCR

ITS region PCR was performed to confirm *H. capsulatum* specie. It was performed using specific primers: ITS5 (5'-TCCGTAGGTGAACCTGCG G-3') and ITS4 (5'-TCCTCCGCTTATTGATATG-3') in a 25-µl reaction mixture, containing 200µM each deoxynucleoside triphosphate (dNTP) (Applied Biosystems Inc., Foster City, CA, USA), 2.0 mM $MgCl_2$, 50ng of each primer, 1.0 U *Taq* DNA polymerase (New England BioLabs Inc., MA, USA), 1 X *Taq* commercial buffer, and 20 ng/µl of each DNA sample. The H2 strain from USA (AF322377.1) was used as positive control.

PCR assays were performed in a Thermal iCycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) programmed as follows: (a) 3 min at 95°C; (b) 32 cycles, consisting of 15 sec at 94°C, 30 sec at 65ºC in the first cycle, which was subsequently reduced by 0.7ºC/cycle for next 12 cycles, and 1 min at 72ºC. The remaining 20 cycles, the annealing temperature was continued at 56ºC; (c) a final extension cycle of 5 min at 72ºC (Kasuga, Taylor, & White, 1999). Amplicons were resolved by 1.5% agarose gel electrophoresis. The 100-bp DNA ladder (Invitrogen) was used as a molecular marker. The amplicons were purified using a QIAquick PCR purification Kit (Qiagen AG, Basel, Switzerland). Automated sequencing was done using the Sequencing Platform at the High-Throughput Genomics Center (University of Washington, Seattle, WA, USA). The sequences were analyzed by BLASTn (Altschul, Gish, Miller, Myers, & Lipman, 1990) in GenBank database (http://www.ncbi.nlm.nih.gov).

(GA)n microsatellite PCR and DNA Sequencing

The HSP-TC microsatellite PCR was performed using specific primers: HSP-TC/U (5'-GACGACGAGTGGTTCCCGAA-3') and HSP-TC/L (5'-GAAGCCCTGGAGGTAGACGA-3') in a 25-µl reaction mixture, containing 200µM each dNTP (Applied Biosystems Inc., Foster City, CA, USA), 2.0 mM $MgCl_2$, 100pmol/ µl of each primer, 1.0 U *Taq* DNA polymerase (New England BioLabs Inc., MA, USA), 1 X *Taq* commercial buffer, and 20 ng/µl of each DNA sample. The G-217B from USA reference strain was used as positive control. PCR assays were performed in a Thermal iCycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) programmed as follows: (a) 94°C for 5 min; (b) 30 times at 94°C for 1 min, and (c) 72°C for 1 min, with a final extension cycle of 5 min at 72ºC (Carter, et al., 1997; Taylor, et al., 2012). Amplicons were resolved by 1.5% agarose gel electrophoresis. The 100-bp DNA ladder (Invitrogen) was used as a molecular marker. The amplicons were purified using a QIAquick PCR purification Kit (Qiagen AG, Basel, Switzerland). Automated sequencing was done using the Sequencing Platform at Fundação Oswaldo Cruz, PDTIS/FIOCRUZ, Brazil (Otto, et al., 2008).

Phylogenetic Analysis

The amplified products by (GA)n PCR were edited and aligned with G-217B reference strain using Clustal-W (Thompson, Higgins, & Gibson, 1994) in MEGA 6.06 software (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The alignment was performed for 304 nucleotides (nt), from sites 1971 to 2274, which contain the (GA)n microsatellite and its flaking regions. All the nucleotide sequences obtained in this study were deposited in GenBank database. Phylogenetic analysis was performed using 8 fungal strains [EH-53 (GQ223268.1), EH-374 (GQ223271.1), EH-655P (GQ180985.1), EH-384P (GQ180982.1), EH-575B (GQ131797.1), 01869 (GQ131798.1), 156-05 (FJ977625.1), and 376-04 (FJ977619.1)] retrieved from GenBank database (http://www.ncbi.nlm.nih.gov). Nucleotide sequence of G-217B strain (L11390.2) was used as outgroup. The phylogenetic analysis were conducted by maximum likelihood (ML) in Phyml ver. 3.1 (Guindon, et al., 2010) and Neighbor Joining (NJ) in MEGA 6.06 (Tamura, et al., 2013). According to results of Bayesian Information (BI) Criterion test (Schwarz, 1978) implemented in Jmodeltest ver. 2.1.6, HKY+G (*Hasegawa-Kishino-Yano*) and TNr (*Tamura Nei*) substitution model gamma distribution was the model selected (Darriba, Taboada, Doallo, & Posada, 2012), respectively for ML and NJ. Bootstrap values (bt) analyses were based on 1,000 heuristic search replicates.

Nucleotide diversity and haplotypes

Estimation of nucleotide diversity of fungal isolates from Ceará of the 304-nt fragment containing the (GA)n microsatellite and its flaking region was performed using the DnaSP 5.10 (Rozas & Rozas, 1995). Haplotypes were generated using all sequences of fungal isolates by Network 5.0 (http://www.fluxus-engineering.com). A map was constructed using the haplotypes identified with sequences length evolution.

Results

Fifty-one fungal isolates were obtained of 40 patients with disseminated histoplamosis from HSJ in the period 2011-2014. Thirty-nine patients were HIV positive and only one case occurred in immunocompetent patient (CE1714). Forty-one fungal isolates were from residents of areas with humid tropical climate. However, 10 strains occurred in individuals that living in regions with semi-arid tropical climate. The biologic samples were obtained from buffy coat, bone marrow, blood and trachea-bronchial secretion (table 1).

All fungal isolates were identified as *H. capsulatum* by amplification and sequencing of ITS region by BLASTn analysis (Altschul, et al., 1990), which revealed that the isolates had 96-100% of similarity with *Ajellomyces capsullatus* (anamorph form of *H. capsulatum)* H2 reference strain. The accession numbers were deposited in GenBank (table 1).

Phylogentic analysis by both methods, ML and NJ, of (GA)n microsatellite identified three majors clusters: cluster I (bt=63/73) represented for isolates from Argentina (bat and human isolates) and São Paulo/Brazil (bat isolates); cluster II (bt=100/92) represented for bat strains from Mexico; and cluster III (bt=93/94) which contained two sub-cluster – IIIa (bt=92/87) represented for isolates from Mexico (bat and human isolates) and IIIb (bt=93/97) represented for isolates from Ceará/Brazil. In addition, it's possible to observe two subgroups in the fungal population of study (figure 1). The subgroup I (bt=65/62) was represented for 10 sequences and subgroup II (bt=87/86) for 5 sequences (table 2).

Eleven haplotypes was identified in the fungal sequences of this study (table 2). On base of G-217B reference strain, the nucleotide substitutions in the microsatellite sequences length from Ceará show that a guanine by cytosine at position 2031, and an adenine by guanine at position 2032 were observed in the haplotype 8 and 9, similar to haplotype 2 that contain strains from Mexico. However, in the haplotype 10 and 11, the point of mutation was observed at position 2039 (guanine or timine by cytosine) and 2040 (adenine by guanine). The map of haplotypes shows the dispersion of genetic population this research (figure 2). The overall nucleotide diversity of genetic population from Ceará was of 0.00585. The subgroup II ($\pi = 0.02590$) had major nucleotide diversity than subgroup I ($\pi = 0.00000$).

Discussion

This is a first study to demonstrate that there is a specific cluster of *H. capsulatum* circulating in Ceará State/Brazil by microsatellites typing. In addition, a great genetic variability was identified in this fungal population based in four different haplotypes.

Distinct genetic populations of *H. capsulatum* from different geographic regions have been observed by biallelic and multiallelic microsatellites analysis (Carter, et al., 1997; Carter, Burt, Taylor, Koenig, & White, 1996; Carter, et al., 2001; McEwen, et al., 2000). In other fungal species, the microsatellites typing have also been used successfully in studies about genetic diversity and population structures, due to a high discriminatory power (Li, et al., 2015; Vanhee, Symoens, Nelis, & Coenye, 2008).

Analysis based in a unique microsatellite marker has found distinct clusters according with geographic region and source of their isolations (Naumova, Naumov, Michailova, Martynenko, & Masneuf-Pomarede, 2011; Taylor, et al., 2012). Typing of (GA)n microsatellites of 47 *H. capsulatum* isolates from infected bats captured in Mexico, Brazil and Argentina demonstrated by phylogenetic analysis that all fungal isolates were grouped according with geographic origin or bat migration behavior of animals. In addition, these

authors also observed that fungal isolates from Mexico, Brazil and Argentina were clearly different of G-217B strain from USA (Taylor, et al., 2012).

In this study, we identified a genetic population of *H. capsulatum* obtained of patients from Ceará noticeably different of other fungal isolates from Argentina, São Paulo/Brazil, Mexico and G-217B reference strain from USA. Despite this, two haplotypes identified in the genetic population from Ceará, were similar the (GA)n sequences length of subcluster IIIa from Mexico. In addition, it is important highlight that were observed two news (GA)n sequence length, represented for GACG$(GA)_5$GA and GATG$(GA)_5$GA, restrict in this geographic area of Brazil. This observation is especially important to characterize the circulating genetic profile of *H. capsulatum* in this region of northeastern of Brazil, because the genetic variability probably is associated with the pathogenesis of histoplasmosis.

Epidemiologic and molecular studies about histoplasmosis have identified different clinical manifestations and outcomes of this mycosis in individuals from different endemic area of histoplamosis. Skin lesions, disseminated form, high mortality and relapse rate are more frequent in histoplasmosis individuals from Latin America than North America (Dasmasceno, et al., 2013; Durkin, et al., 2004; Karimi, et al., 2002).

Sepulveda et al. (2014) also observed differences in fungal burden, disease kinetics, symptomology, and cytokine responses when studied strains of different phylogenetics groups from USA (G-217B – Nam 1 clade) and Panama (G186-AR – linage) by experimental study. These authors conclude that there is a sophisticated relationship between host and fungus that drives the development and progression of histoplasmosis (Sepulveda, Williams, & Goldman, 2014). Thus, more studies about molecular epidemiology need be realized to clarify better these issues.

Moreover, Ceará State is a geographic region characterized for to present diverse environmental conditions. There is a great area with the semi-arid tropical climate, which present temperature extremely hot and low precipitation indices (IPECE, 2016). Autochthones cases of histoplasmosis in human and in selvage animal also were described in environmental and atypical climates as Canada and Patagonia (Burek-Huntington, Gill, & Bradway, 2014; Calanni, et al., 2013). Thus, it demonstrates a high capacity of adaptation and reproduction of this fungus in different climates and microniches.

Conclusion

Although, we can not affirm that all patients acquired *H. capsulatum* primary infection in this region, the phylogenetic analysis and haplotypes of (GA)n microsatellite helped to clarify and to understand better the dispersion of fungal in this region of Brazil.

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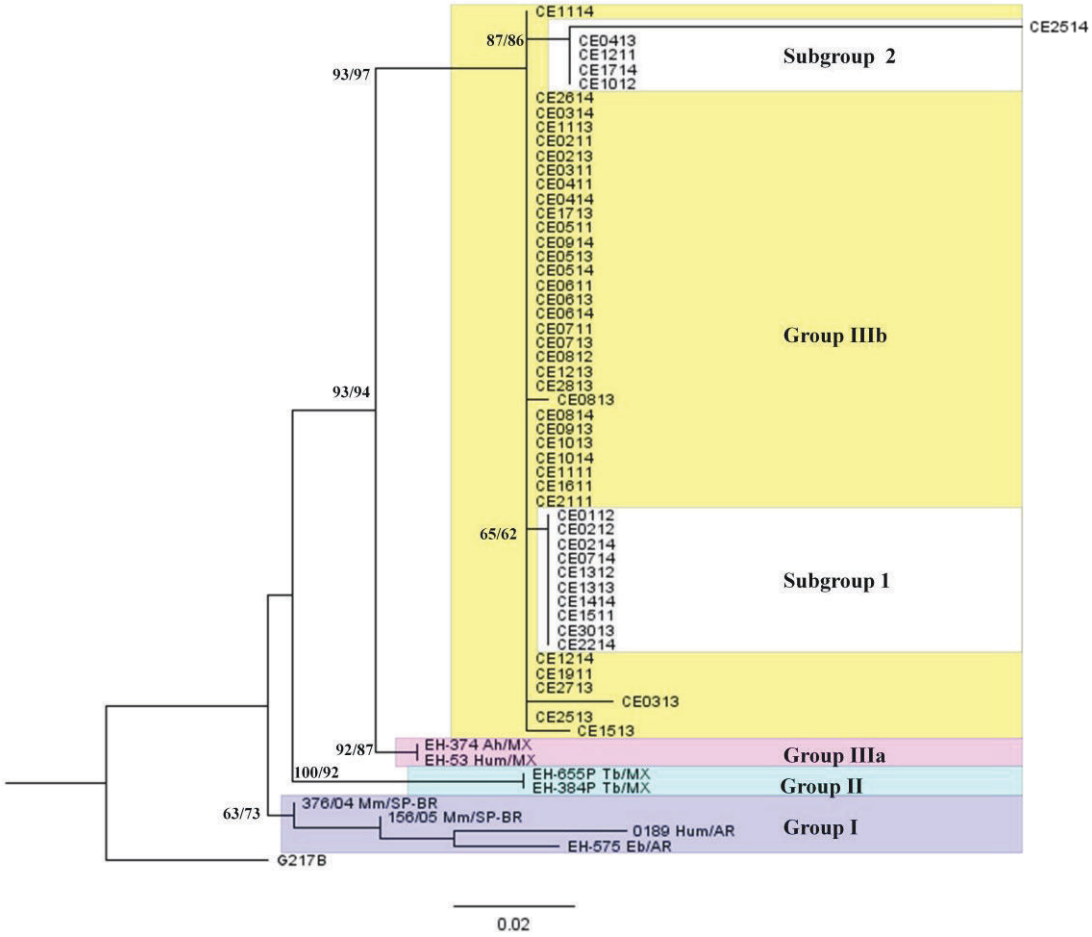

Figure 1: Phylogenetic tree of *H. capsulatum* isolates based in the sequence (GA)n microsatellite and its flaking region considering 304-nt. The tree was performed with 51 sequences of fungal isolates from this study and 8 strain retrieved by GenBank. G-217B reference strain (L11390.2) was used as outgroup. The ML tree was generated using HKY+G model, and NJ tree was generated using TNr model as detailed in the Material and Methods section. The values of bt/bt are indicated on their corresponding tree nodes, respectively. AR = Argentina, BR = Brazil, CE = Ceará, SP = São Paulo, MX = Mexico, Hum = human, Eb = *Eumops bonariensis*, Ah = *Artibetus hirsutus*, Mm = *Molossus molossus*, Tb = *Tadarida brasiliensis*.

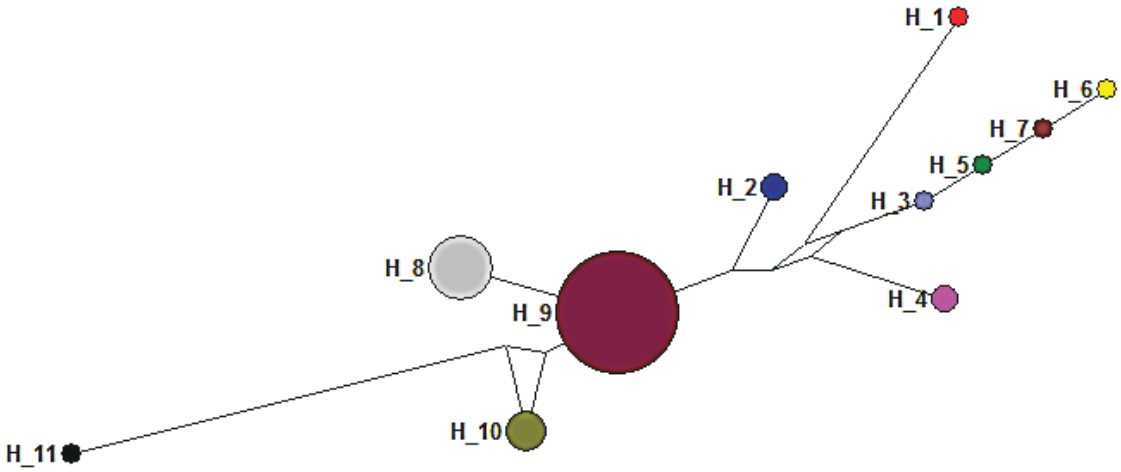

Figure 2: Haplotypes distribution of *H. capsulatum* isolates associated with (GA)n microsatellite and its flaking region haplotypes based in 304-nt. This map was constructed in Network 5.0 and the genetic distance was represented by each depicted node.

Table 1. Characteristics of *H. capsulatum* isolates from Ceará used in this study.

Isolates	Source of sample	Year of Isolation	HIV status	(GA)n microsatellite (Acession number)
CE 0211	Buffy coat	2011	HIV +	KX756753
CE 1911	Blood	2011	HIV +	KX756721
CE 0311	Buffy coat	2011	HIV +	KX756749
CE 0411	Bone marrow	2011	HIV +	KX756748
CE 0511	Buffy coat	2011	HIV +	KX756762
CE 0611	Blood	2010	HIV +	KX756741
CE 0711	Buffy coat	2011	HIV +	KX756732
CE 1111	Bone marrow	2010	HIV +	KX756759
CE 1211	Buffy coat	2011	HIV +	KX756725
CE 1511	Buffy coat	2010	HIV +	KX756724
CE 1611	Buffy coat	2011	HIV +	KX756723
CE 2111	Blood	2011	HIV +	KX756720
CE 0112	Blood	2012	HIV +	KX756754
CE 0212	Buffy coat	2012	HIV +	KX756752
CE 0812	Buffy coat	2012	HIV +	KX756738
CE 1012	Buffy coat	2012	HIV +	KX756758
CE 1312	Buffy coat	2012	HIV +	KX756728
CE 0913	Blood	2013	HIV +	KX756735
CE 0213	Buffy coat	2013	HIV +	KX756751
CE 0313	Buffy coat	2013	HIV +	KX756763
CE 0713	Bone marrow	2013	HIV +	KX756740
CE 1013	Buffy coat	2013	HIV +	KX756734
CE 2713	Buffy coat	2013	HIV +	KX756716
CE 0413	Blood	2013	HIV +	KX756757
CE 0513	Buffy coat	2013	HIV +	KX756746
CE 0914	Blood	2013	HIV +	KX756761
CE 0814	Buffy coat	2013	HIV +	KX756736
CE 0613	Buffy coat	2013	HIV +	KX756743
CE 0813	Bone marrow	2013	HIV +	KX756737
CE 1113	Bone marrow	2013	HIV +	KX756731
CE 1513	Buffy coat	2013	HIV +	KX756724
CE 1213	Buffy coat	2013	HIV +	KX756730
CE 1313	Buffy coat	2013	HIV +	KX756727
CE 0414	Buffy coat	2013	HIV +	KX756747
CE 1414	Blood	2013	HIV +	KX756726
CE 1713	Buffy coat	2013	HIV +	KX756722
CE 2513	Buffy coat	2013	HIV +	KX756718
CE 2813	Buffy coat	2013	HIV +	KX756715
CE 3013	Buffy coat	2013	HIV +	KX756714
CE 0214	Blood	2013	HIV +	KX756750
CE 0314	Buffy coat	2012	HIV +	KX756713
CE 0514	Buffy coat	2013	HIV +	KX756745
CE 0614	Blood	2013	HIV +	KX756742
CE 1014	Bone marrow	2013	HIV +	KX756733
CE 0714	Buffy coat	2013	HIV +	KX756739
CE 1114	Blood	2013	HIV +	KX756755
CE 1214	Blood	2013	HIV +	KX756729
CE 1714	Blood	2014	HIV -	KX756756
CE 2214	Buffy coat	2014	HIV +	KX756719
CE 2514	Trachea- bronchial secretion	2014	HIV +	KX756760
CE 2614	Blood	2014	HIV +	KX756717

Table 2. Allocation of cluster according with haplotypes and sequence length of (GA)n of *H. caspulatum* strains/isolates used in this study.

Haplotypes	Nº	Strains/Isolates	Group	(GA)n length haplotypes	Origin
H-1	1	G-217B	Outgroup	GAGAGAGA$(GA)_{10}$GA	USA
H-2	2	EH-53, EH-374	IIIa	GACG$(GA)_7$GA	Mexico
H-3	1	376-04	I	GAGG$(GA)_6$GA	SP-Brazil
H-4	2	EH-655P, EH-384P	II	GACG$(GA)_{11}$GA	Mexico
H-5	1	156-05	I	GAGG$(GA)_6$GA	SP-Brazil
H-6	1	01869	I	GAGG$(GA)_6$AA	Argentina
H-7	1	EH-575B	I	GAGG$(GA)_6$AA	Argentina
H-8	10	CE3013, CE2214, CE1511, CE1414, CE1313, CE1312, CE0714, CE0214, CE0212, CE0112	IIIb (Subgroup-1)	GACG$(GA)_7$GA	CE-Brazil
H-9	36	CE2513, CE2614, CE2713, CE2813, CE0314, CE0914, CE0313, CE0511, CE2111, CE1911, CE1713, CE1611, CE1513, CE1214, CE1213, CE1113, CE1111, CE1014, CE1013, CE0913, CE0814, CE0813, CE0812, CE0713, CE0711, CE0614, CE0613, CE0611, CE0514, CE0513, CE0414, CE0411, CE0311, CE0213, CE0211, CE1114	IIIb	GACG$(GA)_7$GA	CE-Brazil
H-10	4	CE0413, CE1012, CE1211, CE1714	IIIb (Subgroup-2)	GACG$(GA)_5$GA	CE-Brazil
H-11	1	CE2514	IIIb (Subgroup-2)	GATG$(GA)_5$GA	CE-Brazil

SP = São Paulo; CE = Ceará.

4.7 Capítulo 7

Emerging clinical genotypes of *Histoplasma capsulatum* in Northeast Brazil

Manuscrito em elaboração

Histoplasmose é uma micose sistêmica causada por várias espécies crípticas dentro do complexo *Histoplasma capsulatum*. O nordeste do Brasil, especialmente o estado do Ceará, é uma área endêmica de histoplasmose. Esta região tem contribuindo com uma grande frequência de casos desta micose anualmente. O objetivo deste estudo foi caracterizar genotipicamente os isolados clínicos de *H. capsulatum* obtidos de pacientes com histoplasmose oriundos do Ceará/Brasil. Além disso, também foi investigada a correlação entre as populações genéticas de *H. capsulatum* do nordeste do Brasil, e os dados clínicos e fenotípicos obtidos desses isolados fúngicos. DNA's de 51 isolados fúngicos foram recuperados de várias amostras biológicas de pacientes com histoplasmose. Amplificação parcial através de reação em cadeia da polimerase (PCR), e sequenciamento de quatro genes foram realizados. Avaliação por meio da análise do sequenciamento multilocus e análise bayesiana de estrutura de populações detectaram a presença de uma nova população genética de *H. capsulatum* no nordeste do Brasil. Além disso, três subpopulações foram identificadas: cluster I – constituído por 35 isolados do Ceará, três isolados do Rio de Janeiro (84476, 84564 e 84502) e o isolado H151 de São Paulo; cluster II – formado por 16 do Ceará e o isolado H146 de região desconhecida do Brasil; e, cluster III – constituído por JIEF (Ceará) e dois isolados de Pernambuco (RE 9463 e RE 5646). Isolados de *H. capsulatum* foram geneticamente diferentes de outras populações genéticas do Brasil e outros países da América Latina, demonstrando haver uma alta diversidade genética deste patógeno.

Emerging clinical genotypes of *Histoplasma capsulatum* in Northeast Brazil

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Abstract

Histoplasmosis is a systemic mycosis caused by several cryptic species nested within *Histoplasma capsulatum complex.* The Northeast of Brazil, specially the Ceará state, is an endemic area of histoplasmosis, and this region has contributed with several cases annually. The aim of this study was to characterize genotypically the clinical isolates of *H. capsulatum* obtained from patients with histoplasmosis from Ceará/Brazil. In addition, the co-relation between the identified *H. capsulatum* populations in Northeast with clinical and phenotypic data obtained on theses fungal isolates was also investigated. Fifty-one fungal isolates DNA's were recovered from various biologic samples of histoplasmosis patients. Partial amplification by polymerase chain reaction (PCR) and sequencing of 4 nuclear gens (arf, ole1, H-anti, and tub1) were performed. Evaluation by multi locus sequencing type (MLST) analysis and Bayesian Analysis of Population Structure (BAPS) detected the presence of a new genetic population of *H. capsulatum* isolates in Northeast of Brazil. In addition, tree subpopulations in the Northeast group were identified: cluster I – constituted for 35 fungal isolates from Ceara, 3 *H. capsulatum* isolates from Rio de Janeiro (84476, 84564 and 84502) and H151 from São Paulo; cluster II – formed for 16 clinical isolates from Ceara and H146 from Brazil; and, cluster III – constituted for JIEF (Ceara) and 2 fungal isolates from Pernambuco (RE 9463 and RE 5646). *H. capsulatum* clinical isolates from Ceará were genetically different of other genetic population from Brazil and Latin America what demonstrates a high genetic diversity this pathogen.

Key words. *Histoplasma capsulatum;* multilocus sequence typing; histoplasmosis.

Introduction

Histoplasmosis is a systemic mycosis caused by several cryptic species nested within *Histoplasma capsulatum complex. Histoplasma* is a dimorphic fungus that developed in the environment or *in vitro* at 25-28ºC under mycelia (M) phase. The yeast (Y) phase occurs after an infection in susceptible hosts or by cultivating the fungus at 34-37ºC. The M-phase of the fungus is developed essentially in settings that have a high concentrations of nitrogen and phosphorus, humidity > 60%, darkness and around watercourses (1). *Histoplasma* infections have been reported in all continents with exception of Antarctica. The disease is predominantly distributed in tropical and subtropical areas of the Americas and the disease range keep expanding since this mycosis has also been found in unusual regions of Canada and in the Patagonia desert in Argentina between 57º North and 38º South (2, 3).

This pathogen is also able to cause disease in different animals, as bats, cats, birds and selvage animal (1, 3, 4). In humans, histoplasmosis outbreaks have been described mainly in individuals linked to exploratory activities such visiting caves and archeological sites or even working on constructions for example. These environments are usually highly loaded with bat guano or bird droppings (5).

Despite this, in the last years, an increase of disseminated histoplamosis has been reported, mainly associated in AIDS-patients throughout Americas (6, 7). It's very important highlight that the rate of clinical events such as skin lesion, mortality and relapse this mycosis varies in individuals from distict geographic area of the disease (8, 9). In addition, experimental studies have demonstrated that the virulence of pathogen may be associated to different genetic lineages of *H. capsulatum* (10).

Regardless this fungus has been erroneously classified by biological, clinical, and geographical aspects into three varieties (*H. capsulatum* var. *capsulatum*, *H. capsulatum* var. *duboisii*, and *H. capsulatum* var. *farciminosum*), a broad genetic variability has been found in

H. capsulatum by using molecular typing techniques (11). Actually, multilocus sequence typing (MLST) is the main molecular test used to evaluate at species level the genetic diversity of *H. capsulatum* (11). The first studies were performed by Kasuga et al. that found 8 phylogenetic clades worldwide distributed (12, 13). LAm A and LAm B clades harbors isolates originated mainly Latin America; Nam 1 and Nam 2 clades from North America; Africa clade restrict to Africa; Euroasian clade constituted from fungal strains from Egypt, India, China, Thailand, and England; Netherlands clade; and, Australian clade (13). Seven of eight clades represented phylogenetic species geographically isolated. The Eurasian clade is a monophyletic clade that emerged from the LAm A phylogenetic species. However, a more actual and robust study performed with 234 *H. capsultum* strain identified a more complex phylogenetic distribution of of *H. capsulatum*. The former LAm A and LAm B species were splitted into four different genetic cluster as follows: LAm A1, LAm A2, LAm B1 and LAm B2. Two new phylogenetic species, RJ (Southeast of Brazil) and BAC-1 (Mexico), and four different monophyletic clades from Brazil (BR1-4) were also identified (14).

Brazil contributes to one of the highest incidences of histoplasmosis in the globe as well represents the greatest genetic variability of the *Histoplasma* and the potential center of propagation of this important pathogen (14, 15). However, the real incidence of this mycosis in Brazil is unknown, because it is not notifiable disease. Is estimated that 2,19 individuals had histoplasmosis diagnosis each 1,000 hospitalizations in Brazil (16).

Studies performed by histoplasmin skin-test, between 1950 and 1990 years, have found diverse prevalence of histoplasmosis in Brazil (17). The higher prevalence rate was observed in the Southeast of Brazil (93.2%) (17, 18). In Ceará, Northeast of Brazil, the prevalence rates varied from 23.6% to 61.5% of *Histoplasma* infection in individual's residents in rural area (19, 20). However, among HIV-positive individuals without severe immunossupression (lymphocyte T CD4+ > 350 cels/mm3), the histoplasmosis prevalence reached 11.8% (21). In

the last three decades, the Ceará State have experienced a large number of cases of disseminated histoplasmosis (DH) described mainly in AIDS-patients (8). Between 1995 – 2004 years, De Franchesco Daher et al. (2006) identified 164 cases of co-infection of DH and AIDS in a single hospital of infectious disease (22). Moreover, Pontes et al. (2010) identified 134 cases in a 7 year medical surveillance (23); Brilhante et al. (2012) found 208 cases in 5 years (7) and Damasceno et al. (2013) identified 264 cases in 7 years (8) in the same state. These studies show that the Northeast of Brazil, specially the Ceará state, is an endemic area of histoplasmosis contributing for several cases annually. In Fortaleza (capital of Ceará), the major frequency of cases of histosplamosis has been found in regions with low sanitation capacity, and in preserved area often linked ecotourism and fishing (24).

In the other hand, pacoccidioidomycosis is the more frequent systemic mycosis in Brazil (25, 26), and the major mortality rate is found in South Brazil (6.9%) (26). However, in Northeast of Brazil, deaths for paracoccidioidomycosis are uncommon, especially in Ceará (1.8%) (26). In Brazilian AIDS-patients, cryptococosis, candidiasis and histoplasmosis are the most frequent mycosis that causes death in these individuals. In addition, Ceará is the main responsible region of deaths due histoplasmosis in Brazil (25).

Although, the highly active antiretroviral therapy has modified the course of AIDS, a high mortality associated of histoplasmosis was observed in this region of Brazil (33-42%) (7, 8), contrarily other endemic regions such as Panama (12.5%) (27) and French Guiana (8%) (25, 28). In this study, the Ceará state was the main responsible area of deaths due histoplasmosis in Brazil. Up to date, any molecular epidemiological study about the genetic distribution of *Histoplasma* in the Northeast of Brazil was conceived which restrain the distribution and determinants of histoplasmosis in this particular endemic area.

The aim of this study was to characterize genotypically the clinical isolates of *H. capsulatum* obtained from patients with histoplasmosis from Ceará/Brazil by MLST analysis. In addition,

the co-relation between the identified *H. capsulatum* populations in Northeast of Brazil with clinical and phenotypic data obtained on theses fungal isolates was also investigated.

Methods

Fungal isolates

Fifty-one fungal isolates were obtained from patients recruited at São José Hospital, located in Fortaleza (Ceara, Brazil), a reference institution in infectious diseases, between 2011 to 2014. Yeast cells were cultured in Ham's F12 medium. It was pelleted by centrifugation and washed with distilled deionized water, and were kept at - 4ºC. A total of 500 µl of yeast were used for DNA extractions as previously established (29). DNA's were quantified by spectrophotometry using the Epoch™ Multi-Volume Spectrophotometer System (Biotek Instruments, Inc., USA).

Clinical/epidemiological data and phenotypical aspects

The data about the epidemiological (sex, origin, and co-infection with tuberculosis), clinical (dyspnea, hemorrhagic manifestation, skin lesion, acute renal failure – ARF and death) were obtained by review of medical registers. Mycological aspects (pigmentation, texture, and sporulations structures), exoantigens profile by western blot, and mating type – PCR were retrieved of previously study published (Damasceno et al., 2016 – Capítulo 3).

Multi locus sequencing type

Amplification of partial DNA sequences of four nuclear genes (*arf, H-anti, ole1,* and *tub1*) was performed according to the protocol described by Kasuga *et al.* (12), with some modifications. The PCR was performed in a 25 µl reaction mixture, containing 200 µM each deoxynucleoside triphosphate (dNTP) (Applied Biosystems Inc., Foster City, CA, USA), 2.0 mM $MgCl_2$, 50 ng/µl of each primer, 1.0 U *Taq* DNA polymerase (New England BioLabs Inc., MA, USA), 1 X *Taq* commercial buffer and 20 ng (10 ng/µl) of each DNA template. The G-217B references strain was used as positive control for the PCR reactions. PCR assays

were performed in a Thermal iCycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) programmed as follows: (a) 3 min at 95°C; (b) 32 cycles, consisting of 15 sec at 94°C, 30 sec at 65ºC in the first cycle, which was subsequently reduced by 0.7ºC/cycle for next 12 cycles, and 1 min at 72ºC. The remaining 20 cycles, the annealing temperature was continued at 56ºC; (c) a final extension cycle of 5 min at 72ºC (touchdown PCR) (30). Generated amplicons were also sequenced by Sanger method at the High-Throughput Genomics Center (University of Washington) and the sequences were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) – (Table 1).

Phylogenetic analysis

The deciphered sequences were first checked by BLASTn (31) for evaluate the genetic similarity with *H. capsulatum* sequences from GenBank database (http://www.ncbi.nlm.nih.gov). The sequences were aligned using the Clustal W (32) algorithm implemented in the Mega 6.0 software (33). We included the same dataset evaluated by Teixeira et al. (2016) (14) in order to include the whole diversity of the genus *Histoplasma* (Table 2).

The combined matrix resulted in 251 taxa and 1582-nt that was analyzed through two methods: 1) Maximum likelihood (ML) trees were generated using the IQ-TREE program (34) using the –m MODEL function that allowing an automatic best-fit model selection (ModelFinder – K2P + Inv Gamma was used in all tested phylogenies). The ultrafast bootstrap (UFBoot) approximation described by Minh et al. (35) was employed to test branch confidence; 2) Bayesian inference (BI) were conducted using the MrBayes ver. 3.2 software (36). Bayesian analysis were performed through 300.000 generations and samples were collected every 100 generations using 4 Markov chain Monte Carlo (MCMC) chains. Twenty-five percent of the initial samples were discarded as burnin. The consensus tree obtained by both methods was visualized in FigTree v1.3.1 (37).

Population structures analysis

Population distribution of the Northeast *H. capsulatum* isolates was inferred using a Bayesian Analysis of Population Structure (BAPS) (38). We conducted admixture and mixture analysis setting up the Kmax to 50. In addition, fixed K model analysis (K=2-5) was used for admixture analysis in order to infer the population sub-structuring within the Northeast population.

Statistical analysis

The statistical analysis was carried out using the software program STATA 11.2 (StataCorp LP, College Station, TX, USA). The univariate analysis were performed to evaluate the relationship between genetic populations found and clinical manifestation or phenotype aspects, using the Chi-squared test or Fisher exact test, if any value in the cells of the contingency table was less than five, where it was applied a significance level of 5% (α = 0.05). The dependent variables considered were the presence of skin lesion, hemorrhagic manifestations, dyspnea, ARF and death. Individuals who had more than a fungal isolate that are not in the same cluster were not considered in the statistical analysis. The study was approved in the Research Ethical Committee of INI/FIOCRUZ (No. 19342513.2.0000.5262).

Results

A total of 51 clinical isolates were obtained during 43 hospitalizations of individuals with disseminated histoplasmosis, in the period from 2011 to 2014 in HSJ. By phylogenetic analysis was possible to identify a new monophyletic clade within Latin America population constituted mainly for fungal isolate from this study, which was denominated Northeast clade. Moreover, two genetic subpopulations were identified (Figure 1 and 2).

Based on Bayesian Analysis of Population Structure (BAPS) was possible to confirm the presence of one restrict genetic population from Northeast of Brazil (Figure 2). In addition, tree subpopulations in the Northeast group were identified: cluster I – constituted for 35

fungal isolates from Ceara, 3 *H. capsulatum* isolates from Rio de Janeiro (84476, 84564 and 84502) and H151 from São Paulo; cluster II – formed for 16 clinical isolates from Ceara and H146 from Brazil; and, cluster III – constituted for JIEF (Ceara) and 2 fungal isolates from Pernambuco (RE 9463 and RE 5646). By recombination genetic analysis a cluster rede was generated with only fungal isolates from Ceará (cluster I and cluster II), and it was possible to observe that there are aleles interchange between both clusters (Figure 3).

Regarding the didtribution of cluster I and II from Northeast isolates reveled that there are a genetic population more homogenea at the Fortaleza metropolitan region than other regions of Ceará (Figure 4).

In regard the relationship between the genetic subpopulations from Ceara and the epidemiological and clinical features, there is no observed association (Table 3). However, MAT1-1 was predominance in the cluster II, and MAT1-2 in the cluster I (p=0.036). Despite this, the others phenotypic aspects have not statistical association with genetic subpopulation from Ceara (Table 4).

Discussion

This was the largest and most complete study about population of *H. capsulatum* isolates from Ceará. A new genetic population was identified with two subclusters, which presented a high rate of genetic recombination. In addition, different mating type was found in both clusters.

MLST analysis is a molecular technique with a high discriminatory power between *H. capsulatum* isolates. Based in previous MLST studies a great and diverse genetic population of *H. capsulatum* has been found in the world (14). Currently, *H. capsulatum* is a pathogen considered a fungal species complex, which is constituted for 11 phylogenetic species, and some clusters and linages according to Teixeira et al. (14). Latin America is the region with a larger mixture of fungal isolates than other endemic area of world (14, 39). Up to date, the

majority of *H. capsulatum* clinical isolates studied of Brazil were obtained from residents of Southeast of Brazil (14, 40). In our study, we evaluated a large number of fungal isolates from Northeast of Brazil, and similar to other phylogenetic species as RJ and BAC-1, identified in Southeast of Brazil and Mexico, respectively, the Northeast clade represented a new and restricts genetic population supported by BAPS. Molecular study based in the (GA)n microsatellite typing have also identified one restrict genetic population from Ceará (data not published), which corroborate these results.

In addition, it is the first study that evaluated the relationship among the genetic subpopulations and epidemiological, clinical and phenotypic aspects of *H. capsulatum* isolates. It is known that the disseminated histoplasmosis presented with different clinical characteristics in individuals from diverse endemic area. For example, skin lesions and death are more frequent in individuals from Brazil than USA patients (9). Besides, experimental studies have also found differences in the virulence of pathogen, genetic expression and pathogenesis of disease among *H. capsulatum* from different phylogenetic clades (41-43). Despite this, here, we did not find any association among the genetic subpopulations (cluster I and II) with the epidemiological or clinical features. Nevertheless, in this study all the fungal isolates were obtained mainly of AIDS-patients with disseminated disease and from only one geographic region of Brazil, which does these evaluations more difficult.

Morphology and exoantigen profile were also not associated with clusters from Ceará. Nevertheless, as a new data, mixed mating types were found in both clusters with a predominance of determined mating type in each cluster. Sexual reproduction is responsible for increased genetic diversity. The consequences of this event for pathogenesis can lead to development of fungal isolates with ability to evade the host immune responses and with greater resistant to antifungal drugs; to format biofilms; and to develop hypervirulent strains (44). Thus, recombination events can determinate shifts in the lifestyles of these species,

which can persist in successive lineages (45). Genetic recombination was clearly observed in this study based in the interchange of alleles within genetic subpopulations of Ceará. However, more studies are necessary to characterize better the influence and the impact of mating types of *H. capsulatum* isolates in the virulence of pathogen and in the pathogenesis of disease.

In summary, *H. capsulatum* clinical isolates from Ceará are genetically different of other fungal isolates from Brazil and Latin America what demonstrates a high genetic diversity this pathogen. It's possible that event can influence the development of disease. However, more studies about virulence of pathogen are necessary to elucidate this.

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Table 1: Acession number of sequences of fungal isolates from Ceará used in this study.

	GenBank accession number			
Isolates	***arf***	***H-anti***	***ole1***	***tub1***
CE0112	KX756766	KX756853	KX756900	KX756945
CE0211	KX756787	KX756852	KX756899	KX756946
CE0212	KX756788	KX756851	KX756898	KX756903
CE0213	KX756789	KX756850	KX756897	KX756944
CE0214	KX756767	KX756855	KX756896	KX756918
CE0313	KX756768	KX756849	KX756895	KX756904
CE0314	KX756769	KX756848	KX756894	KX756943
CE0413	KX756770	KX756847	KX756893	KX756905
CE0414	KX756783	KX756846	KX756892	KX756906
CE0513	KX756784	KX756845	KX756891	KX756935
CE0514	KX756785	KX756814	KX756890	KX756939
CE0611	KX756786	KX756844	KX756889	KX756914
CE0613	KX756771	KX756843	KX756888	KX756907
CE0614	KX756772	KX756842	KX756887	KX756917
CE0711	KX756765	KX756813	KX756886	KX756940
CE0713	KX756773	KX756841	KX756885	KX756908
CE0714	KX756790	KX756840	KX756884	KX756941
CE0812	KX756774	KX756839	KX756883	KX756909
CE0813	KX756775	KX756838	KX756882	KX756936
CE0814	KX756791	KX756837	KX756881	KX756916
CE0913	KX756792	KX756836	KX756880	KX756910
CE0914	KX756793	KX756835	KX756879	KX756942
CE1012	KX756794	KX756834	KX756878	KX756937
CE1013	KX756776	KX756856	KX756877	KX756921
CE1014	KX756795	KX756833	KX756876	KX756919
CE1111	KX756796	KX756832	KX756875	KX756920
CE1113	KX756797	KX756831	KX756874	KX756911
CE1114	KX756798	KX756830	KX756873	KX756922
CE1211	KX756799	KX756829	KX756901	KX756923
CE1213	KX756777	KX756828	KX756872	KX756926
CE1214	KX756778	KX756827	KX756871	KX756947
CE1312	KX756779	KX756854	KX756870	KX756924
CE1313	KX756800	KX756826	KX756869	KX756925
CE1414	KX756801	KX756825	KX756868	KX756927
CE1511	KX756780	KX756824	KX756867	KX756915
CE1513	KX756802	KX756823	KX756866	KX756912
CE1611	KX756803	KX756822	KX756865	KX756929
CE1713	KX756804	KX756812	KX756902	KX756938
CE1714	KX756781	KX756821	KX756864	KX756930
CE1911	KX756805	KX756820	KX756863	KX756948
CE2111	KX756806	KX756819	KX756862	KX756913
CE2214	KX756807	KX756818	KX756861	KX756931
CE2514	KX756808	KX756817	KX756860	KX756934
CE2614	KX756809	KX756816	KX756859	KX756932
CE2713	KX756782	KX756815	KX756858	KX756933
CE3013	KX756810	KX756811	KX756857	KX756928
CE0311	KX058302	KX058322	KX058307	KX058312
CE0411	KX058301	KX058321	KX058306	KX058311
CE0511	KX058300	KX058320	KX058305	KX058310
CE2813	KX058298	KX058318	KX058303	KX058309
CE2513	KX058299	KX058319	KX058304	KX058308

Table 2: Dataset of *H. capsulatum* strain/isolate used in the phylogenetic analysis and BAPS.

Isolate	State/Country	Species	*arf*	*H-anti*	*ole1*	*tub1*
2761	Alabama/USA	?	JX443639.1	JX458497.1	NI	NI
385BG	MS/Brazil	?	GU320865.1	GU320903.1	GU320993.1	GU321043.1
H64	Bogota/Colombia	?	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H67	Medellin/Colombia	?	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H145	Surinam	?	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
Hond10	Honduras	?	JQ218428.1	JQ218367.1	JQ218408.1	JQ218382.1
Hond13	Honduras	?	JQ218431.1	JQ218370.1	JQ218411.1	JQ218396.1
Hond18	Honduras	?	NI	JQ218374.1	JQ218415.1	JQ218397.1
Hond19	Honduras	?	NI	NI	JQ218416.1	NI
SP2414	SP/Brazil	?	GU320867.1	GU320901.1	GU320995.1	GU321045.1
EH317	Morelos/Mexico	?2	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH325	Chiapas/Mexico	?2	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
Hond3	Honduras	?2	JQ218422.1	JQ218362.1	JQ218401.1	JQ218393.1
Hond7	Honduras	?2	JQ218425.1	NI	JQ218405.1	JQ218381.1
Hond9	Honduras	?2	JQ218427.1	JQ218366.1	JQ218407.1	JQ218395.1
Hond11	Honduras	?2	JQ218429.1	JQ218368.1	JQ218409.1	NI
H149	Sao Paulo/Brazil	BR1 (SP)	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
SP49	SP/Brazil	BR1 (SP)	GU320866.1	GU320902.1	GU320994.1	GU321044.1
84476	Rio de Janeiro/Brazil	BR2	GU320841.1	GU320890.1	GU321008.1	GU321084.1
84502	Rio de Janeiro/Brazil	BR2	GU320840.1	GU320888.1	GU321006.1	GU321068.1
84564	Rio de Janeiro/Brazil	BR2	GU320842.1	GU320889.1	GU321007.1	GU321085.1
H151	Sao Paulo/Brazil	BR2	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
JIEF	CE/Brazil	BR2	GU320862.1	GU320906.1	GU320990.1	GU321040.1
ES62	Espirito Santo/Brazil	BR3	GU320871.1	GU320897.1	GU320999.1	GU321049.1
H154	Sao Paulo/Brazil	BR3	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063

H196	Rio de Janeiro/Brazil	BR3	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H146	Brazil	BR4	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
RE5646	PE/Brazil	BR4	GU320860.1	GU320908.1	GU320988.1	GU321038.1
RE9463	PE/Brazil	BR4	GU320861.1	GU320907.1	GU320989.1	GU321039.1
H90	Egypt/Africa	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H95	Egypt/Africa	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H96	India	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H142	England	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H148	NI	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H174	Poland	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H175	Poland	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H177	Beijing/China	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H178	Beijing/China	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H190	NI	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H191	NI	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H192	India	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H193	Egypt	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H194	Egypt	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H204	India	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H205	Thailand	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H206	Thailand	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H207	Thailand	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H208	Thailand	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H209	Thailand	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H210	Thailand	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H212	Algeria	Eurasia	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
Hond1	Honduras	Eurasia	NI	JQ218360.**1**	JQ218399.**1**	JQ218379.**1**
Hond2	Honduras	Eurasia	JQ218421.1	JQ218361.1	JQ218400.1	JQ218386.1

Hond8	Honduras	Eurasia	JQ218426.1	JQ218365.1	JQ218406.1	JQ218388.1
Hond12	Honduras	Eurasia	JQ218430.1	JQ218369.1	JQ218410.1	JQ218389.1
Hond14	Honduras	Eurasia	JQ218432.1	JQ218371.1	JQ218412.1	NI
Hond25	Honduras	Eurasia	JQ218435.1	JQ218377.1	JQ218419.1	JQ218385.1
Meles	Germany	Eurasia	JX093565.1	JX093566.1	NI	JX093567.1
H91	Guinea-Liberian Border/Africa	H140 clade	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H140c	Maryland/USA/Peru	H140 clade	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H185c	Maryland/USA/Peru	H140 clade	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H153	Sao Paulo/Brazil	H153 ligneage	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H167	Argentina	H167 ligneage	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH-383IA	Morelos/Mexico	LAmA1	AF495619.1	NI	NI	NI
EH-383PA	Morelos/Mexico	LAmA1	AF495623.1	NI	NI	NI
EH-393[a]	Oaxaca/Mexico	LAmA1	AF495635.1	NI	NI	NI
EH-408HA	Puebla/Mexico	LAmA1	AF495644.1	NI	NI	NI
EH-408PA	Puebla/Mexico	LAmA1	AF495647.1	NI	NI	NI
H60	Bogota/Colombia	LAmA1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H61	Bogota/Colombia	LAmA1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H62	Bogota/Colombia	LAmA1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H63	Bogota/Colombia	LAmA1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H211	Thailand	LAmA1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH304	Guatemala	LAmA1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH319	Mexico City/Mexico	LAmA1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH332	Guatemala	LAmA1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH359	Oaxaca/Mexico	LAmA1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH374	Morelos/Mexico	LAmA1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH375	Morelos/Mexico	LAmA1	AF495607.1	NI	NI	NI
EH376	Morelos/Mexico	LAmA1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH379	Mexico	LAmA1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063

EH391	Morelos/Mexico	LAmA1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH394	Oaxaca/Mexico	LAmA1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
Hond4	Honduras	LAmA1	JQ218423.1	JQ218363.1	JQ218402.1	JQ218380.1
Hond5	Honduras	LAmA1	JQ218424.1	JQ218364.1	JQ218403.1	JQ218387.1
Hond15	Honduras	LAmA1	JQ218433.1	JQ218372.1	JQ218413.1	JQ218383.1
Hond16	Honduras	LAmA1	NI	JQ218373.1	JQ218414.1	JQ218390.1
Hond21	Honduras	LAmA1	NI	NI	JQ218417.1	JQ218384.1
Hond22	Honduras	LAmA1	JQ218434.1	JQ218375.1	NI	JQ218391.1
Hond23	Honduras	LAmA1	NI	JQ218376.1	JQ218418.1	JQ218398.1
Hond26	Honduras	LAmA1	JQ218436.1	JQ218378.1	JQ218420.1	JQ218392.1
H141	Indonesia	LAmA1/H141 clade	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H188	Panama	LAmA1/H141 clade	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
2134	Texas/USA	LAmA2	JX443630.1	JX458485.1	JX458503.1	JX431896.1
H71	Medellin/Colombia	LAmA2	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H73	Bogota/Colombia	LAmA2	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H74	Medellin/Colombia	LAmA2	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H76	Medellin/Colombia	LAmA2	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH46	Guerrero/Mexico	LAmA2	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH53	Hidalgo/Mexico	LAmA2	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH303	Guatemala	LAmA2	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH316	Guerrero/Mexico	LAmA2	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH333	Guatemala	LAmA2	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH362	Guatemala	LAmA2	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH363	Guatemala	LAmA2	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH364	Guatemala	LAmA2	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH372	Morelos/Mexico	LAmA2	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH373	Morelos/Mexico	LAmA2	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063

EH377	Morelos/Mexico	LAmA2	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
EH378	Morelos/Mexico	LAmA2	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
190CLC	RS/Brazil	LAmB	GU320877.1	GU320885.1	GU320987.1	GU321058.1
B670	RJ/Brazil	LAmB	GU320882.1	GU320935.1	GU321035.1	GU321086.1
GO1820	Goias/Brazil	LAmB	GU320864.1	GU320904.1	GU320992.1	GU321042.1
GO764	Goias/Brazil	LAmB	GU320863.1	GU320905.1	GU320991.1	GU321041.1
H66	Medellin/Colombia	LAmB	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H69	Medellin/Colombia	LAmB	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
Hond6	Honduras	LAmB	NI	NI	JQ218404.1	JQ218394.1
H59	Bogota/Colombia	LAmB/LAmB1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H68	Medellin/Colombia	LAmB/LAmB1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H70	Medellin/Colombia	LAmB/LAmB1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H75	Medellin/Colombia	LAmB/LAmB1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H85	Argentina	LAmB/LAmB1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H162	Argentina	LAmB/LAmB1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H163	Argentina	LAmB/LAmB1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H164	Argentina	LAmB/LAmB1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H166	Argentina	LAmB/LAmB1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H168	Argentina	LAmB/LAmB1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H169	Argentina	LAmB/LAmB1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H171	Argentina	LAmB/LAmB1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H172	Argentina	LAmB/LAmB1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
MS53	MS/Brazil	LAmB/LAmB1	GU320847.1	GU320934.1	GU321036.1	GU321076.1
157CS	RS/Brazil	LAmB/LAmB2	GU320875.1	GU320887.1	GU321009.1	GU321056.1
177CS	RS/Brazil	LAmB/LAmB2	GU320884.1	GU320933.1	GU321037.1	GU321087.1
187LCT	RS/Brazil	LAmB/LAmB2	GU320876.1	GU320886.1	GU321010.1	GU321057.1
184PRS	RS/Brazil	LAmB/LAmB2	GU320883.1	GU320932.1	GU321011.1	GU321088.1
ES55	Espirito Santo/Brazil	LAmB/LAmB2	GU320868.1	GU320900.1	GU320996.1	GU321046.1

ES56	Espirito Santo/Brazil	LAmB/LAmB2	GU320869.1	GU320899.1	GU320997.1	GU321047.1
ES60	Espirito Santo/Brazil	LAmB/LAmB2	GU320870.1	GU320898.1	GU320998.1	GU321048.1
EH-384IA	Oaxaca/Mexico	MBC1	AF495627.1	NI	NI	NI
EH-384PA	Oaxaca/Mexico	MBC1	AF495631.1	NI	NI	NI
EH315	Guerrero/Mexico	MBC1	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H81	Panamá	Panama	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H82	Panamá	Panama	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H83	Panamá	Panama	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
3356	Rio de Janeiro/Brazil	RJ	GU320879.1	GU320909.1	GU321033.1	GU321059.1
3688	Rio de Janeiro/Brazil	RJ	GU320834.1	GU320929.1	GU321025.1	GU321062.1
4334	Rio de Janeiro/Brazil	RJ	GU320835.1	GU320912.1	GU321026.1	GU321063.1
6406	Rio de Janeiro/Brazil	RJ	GU320837.1	GU320914.1	GU321034.1	GU321065.1
9414	Rio de Janeiro/Brazil	RJ	GU320873.1	GU320892.1	GU321004.1	GU321054.1
AC02	RJ/Brazil	RJ	GU320858.1	GU320916.1	GU321013.1	GU321069.1
AC05	RJ/Brazil	RJ	GU320859.1	GU320917.1	GU321021.1	GU321070.1
CAO4	RJ/Brazil	RJ	GU320844.1	GU320919.1	GU321022.1	GU321072.1
EP02	Rio de Janeiro/Brazil	RJ	GU320878.1	GU320920.1	GU321015.1	GU321073.1
H150	Sao Paulo/Brazil	RJ	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H152	Sao Paulo/Brazil	RJ	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H155	Sao Paulo/Brazil	RJ	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H156	Sao Paulo/Brazil	RJ	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H198	Rio de Janeiro/Brazil	RJ	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H199	Rio de Janeiro/Brazil	RJ	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H200	Rio de Janeiro/Brazil	RJ	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H201	Rio de Janeiro/Brazil	RJ	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H202	Rio de Janeiro/Brazil	RJ	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
H203	Rio de Janeiro/Brazil	RJ	Tree base #1063	Tree base #1063	Tree base #1063	Tree base #1063
IGS19	Rio de Janeiro/Brazil	RJ	GU320855.1	GU320895.1	GU321001.1	GU321051.1

IGS4/5	Rio de Janeiro/Brazil	RJ	GU320845.1	GU320922.1	GU321029.1	GU321074.1
IT04	Rio de Janeiro/Brazil	RJ	GU320846.1	GU321074.1	GU321020.1	GU321075.1
RS01	Rio de Janeiro/Brazil	RJ	GU320853.1	GU320921.1	GU321032.1	GU321080.1
RS09	Rio de Janeiro/Brazil	RJ	GU320854.1	GU320894.1	GU321002.1	GU321052.1
3416	Rio de Janeiro/Brazil	RJ/RJ1	GU320880.1	GU320910.1	GU321023.1	GU321060.1
3612	Rio de Janeiro/Brazil	RJ/RJ1	GU320881.1	GU320911.1	GU321024.1	GU321061.1
4631	Rio de Janeiro/Brazil	RJ/RJ1	GU320836.1	GU320913.1	GU321027.1	GU321064.1
9291	Rio de Janeiro/Brazil	RJ/RJ1	GU320874.1	GU320891.1	GU321005.1	GU321055.1
CADAM	RJ/Brazil	RJ/RJ1	GU320843.1	GU320918.1	GU321014.1	GU321071.1
6503	Rio de Janeiro/Brazil	RJ/RJ2	GU320838.1	GU320930.1	GU321028.1	GU321066.1
RPS51	Rio de Janeiro/Brazil	RJ/RJ2	GU320848.1	GU320924.1	GU321030.1	GU321077.1
RPS86	Rio de Janeiro/Brazil	RJ/RJ2	GU320856.1	GU320925.1	GU321031.1	GU321078.1
TI01	Rio de Janeiro/Brazil	RJ/RJ3	GU320850.1	GU320926.1	GU321017.1	GU321081.1
TI05	Rio de Janeiro/Brazil	RJ/RJ3	GU320851.1	GU320927.1	GU321018.1	GU321082.1
TI14	Rio de Janeiro/Brazil	RJ/RJ3	GU320852.1	GU320928.1	GU321019.1	GU321083.1

Table 3: Relationship between epidemiological/clinical features and *H. capsulatum* cluster of patients from Ceará, 2011-2014.

Epidemiological/Clinical features	Number of patients in each cluster		
	Cluster I (n=30)	Cluster II (n=12)	*p*-value
Man sex	24 (80%)	9 (75%)	0.699
Metropolitan region	26 (86.7%)	7 (58.3%)	0.090
Co-infection with tuberculosis	3 (10%)	2 (16.7%)	0.613
Dyspnea	20 (66.7%)	7 (58.3%)	0.726
Acute renal failure	5 (16.7%)	1 (8.3%)	0.655
Hemorrhagic manifestation	8 (26.7%)	2 (16.7%)	0.696
Skin lesion	7 (23.3%)	2 (16.7%)	1.000
Death	9 (30%)	5 (41.7%)	0.491

Table 4: Relationship between phenotypical aspects features and cluster of *H. capsulatum* isolates from Ceará, 2011-2014.

Phenotypical aspects	Distribution of *H. capsulatum* isolates		*p*-value
	Cluster I (n=35)	Cluster II (n=16)	
Pigmentation			
Pallid*	25 (71.4%)	11 (68.7%)	0.846
Dark**	10 (28.6%)	5 (31.3%)	
Texture			
Cotton	30 (85.7%)	12 (75%)	0.436
Powdery	5 (14.3%)	4 (25%)	
Sporulation structures			
Yes	26 (74.3%)	12 (75%)	1.000
No	9 (25.7%)	4 (25%)	
M and H exoantigens			
Yes	20 (57.1%)	9 (56.2%)	0.952
No	15 (42.9%)	7 (43.8%)	
Mating type			
MAT1-1	13 (37.1%)	11 (68.7%)	**0.036**
MAT1-2	22 (62.9%)	5 (31.3%)	

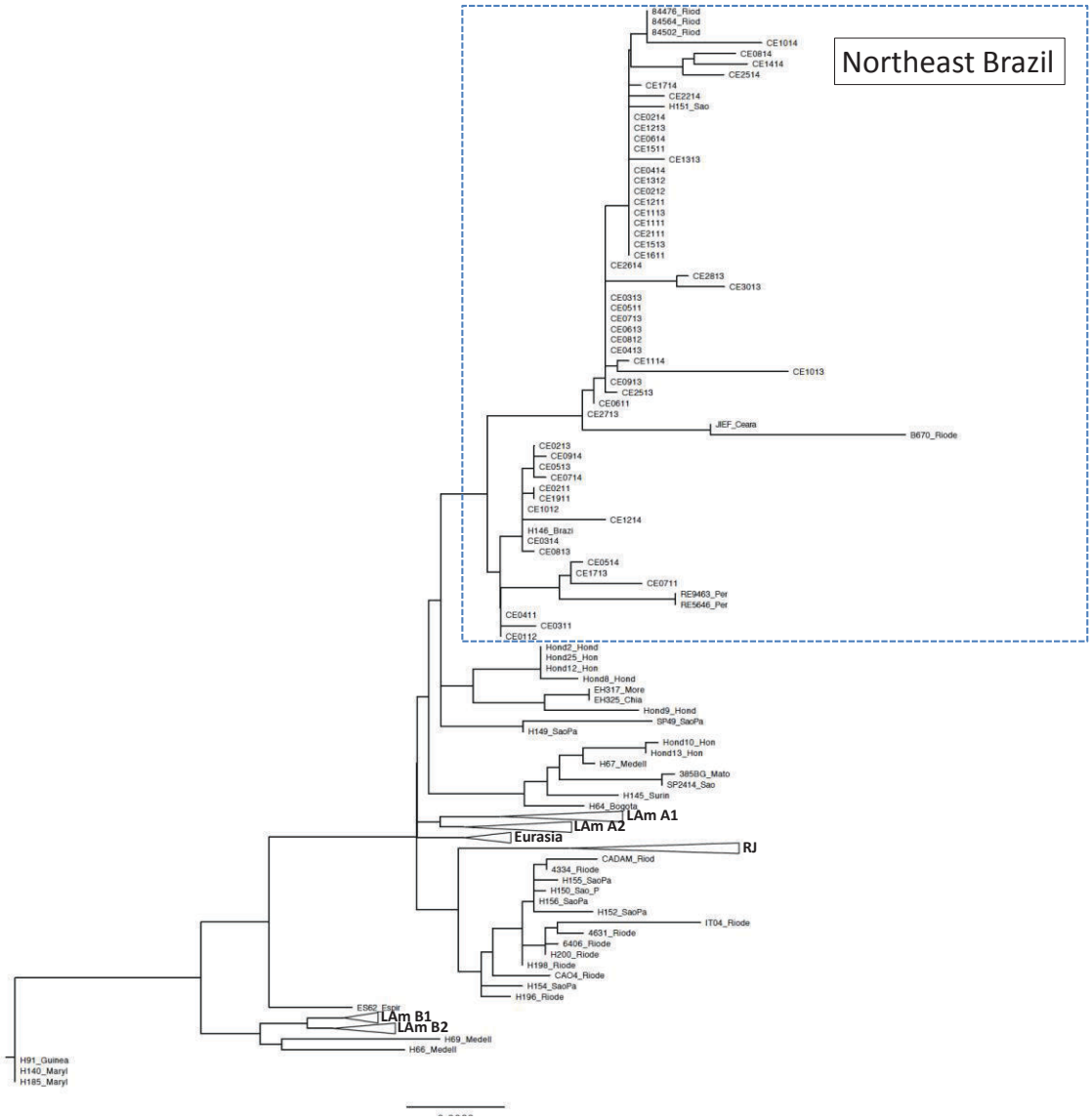

Fig. 1. Maximum Likelihood (ML) tree of *Histoplasma capsulatum* generated by IQ-TREE software for 251 taxa through 4 different loci (arf, ole1, tub and anti-H loci) reveals at least monophyletic braches as following: LAm A1, LAm A2, RJ, LAm B1, LAm B2, and Euroasia. Monophyletic branches that were supported by two methods (Bootstrap≥70/Posterior Probabilities≥0.95) were designated high confidence clades. We also identified possible in-group variation that may be associated with specific niches. Low supported clades such as Eurasia and Northeast were detected but do not follow our monophyletic branches supporting criteria.

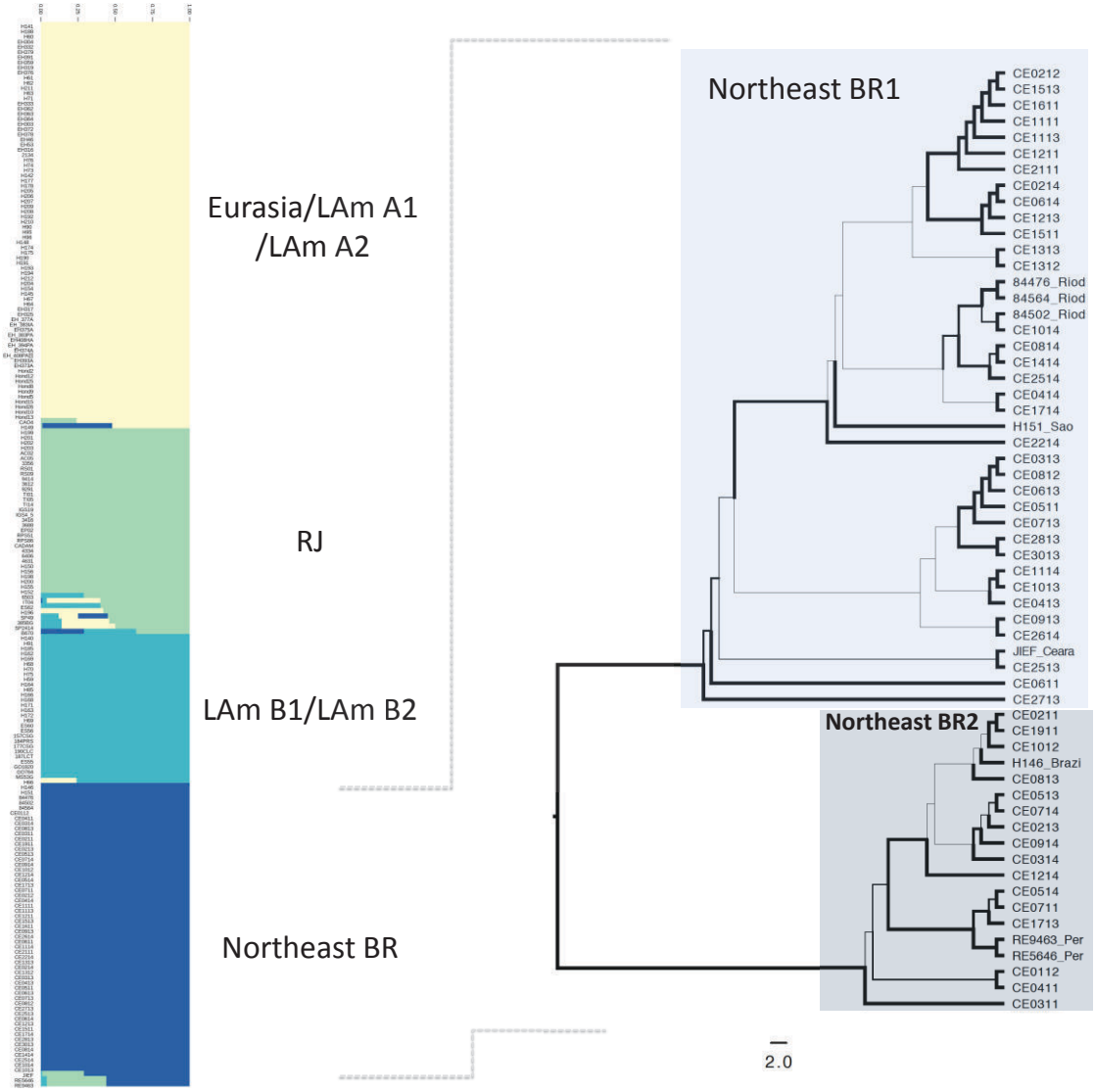

Fig. 2. Population structure of *Histoplasma capsulatum* deduced by Bayesian Analysis of Population Structure (BAPS). Structure plots of 205 isolates revealing 4 different major populations (Clusters 1–4). Phylogenetic species were assigned to each of the four deduced populations as follows: Cluster 1 constituted by LAm A1, LAm A2, BR1-4, and the paraphyletic low supported clades Eurasia; Cluster 2 representing the phylogenetic species RJ; Cluster 3 containing LAm B1 and LAm B2; Cluster 4 constituted by phylogenetic species Northeast BR.

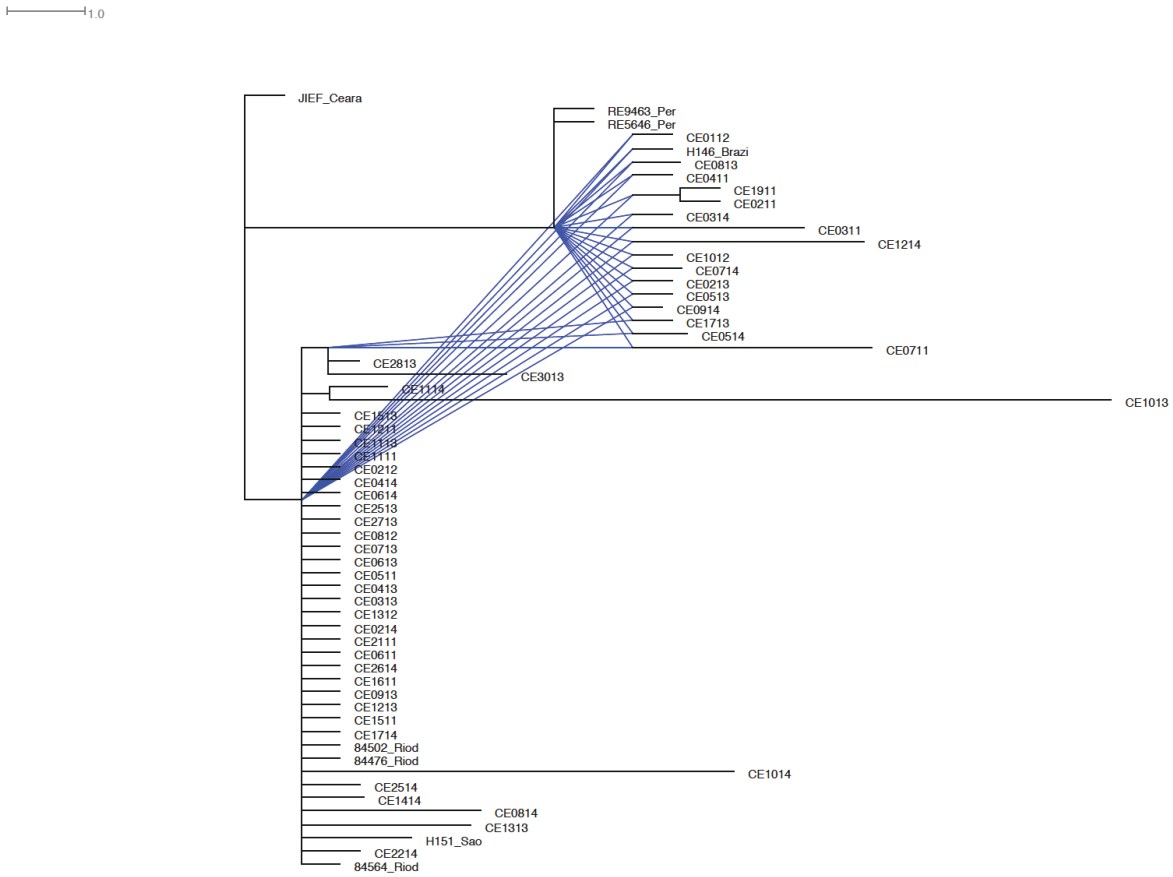

Fig. 3. Clusters rede generated by genetic recombination analysis deduced by phi-test (p=2.345E-12). Aleles interchange can occur between cluster I and II of *Histoplasma capsulatum* from Ceará.

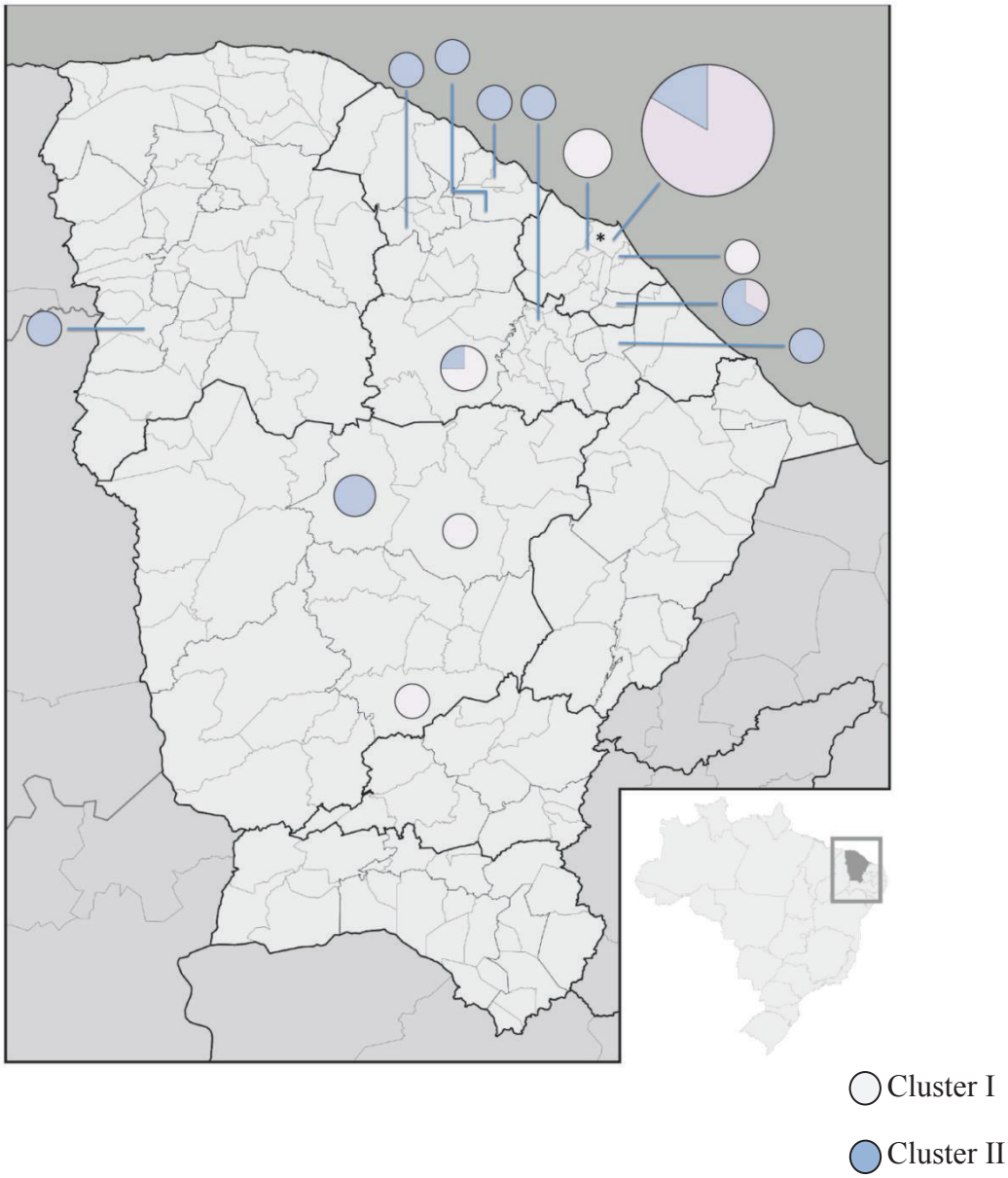

Fig. 4. Geographic distribution of cluster I and cluster II of *H.capsulatum* from Ceará. (*) Fortaleza City.

5 DISCUSSÃO

A histoplasmose é uma micose sistêmica grave, que necessita de um diagnóstico rápido e precoce. No estado do Ceará é uma doença endêmica e acomete principalmente PVHA. Neste estudo, a forma disseminada, o acometimento predominante em indivíduos do sexo masculino, elevada frequência de acometimento cutâneo e alta taxa de mortalidade foram características semelhantes observadas em outros estudos locais (RAMOS, 2008; PONTES *et al.*, 2010; BRILHANTE; FECHINE *et al.*, 2012; DAMASCENO *et al.*, 2013). Também identificamos aqui, alta taxa de coinfecção com tuberculose, a qual é a principal patologia considerada no diagnóstico diferencial desta micose, devido à semelhança dos sintomas entre essas duas doenças (ADENIS; NACHER; HANF; BASURKO *et al.*, 2014). Além disso, tuberculose, no Brasil, continua tendo alta taxa de prevalência, principalmente em PVHA (WHO, 2015).

O diagnóstico da histoplasmose, muitas vezes é tardio e negligenciado pela equipe de saúde, que desconhece a patologia (NACHER *et al.*, 2013). Na Colômbia, após treinamento de um grupo de micologistas, epidemiologistas e clínicos sobre a doença, verificou-se um aumento do diagnóstico de histoplasmose (CACERES *et al.*, 2015). Em países com poucos recursos financeiros, o diagnóstico usualmente é realizado através do isolamento e/ou identificação do *H. capsulatum* (SCHEEL; GÓMEZ, 2014; MURPHY *et al.*, 2015). Entretanto, trata-se de um método laborioso e que consome muito tempo (HAGE *et al.*, 2015), já que se faz necessário demonstrar o dimorfismo do fungo. Além disso, o isolamento depende da carga fúngica dos indivíduos. Em PVHA, em geral as culturas são positivas em 75% nas formas disseminadas (ARANGO-BUSTAMANTE *et al.*, 2013; SCHEEL; GÓMEZ, 2014).

A técnica convencional de identificação de *H. capsulatum* baseia-se na observação das características morfológicas e no teste de conversão, para caracterização do dimorfismo. Em nosso estudo, cinco tipos de colorações do morfotipo-M foram observadas ao contrário do que havia sido descrito por Berliner et al. (1968), onde apenas dois padrões de colônias foram demonstrados: as do tipo A ou albinas, e as do tipo B ou marrom (BERLINER, 1968). Entretanto, Freitas (2005) já havia encontrado uma maior variação fenotípica, quando observou que 12 isolados de *H. capsulatum* semeados no meio PDA e Agar Sabouraud Dextrose adquiriam textura e pigmentação diferentes, com o mesmo tempo de cultivo (FREITAS, 2005). Assim como em nosso estudo, os autores encontraram colônias com

pigmentação variando do branco ao bege-marrom. Alguns fatores que podem influenciar a pigmentação e a conidiação são: sucessivos cultivos, que favorecem o aparecimento de colônias tipo A (PINE; PEACOCK, 1957; LIMA; BORBA, 2001; IMANO, 2005), e isolados provenientes de solo ricos em fezes de pássaros ou morcegos, que tendem a apresentar colônias do tipo B (EISSENBERG; GOLDMAN, 1991).

A relação da pigmentação com as estruturas de esporulação foi encontrada por alguns autores, que observaram que em linhagens do tipo B havia pouca quantidade de microconídios e geralmente apresentavam "massas" de macroconídios tuberculados, e em colônias do tipo A havia pouco ou nenhum macroconídio e microconídios abundantes (BERLINER, 1968; FREITAS, 2005; DIAS, 2009). Assim como esses autores, observamos que 12 isolados fúngicos do tipo A (branco) apresentavam somente microconídios em abundância. Apesar de não usarmos nenhum método padronizado para quantificar os macroconídios de cada isolado, podemos observar uma estreita relação entre pigmentação e a presença de macroconídios, pois isolados do bege-marrom (Capítulo 3), tinham abundância de macroconídios tuberculados. Entretanto, uma única colônia totalmente marrom (tipo B) não apresentou macroconídios. Este foi sem dúvida um achado interessante, em que acreditamos que possa ser uma característica própria deste isolado (CE0112). A perda da conidiação ocorre em aproximadamente 25% dos isolados provenientes de culturas "velhas" de *H. capsulatum* (PINE; PEACOCK, 1957; IMANO, 2005). Entretanto, esta condição não se aplica ao isolado CE0112 ou colônias de coloração branca somente com microconídios, já que cada linhagem deste estudo foi submetida a apenas um subcultivo para observação das características morfológicas do morfotipo-M.

Embora alguns estudos tenham descrito que o tipo B (marrom) de colônias seja mais patogênico (EISSENBERG; GOLDMAN, 1991), no presente estudo, nenhuma associação com o tipo de colônia foi relacionado ao quadro clínico. Prévios estudos experimentais também não observaram haver diferença na virulência em relação às colônias brancas e marrons (TEWARI; BERKHOUT, 1965; CAMPBELL; BERLINER, 1973).

A realização do teste de conversão é fundamental para confirmação da presença de *H. capsulatum*, devido a outros fungos saprófitos dos gêneros *Chrysosporium* e *Sepedonium* apresentarem estruturas de propagação semelhantes (DEEPE JR, 2005). Em nosso estudo, observamos uma elevada taxa de conversão micélio-levedura (98%). Taxas de conversão semelhante foram obtidas em outros estudos (CHÁVEZ-TAPIA *et al.*, 2005; DIAS, 2009; RIBEIRO, 2012). Entretanto, alguns autores têm encontrado baixas taxas (25-38%) de conversão mesmo usando meios enriquecidos (BERLINER, 1968; IMANO, 2005). Tais

autores acreditam que a perda da capacidade de conversão também esteja relacionada com sucessivos cultivos e possivelmente a perda da capacidade de esporulação.

A conversão no primeiro ou segundo subcultivo, geralmente ocorre em isolados de pigmentação marrom. Isolados de coloração branca podem converter mais tardiamente, até no sétimo ou oitavo subcultivo (BERLINER, 1968). Neste estudo, também foi observado que isolados que não converteram no primeiro subcultivo, eram colônias brancas, as quais precisaram de mais dois ou três subcultivos para realizarem a transição dimórfica. Apenas um isolado, CE0613, não converteu em três subcultivos no meio ML-Gema.

Entretanto, sabemos que o dimorfismo é um processo complexo, cepa-dependente, e que envolve várias condições físico-químicas e biológicas para que possa ocorrer. Sabe-se que cepas do tipo Downs realizam a transição dimórfica em temperaturas a 34ºC, sendo consideradas cepas termossensíveis, já que a 37ºC (temperatura usualmente usada para conversão) essas cepas sofrem degeneração celular (MEDOFF *et al.*, 1986; KEATH *et al.*, 1989). Cepas termotolerantes iniciam o processo de degeneração celular somente em temperaturas acima de 40ºC, e por isso são consideradas mais virulentas que cepas do tipo Downs (MEDOFF *et al.*, 1987). Spitzer et al. (1990) descreveram três isolados clínicos termossensíveis procedentes de pacientes com aids, semelhante à cepa Downs (SPITZER *et al.*, 1990). Entretanto, o isolado CE0613, não realizou conversão dimórfica mesmo em temperatura de 34ºC. Apesar de este isolado fúngico ter estruturas de esporulação, banda M ao WB, e ter sido confirmado como *H. capsulatum* através da amplificação da região ITS, é possível que alguma condição *in vitro* tenha impossibilitado a não realização do dimorfismo.

Técnicas de imunoidentificação de isolados fúngicos são mais rápidas, mais simples, específicas e menos laboriosas do que as técnicas convencionais. Buscam identificar antígenos específicos, H e M, produzidos por *H. capsulatum* (STANDARD; KAUFMAN, 1976; SEKHON *et al.*, 1986). Têm a vantagem de poderem ser usadas em culturas contaminadas com outros fungos saprófitos ou por bactérias, além de conseguirem identificar isolados fúngicos com morfologia atípica ou isolados sem estruturas de esporulação (DISALVO *et al.*, 1980; DISALVO; TERRENI; WOOTEN, 1981; SEKHON *et al.*, 1984; PADHYE *et al.*, 1992).

A produção e detecção de exoantígenos de *H. capsulatum* foi padronizada em 1976 (STANDARD; KAUFMAN, 1976). Os exoantígenos H e M podem ser obtidos tanto por meio de cultivos do fungo em meio líquido, sob agitação, durante três a seis dias; ou através de culturas estacionárias em tubos com meios sólidos durante no mínimo 15 dias (STANDARD; KAUFMAN, 1976; KAUFMAN; STANDARD, 1978). Para a detecção desses

exoantígenos de *H. capsulatum* a técnica mais utilizada é a ID, que apresenta uma sensibilidade 95-100% (DISALVO *et al.*, 1980; ANDREU *et al.*, 1990; PADHYE *et al.*, 1992; CHÁVEZ-TAPIA *et al.*, 2005).

Nossos resultados contrariam estudos prévios, já que encontramos uma sensibilidade baixa com o uso da ID (18%) para imunoidentificação dos isolados fúngicos deste estudo. No entanto, outros estudos também identificaram baixa sensibilidade (33-58%) da ID para identificação de *H. capsulatum* (REISS *et al.*, 2000; FREITAS, 2005). Alguns fatores são implicados na detecção das bandas de precipitinas de isolados de *H. capsulatum* por meio de ID. As melhores condições associadas à detecção dos antígenos H e M, por essas técnicas são o uso de agitação durante o cultivo das linhagens com tempo de crescimento maior que três dias, quantidade do inóculo de 2-4cm^2, e concentração de 50x dos antígenos. Colônias com atraso no crescimento devem ser mantidas em cultivo por mais dias do que o previamente padronizado (EHRHARD; PINE, 1972a;b; STANDARD; KAUFMAN, 1976; DISALVO *et al.*, 1980).

A produção de exoantígenos de nossos isolados foi realizada conforme as condições estabelecidas por Standard e Kaufman (1976) e, portanto, os fatores referentes ao cultivo como concentração e quantidade do inóculo, não interferiram no resultado da ID. Entretanto, a observação sobre o volume da massa celular e o retardo no crescimento das colônias não foram parâmetros controlados para a realização da ID. Apesar disso, vale ressaltar, que quando buscamos identificar a presença dos antígenos H e M por meio da técnica WB, verificamos uma sensibilidade de 100% na identificação dos exoantígenos. WB é uma técnica mais sensível, já que detecta baixas quantidades de antígenos individuais em um extrato, usando um anticorpo marcado por uma enzima (KURIEN; SCOFIELD, 2006). Reiss et al. (2000) também observou uma maior sensibilidade no WB quando comparado com ID na identificação de 12 isolados de *H. capsulatum* (REISS *et al.*, 2000).

Estudos prévios de imunoidentificação de *H. capsulatum* por ID, usualmente observavam a presença de bandas H e M concomitantemente (na maioria dos isolados), ou banda H única (STANDARD; KAUFMAN, 1976; DISALVO; TERRENI; WOOTEN, 1981; ANDREU *et al.*, 1990; CHÁVEZ-TAPIA *et al.*, 2005). A detecção de isolados fúngicos produtores somente do antígeno M havia sido identificado em único trabalho (FREITAS, 2005). Em nosso estudo dos nove isolados fúngicos com ID positiva, oito tinham apenas banda M e somente um isolado tinha os antígenos H e M (CE0611).

Em relação ao perfil de exoantígenos detectados por WB, observamos um grande número de amostras produtoras de antígenos H e M, assim como, de somente antígeno M.

Sabe-se que anticorpos contra o antígeno-H aparecem mais tardiamente em infecções agudas em cerca de somente 20% dos pacientes (WHEAT *et al.*, 1982), e que durante o processo de produção da histoplasmina (complexo antigênico que contém os antígenos H e M), o tempo de cultivo mínimo deve ser de 15 dias, a fim de garantir a produção dos antígenos H e M (EHRHARD; PINE, 1972b). Portanto, é possível que essas linhagens produtoras somente de antígeno M ocorra devido a uma metabolização e excreção do antígeno H mais tardia, ou ainda, que isso seja uma característica própria destes isolados.

Em relação ao tipo de compatibilidade sexual, estudos prévios observaram uma maior frequência de *MAT1-2* (7:1) em isolados clínicos, e a ocorrência destes principalmente em indivíduos imunocomprometidos (KWON-CHUNG, 1973; KWON-CHUNG; WEEKS; LARSH, 1974; KWON-CHUNG; BARTLETT; WHEAT, 1984). *MAT1-2* também era sugerido ser mais virulento pelo simples fato de ter maior frequência de conversão dimórfica, do que os isolados *MAT-1*. Entretanto, ensaios de virulência não sustentaram essa hipótese, já que os dois tipos de compatibilidade sexual apresentaram habilidade em realizar a transição dimórfica e causar infecção (KWON-CHUNG, 1981). No Brasil, estudo realizado com isolados fúngicos do Rio de Janeiro identificou apenas *MAT1-1* entre isolados clínicos e ambientais (RODRIGUEZ-ARELLANES *et al.*, 2013). No presente estudo, a proporção de *MAT1-2* e *MAT1-1* foi semelhante ocorrendo 98% de conversão dimórfica dentre os 51 isolados deste estudo. Além disso, não foi observada nenhuma associação entre o tipo de compatibilidade sexual com as características clínicas dos pacientes, ou morfológicas dos isolados. Em outros fungos patogênicos, como *Cryptococcus neoformans* var. *neoformans* (sorotipo D) (KWON-CHUNG; EDMAN; WICKES, 1992) e *Aspergillus fumigatus* (ALVAREZ-PEREZ *et al.*, 2009; ALVAREZ-PEREZ *et al.*, 2010a), determinados tipos de compatibilidade sexual (α-MAT e *MAT1-1*, respectivamente), têm sido associado com uma maior virulência do patógeno. É possível que futuros ensaios de virulência possam esclarecer se existe relação entre o tipo de compatibilidade sexual e a patogenia da histoplasmose.

A presença de infecção mista por *H. capsulatum* com diferentes tipos de compatibilidade sexual em dois pacientes foi um achado interessante e surpreendente. Este tipo de infecção, já foi observado em indivíduos com infecção por *A. fumigatus* (PAOLETTI *et al.*, 2005; ALVAREZ-PEREZ *et al.*, 2009). Entretanto, até o momento, não sabemos se é possível haver cruzamento dos dois tipos de compatibilidade sexual *in vivo* (ALVAREZ-PEREZ et al., 2010b). Sabe-se que tanto *A. fumigatus* como *H. capsulatum* são capazes de realizar cruzamento *in vitro* (KWON-CHUNG, 1972a; O'GORMAN; FULLER; DYER, 2009), e que as consequências deste cruzamento podem causar modificações no genoma do

fungo, levando ao surgimento de cepas mais virulentas e com maior resistência antifúngica, além de isolados fúngicos de padrões atípicos (ALVAREZ-PEREZ *et al.*, 2010b; ENE; BENNETT, 2014). Coinfecções por diferentes tipos de compatibilidade sexual em *C. neoformans* demonstraram que o tipo α-MAT tem predileção pelo sistema nervoso central (NIELSEN *et al.*, 2005). Mais estudos podem ajudar a esclarecer melhor o significado e a relevância deste tipo de coinfecção na histoplasmose.

Distintas populações genéticas de *H. capsulatum* procedentes de diferentes regiões geográficas já foram observadas através de análise de microssatélites bi e multialélicos (CARTER *et al.*, 1996; CARTER *et al.*, 1997; CARTER *et al.*, 2001). Entretanto, análise de um único microssatélite como (GA)n, também foi capaz de identificar grupos genéticos distintos de acordo com a origem e fonte do isolamento em fungos patogênicos (TAYLOR *et al.*, 2012).

Neste estudo, uma população genética de *H. capsulatum* restrita ao Estado do Ceará foi identificada, e claramente diferente de outros isolados fúngicos do México, EUA, Argentina e São Paulo/Brasil. Além disso, quatro haplótipos diferentes foram identificados na população fúngica do Ceará, sendo que dois haplótipos tinham o comprimento das sequências de (GA)n semelhantes geneticamente a isolados do México. Vale ressaltar, a observação de dois novos haplótipos GACG$(GA)_5$GA and GATG$(GA)_5$GA, pela primeira vez identificado no mundo.

Em relação ao sequenciamento multilocus, nossos isolados foram identificados como uma nova população genética, agrupando-se com outros isolados do Brasil, que até então eram considerados apenas linhagens no estudo realizado por Teixeira et al. (2016). Além disso, é possível destacar dois subgrupos diferentes dentre os isolados fúngicos do Ceará, baseado na análise de estrutura de populações genéticas com variado grau de recombinação genética. Apesar de estudos experimentais e epidemiológicos demonstrarem variações nas características relacionadas à virulência do patógeno e manifestações clínicas, com diferentes clados genéticos (KARIMI et al., 2002; DURKIN et al., 2004; SEPÚLVEDA et al., 2014), em nosso estudo, não foi possível detectar nenhuma associação com os aspectos clínico-epidemiológicos e morfológicos dos isolados fúngicos. Isso pode ter ocorrido devido estarmos avaliando *H. capsulatum* de uma única região geográfica e com somente uma forma clínica. Entretanto, houve associação de determinado *mating type* com as subpopulações genéticas, apesar de encontrarmos ambos os tipos de compatibilidade sexual nos dois subgrupos, achado este que pode possibilitar eventos de recombinação genética. Mais estudos devem ser

realizados com diferentes espécies filogenéticas sobre a virulência e patogenia, a fim de esclarecer melhor estas questões.

6 CONCLUSÕES

- ✓ A forma disseminada da histoplasmose, usualmente com acometimento cutâneo e com elevada taxa de mortalidade, é a principal forma clínica desta micose encontrada em PVHA no Ceará;
- ✓ O diagnóstico de histoplasmose deve ser buscado incessantemente em PVHA que estão em tratamento para tuberculose sem melhora clínica significativa, já que a coinfecção HD/tuberculose é frequente nesta população;
- ✓ Apesar da maioria dos casos de histoplasmose no Ceará ocorrerem em PVHA, as equipes de saúde devem estar atentas para o surgimento dessa micose em indivíduos com imunossupressão transitória ou naqueles que estão em período de recuperação imunológica, como em mulheres no puerpério;
- ✓ Embora técnicas fenotípicas usuais consumam bastante tempo e apresentem baixa sensibilidade, em locais com poucos recursos e com indisponibilidade de técnicas diagnósticas mais rápidas, a caracterização fenotípica segue sendo uma importante metodologia para identificação de *H. capsulatum;* demonstrando em nosso estudo um importante parâmetro para inferir a gravidade clínica dos pacientes.
- ✓ Diferentes perfis genéticos de *H. capsulatum* são observados entre populações oriundas da América do Norte e países da América Latina, sendo demonstrando assim, uma alta variabilidade genética do patógeno, através de distintas técnicas de tipagem molecular;
- ✓ WB pode ser um teste alternativo na rotina laboratorial para imunoidentificação de *H. capsulatum,* por apresentar melhor sensibilidade do que a ID, e por proporcionar um diagnóstico mais rápido do que os testes fenotípicos usuais, especialmente, nos casos em que possivelmente possa estar ocorrendo baixa expressão dos genes M e H;
- ✓ A caracterização dos tipos de compatibilidade sexual é importante parâmetro para inferir recombinação genética dos isolados fúngicos no ambiente, podendo resultar no aparecimento de linhagens hipervirulentas ou com maior resistência antifúngica;
- ✓ A detecção de infecções mistas por diferentes tipos de compatibilidade sexual e com heterogeneidade genotípica, verificada pelo MLST, demonstrou que mecanismos de coinfecção e superinfecção ocorrem no Ceará, provavelmente devido à contínua re-exposição a diferentes micronichos de *H. capsulatum* nesta região endêmica;

- ✓ Até o presente momento, devido à casuística reduzida, mais estudos são necessários para avaliar as consequências clínicas das infecções mistas com diferentes características genéticas na patogênese da histoplasmose;
- ✓ Análise do microssatélite GA(n) demonstra haver uma população genética de *H. capsulatum* restrita à região do Ceará, evidenciando variabilidade genética dentro desta população, caracterizada por diferentes haplótipos;
- ✓ A detecção inédita de dois diferentes haplótipos de *H. capsulatum* foram achados especialmente importantes para caracterizar o perfil genético circulante nesta região do Brasil, já que a variabilidade genética do fungo possivelmente está associada à patogênese da doença;
- ✓ Isolados de *H. capsulatum* do Ceará constituíram uma nova população genética deste fungo, o que caracteriza a presença de micronicho restrito a esta região do Brasil;
- ✓ A identificação de dois diferentes grupos na população fúngica do Ceará (*clado* do Nordeste), detectados através de MLST demonstrou uma alta diversidade genética de *H. capsulatum* dentro dessa população, e sugere que foram eventos associados à recombinação genética;
- ✓ A ausência de associação entre os tipos de compatibilidade sexual, bem como dos genótipos encontrados, com as manifestações clínicas dos pacientes pode ter ocorrido devido à análise dar-se somente com indivíduos que apresentavam a forma disseminada da doença.

OUTRAS PRODUÇÕES CIENTÍFICAS DURANTE O DOUTORAMENTO

Artigo 1

mycoses
Diagnosis, Therapy and Prophylaxis of Fungal Diseases

Original article

Disseminated histoplasmosis in HIV-infected patients: determinants of relapse and mortality in a north-eastern area of Brazil

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Summary

Many relapses and deaths resulting from disseminated histoplasmosis (DH) in acquired immunodeficiency syndrome (AIDS) patients have been observed in an endemic area in north-eastern Brazil. The objective of this study was to evaluate the risk factors associated with the clinical outcomes of DH/AIDS coinfection in patients from the state of Ceará, Brazil. A retrospective cohort of AIDS patients, after their hospital discharge due to first DH episode in the period 2002–2008, was followed until December 31, 2010, to investigate the factors associated with relapse and mortality. A total of 145 patients were evaluated in the study. Thirty patients (23.3%) relapsed and the overall mortality was 30.2%. The following variables were significantly ($P < 0.05$) associated with relapse and overall mortality (univariate analysis): non-adherence to highly active antiretroviral therapy (HAART), irregular use of an antifungal, non-recovery of the CD4+ count and having AIDS before DH; histoplasmosis relapse was also significantly associated with mortality. In the multivariate analysis, non-adherence to HAART was the independent risk factor that was associated with both relapse (Adj OR = 6.28) and overall mortality (Adj OR = 8.03); efavirenz usage was discovered to be significant only for the overall mortality rate (Adj OR = 4.50). Adherence to HAART was the most important variable that influenced the outcomes in this specific population.

Key words: Disseminated histoplasmosis, HIV, relapse, mortality.

Introduction

Disseminated histoplasmosis (DH) is the most frequent presentation of *Histoplasma capsulatum* infection in acquired immunodeficiency syndrome (AIDS) patients with severe immunosuppression.[1] The incidence of this illness ranges from 5 to 25% among the human immunodeficiency virus (HIV)-infected residents of Histoplasma-endemic areas.[2–4] Overall, histoplasmosis is considered the main cause of hospitalisation by systemic mycosis in the United States, with admission rates ranging from 16.7 to 20.6 per one million person-years in endemic areas.[5]

Few studies have investigated the prevalence of this mycosis in Brazil; however, one of the largest studies of DH in AIDS patients published recently was from the state of Ceará in north-eastern Brazil.[6–9] Brilhante *et al.* [9] conducted a retrospective study in 208 HIV patients with DH over a 5-year period in Ceará and identified an overall mortality of 42.3%. Considering

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AIDS to be the underlying cause of death and the systemic mycosis to be an associated condition, Prado *et al.* [10] evaluated the mortality rates attributable to the most important systemic mycoses in Brazil and found histoplasmosis to be the third most common illness (10.1%). In this same study, Ceará had the highest number of deaths reporting histoplasmosis as the primary cause.[11]

The DH prompt treatment initiation is decisive for a better prognosis in severe cases.[7] The 2000 guidelines published by the Infectious Diseases Society of America (IDSA) recommend a DH induction therapy of 12 weeks, followed by suppressive therapy indefinitely.[11] The updated 2007 guidelines recommend a prolonged treatment duration of 12 months (induction therapy), followed by prophylaxis with a possible interruption after a good immunologic response to the highly active antiretroviral therapy (HAART).[12] The rate of relapse was reduced considerably from 80% to approximately 5% when the secondary prophylaxis was coupled with HAART.[2,12] In endemic areas, such as Panama and the U.S., relapse has varied from 0 to 6%[3,13]; however, in Ceará, relapse has reached rates of approximately 22%.[8]We recently conducted study in Ceará and detected rates of recurrence (23.3%) and mortality (30.2%) that were still quite high.[14]

Our objective was to evaluate the risk factors associated with relapse and mortality after hospital discharge due to DH in AIDS patients, an endemic area of Brazil with limited resources.

Patients and methods

The study population consisted of hospital-discharged AIDS patients who had their first episode of DH between 2002 and 2008. Three public reference health units for HIV patients participated in the study: the Hospital São José of Infectious Diseases (HSJ – State Health Secretariat of Ceará), which follows approximately 85% of the HIV patients from Ceará; the Hospital Universitário Walter Cantídeo (HUWC – Federal University of Ceará), which initiated the HIV/AIDS attention in 2002; and the outpatient clinic the Centro de Especialidades Médicas José de Alencar (CEMJA– Health Secretariat of Fortaleza city), which was opened in 2006 and receives the newly diagnosed HIV/AIDS cases from the capital. These institutions are all located in Fortaleza (2.5 million inhabitants), which is the capital of Ceará. Until now, the HSJ was the main reference service for the diagnosis, hospital admission and outpatient care of people living with HIV and AIDS (PLHA) for all the state of Ceará.

This study used the same population of subjects with DH and AIDS who were used in a previously published study.[14] The patients were identified by reviewing the records of the microbiology laboratory of the HSJ and the Specialized Medical Mycology Center, which provides mycology laboratory support to the HUWC. The cohort included male and female AIDS patients (diagnosed according to the Brazilian Ministry of Health criteria)[15] over 18 years of age with confirmed evidence of DH by *H. capsulatum* identification in microscopy (histology or stained smears) and/or culture of the affected tissues or fluids other than or in addition to lung or cervical or hilar lymphnodes,[16] and who had survived their first DH episode.

Notes from all patients' medical visits were collected from the date of the DH diagnosis until December 31, 2010. The variables investigated included epidemiological variables (sex, age, risky occupation for histoplasmosis, origin and date of AIDS diagnosis), laboratory variables [CD4+ count, haemoglobin, leucocytes, platelets, aspartate aminotransferase (AST), lactate dehydrogenase (LDH), urea and creatinine], adherence to HAART and to the prophylactic antifungal agents for DH (maintenance dose), drugs used in the HAART regimen and outcome (relapse and mortality). We excluded patients with no medical records available after the DH diagnosis, patients cared for by other institutions and patients with the first episode of histoplasmosis in the lung tissue and/or ganglion only.

The risk activity for histoplasmosis was defined as a patient who had an occupation that was related to soil tillage (excavation, construction, demolition, or agriculture), worked directly with chickens, or visited caves. A relapse of histoplasmosis was defined in patients who presented with another episode at least 60 days after the first DH event; the relapse was confirmed by the isolation of the fungus in any tissue or body fluids. The patients who stopped clinical follow-up for more than 1 year before the end of the study (December 31, 2010) were excluded from the outcome (relapse and mortality) analyses.

The regular use of a maintenance dose of an antifungal and non-adherence to HAART was evaluated in the medical records according to patient self-reports that were systematically recorded by the attending physician during follow-up and/or by a lack of undetectable viral load (VL) during the HAART regimen. Immunological recovery was considered when a sustained increase in CD4+ count in two consecutive measurements was obtained after HAART initiation, in addition to undetectable VL[17,18]; consequently,

those patients who maintain a decrease in their CD4 counts, (even after initial response), and who failed to suppress plasma VL to undetectable levels until the end of the study were not considered immune recovered.

Epi-Info, version 3.5.1 (Centers for Disease Control and Prevention, Atlanta, GA, USA), was used for data entry. The statistical analyses were performed using the software program STATA 11.0 (StataCorp LP, College Station, TX, USA). The univariate analyses were performed using the *Chi-squared test* or Fisher exact test if any value in the cells of the contingency table was less than five. The Mann–Whitney test was applied to non-parametric variables (laboratory); the multivariate analysis was not performed in this specific variable (laboratory) because the sample size was lower than 30. For all statistical analyses, we used a significance level of 5% ($\alpha = 0.05$). The logistic regression analysis was conducted using the variables that reached $P < 0.2$ or those variables with epidemiologic interest, excluding possible confounding factors. The measures of association we utilised were relative risk (RR) and the odds ratio (OR), with their respective confidence intervals (95% CI); the measures of central tendency we used were median and interquartile range (IQR). This study protocol was reviewed and approved by the ethical boards of the HSJ (protocol number 029/2008) and the HUWC (protocol number 115.09.09).

Results

Patient characteristics

Of the initial 185 cases identified, a sample of 145 patients comprised the final study population. Forty patients were excluded because they received care at another institution or were missing records.

Detailed information concerning the epidemiological, laboratory and treatment characteristics of the studied population was published previously.[14] Briefly, the median age of the sample population was 34 years (IQR = 27–41) and males represented 83.4% of patients; 72% of the participants were living in Fortaleza, and 41% of the patients had AIDS diagnosed prior to DH. Two-nucleoside analogue reverse transcriptase inhibitor (NRTI) combined with efavirenz (EFZ) or lopinavir plus ritonavir were used by 53.6% and 26.8% of the participants, respectively; the other participants used nevirapine (2.2%) or other protease inhibitors (17.4%). The adherence to HAART was noted in 33.8% (49/145) of patients.

Amphotericin B deoxicholate (AB) was used in 97% of the patients during the first DH event and the mean total induction time (AB plus 400 mg of itraconazole) was 67 days. The maintenance dose of itraconazole (200 mg orally) was initiated in 92% of the patients, only 51% of whom used it regularly. The majority of the individuals (95%) had CD4+ counts <100 cells mm^{-3} at the time of the DH diagnosis and a recovered CD4+ count occurred in 42/66 patients in a median time of 399 days (IQR = 252–540). Nineteen individuals interrupted clinical follow-up for more than 12 months before the end of the study and were removed from the outcome analysis.

Risk factors for relapse

Thirty patients (23.3%) had histoplasmosis relapse in a median time of 355 days (IQR = 189–746) since the first episode. The incidence rate of relapse was 6 cases/person-year. Of those who relapsed, 22% were taking an antifungal regularly and 55% had EFZ as a part of the HAART regimen. During the relapse, the median CD4+ count was 28 cells mm^{-3} (IQR = 8–68); 28 patients (93.3%) were non-adherence to HAART and only two were adherent to the antiretroviral (ARV) drugs. Of these two, one had their first DH event in 2004 and the second event in 2007 as pulmonary histoplasmosis. The last recorded CD4+ count (422 cells mm^{-3}) and the VL (undetectable) was in September 2006, with lost of follow-up after that; the fungal prophylaxis had been discontinued in April 2006. The other patient had DH in 2007 and 2009; in the last episode, the CD4+ count was 118 cells mm^{-3} and VL undetectable. This patient received HAART and antifungal without interruption.

Non-adherence to HAART ($P = 0.000$), irregular use of an antifungal ($P = 0.000$), non-recovery of the CD4+ count ($P = 0.000$) and having AIDS before DH ($P = 0.025$) were all associated with relapse (Table 1). The only independent risk factor that was significantly associated with relapse was non-adherence to HAART ($P = 0.028$, Table 1).

An unrecovered CD4+ count was observed in 24/66 patients and was excluded from the initial model because it is a direct consequence of non-adherence to the ARV therapy, and turned out to be a confounding factor. The laboratory results were obtained between 3 and 12 months after the DH diagnosis. The laboratory parameters (median) significantly associated with a relapse were anaemia (haemoglobin < 10.0 g dl^{-1}), leukopenia (leucocytes < 4000 cells mm^{-3}), thrombocytopenia (platelets < 150 000), AST > 3 × normal

Table 1 Risk factors associated with relapse in patients with DH and AIDS, Ceará, Brazil, 2002–2008.

	Univariate			Multivariate		
Risk factors	RR	95% CI	*P*-value	AdjOR[3]	95% CI	*P*-value
Males	0.73	0.26–2.0	0.536	–	–	–
Risk activity for histoplasmosis	0.34	0.64–3.56	0.345	–	–	–
Non-adherence to HAART	1.68	1.37–2.05	**0.000**	6.28	1.22–32.27	**0.028**
Irregular use of antifungal	1.97	1.42–2.74	**0.000**	2.60	0.85–7.97	0.094
Use of EFZ in HAART[1]	1.04	0.70–1.53	0.823	1.54	0.57–4.18	0.388
Absence of recovery of CD4+ count[2]	2.69	1.70–4.23	**0.000**	–	–	–
AIDS prior to DH	1.65	1.10–2.48	**0.025**	2.08	0.78–5.53	0.142

[1]This variable was evaluated in the multivariate analysis due to epidemiologic interest.

[2]This variable was considered a confounding factor and was excluded from the multivariate analysis.

[3]Adj OR = odds ratio adjusted.

Bold values indicates $P < 0.05$.

upper limit (NUL), LDH > 1000 U l^{-1} and a CD4+ count <100 cells mm^{-3} (Table 3).

Risk factors for mortality

During the study period, the overall mortality was 30.2% (38 patients) and the median time from the first DH episode until death was 276.5 days (IQR = 88.5–606). The incidence rate of death was 8 cases/person-year. The majority of those who died did not use an antifungal regularly (72%) and were not HAART adherent (92%). In the univariate analysis, non-adherence to HAART ($P = 0.000$), irregular use of a maintenance dose of an antifungal ($P = 0.000$), absence of a CD4+ count recovery ($P = 0.000$), relapse of histoplasmosis ($P = 0.000$), use of EFZ in the HAART regimen ($P = 0.006$) and having AIDS prior to DH ($P = 0.009$) were all significantly associated with the overall mortality rate (Table 2). After excluding potential confounding variables (Table 2), the independent risk factors associated with mortality were non-adherence to HAART ($P = 0.007$) and EFZ in the HAART regimen ($P = 0.005$). None of the laboratory parameters of the relapsed patients were related with mortality (Table 3).

Discussion

High proportions of deaths and relapses were identified in this study. Non-adherence to HAART was the independent risk factor associated with both evaluated outcomes (relapses and death) in DH/AIDS patients in this endemic area of Brazil. Several studies have demonstrated the benefits of HAART in PLHA, including an important decrease in the mortality rates, a reduction in the incidence of opportunistic infections, an improvement in the quality of life and, mainly, a significant increase in survival.[19–23]

In Brazil, the programme providing the universal availability of ARV drugs to AIDS patients by the

Table 2 Risk factors associated with overall mortality in patients with DH and AIDS, Ceará, Brazil, 2002–2008.

	Univariate			Multivariate		
Risk factors	RR	95% CI	*P*-value	Adj OR[2]	95% CI	*P*-value
Males	0.68	0.27–1.72	0.403	–	–	–
Risk activity for histoplasmosis	1.07	0.44–2.61	0.868	–	–	–
Non-adherence to HAART	1.76	1.41–2.19	**0.000**	8.03	1.76–36.63	**0.007**
Irregular use of antifungal	2.01	1.40–2.88	**0.000**	1.88	0.62–5.67	0.259
Use of EFZ in HAART	1.39	1.02–1.92	**0.006**	4.50	1.59–12.77	**0.005**
Absence of recovery of CD4 + count[1]	3.15	2.02–4.93	**0.000**	–	–	–
Relapse	3.36	1.72–6.56	**0.000**	2.58	0.83–8.05	0.101
Relapsed at 1st year	1.54	0.63–3.77	0.309	–	–	–
AIDS prior to DH	1.75	1.17–2.62	**0.009**	2.51	0.92–6.85	0.071

[1]This variable was considered a confounding factor and was excluded from the multivariate analysis.

[2]Adj OR = odds ratio adjusted.

Bold values indicates $P < 0.05$.

Table 3 Laboratory parameters (Mann–Whitney test) associated with relapse and death by relapse in patients with DH and AIDS, Ceará, Brazil, 2002–2008.

	Relapse			Death by relapse		
Laboratory	Median	IQR	*P*-value	Median	IQR	*P*-value
Haemoglobin (g dl^{-1})	9.3	8.1–9.6	**0.011**	9.55	6.95–12.5	0.653
Leucocytes (cells mm^{-3})	2600	1500–3400	**0.002**	2450	0.970–3500	0.384
Platelets ($\times 10^3$ cells mm^{-3})	128	71–163	**0.005**	91	45–145	0.342
AST (U l^{-1})	158	51–286	**0.011**	57	36–172	0.500
Urea (mg dl^{-1})	33.5	30.5–52	0.932	33	31–48	0.893
Creatinine (mg dl^{-1})	1.0	0.8–1.1	0.772	0.85	0.7–1.0	0.478
DHL (U l^{-1})	2609	1238–6371	**0.005**	2,687	1476–6453	0.909
CD4+ count (cells mm^{-3})	28	8–68	**0.034**	62.7	22.4–103.1	0.648

Bold values indicates $P < 0.05$.

Brazilian Unified Health System (SUS) has had a noticeable impact on the morbidity and mortality of AIDS patients.[24,25] According to the Joint United Nations Programme on HIV/AIDS (UNAIDS [26]), AIDS-related deaths have decreased by 24% in recent years and the life expectancy of AIDS patients currently exceeds 120 months, at the same time, access to treatment is expanding. Nevertheless, opportunistic infections such as oral candidiasis, diarrhoea and *Pneumocystis jiroveci* pneumonia remain the leading causes of morbidity and mortality of PLHA in Brazil in the post-HAART era, particularly in those who irregularly used the ARV drugs.[27]Two cohort (1983–1998 and 1999–2002) studies performed in Brazil in children with AIDS have demonstrated that bacterial infections represented the most common opportunistic event, followed by *P. jiroveci* pneumonia.[28] In French Guiana, an endemic area for histoplasmosis, a high incidence of this mycosis was observed even in the post-HAART era.[29]

The mean rate of adherence to treatment observed in numerous interventions studies reached approximately 50% for all types of treatment in patients with any chronic diseases.[30,31] Several concepts of adhesion can be identified. In a more restricted sense, adhesion may correspond to the concept of obedience to the health team recommendations. In a broader and more comprehensive definition, it can be understood as a dynamic and multifactorial process that requires decisions and coresponsibility shared between PLHA and the health team. Must be understood, as a process of negotiation between the patient and the health professionals, which are recognised the specific responsibilities of each one and that aims to strengthen the autonomy for self-care.[32] In the current study, 41% of the patients had AIDS prior to DH and the majority used HAART irregularly at the end of the observation period. Nachega *et al.* [33], after evaluating 2035 patients, found that 57% had taken 100% of their HAART doses in the past 30 days and that 71% of these patients reported having received practical advice in adherence.

In southeast Brazil, a study evaluating the ARV dispensing registers of a University Hospital in 2009 found an adherence rate of 72.9% in 229 patients.[34] Several factors may contribute to the low rate of compliance and irregular use of prescribed drugs in AIDS patients, such as changes in life routine, daily use of medications, number of pills, side effects, difficulty in understanding the prescription, alcohol use, lack of higher education, low socioeconomic levels and the use of illicit drugs.[35–40] In the Caribbean, a study in 394 PLHA on adherence to HAART reported that the factors associated with good adherence were counselling services, a low consumption of alcohol and drugs and reduced side effects from ARV drugs.[41]

Histoplasmosis is a severe disease with a high mortality in the first presentation and, as recently noted, during the maintenance phase.[8] This information reveals the vulnerability of those patients and the need for programmes directed at treatment adhesion in this specific group. Previously, the HSJ provided the only outpatient services for PLHA in Ceará; this scenario resulted in an excessive number of HIV patients cared for by that institution and, consequently, less frequent follow-up visits. Currently, the state of Ceará has 18 specialised outpatient services for PLHA, nine of which are located in Fortaleza; however, the HSJ remains the main reference hospital responsible for almost all the AIDS hospital admissions for Ceará.

DH typically occurs in patients with advanced AIDS and CD4+ counts less than 150 cells mm^{-3},[2] as demonstrated in this study. Therefore, an early diagnosis of HIV infection is critical to reduce the extreme morbidity and mortality of patients with AIDS and such a diagnosis consequently increases the survival rate after

the HAART initiation.[42] However, a late diagnosis of HIV and AIDS is still a common reality in several states in Brazil.[43–45] Grangeiro *et al.* [43] showed that from 2003 to 2006, patients who had a late detection of HIV in Brazil (CD4+ count < 350 cells mm^{-3}) had a 36% probability of death in the first 12 months after the diagnosis. The absence of an immune recovery was directly associated with relapse and death in the univariate analysis in the current study. In addition, non-adherence to antifungal treatment does not turn out to be an independent risk factor for relapse and death.

Tobón *et al.* [46] comparing DH in patients with and without AIDS and among those with AIDS found that the absence of HAART prevents the response to antifungal treatment, whereas all patients receiving HAART responded ($P = 0.030$); this same study found a significantly higher mortality in the group without HAART, indicating that the immune restoration plays the main role in the illness control. In our study, two of the relapsed patients were adherent to HAART. It is possible that for one of them, the relapsed was due to the secondary antifungal prophylaxis discontinuation, and for the other patient, the histoplasmosis event has been related with the low itraconazole blood levels, although we do not have that information available. Immune reconstitution inflammatory syndrome (IRIS) was not considered in these two cases due to the time elapsed between the HAART initiation and the second DH episode (more than 2 years). IRIS occurs primarily during the first 3 months after HAART initiation, especially in individuals with CD4+ counts lower than 50 cells mm^{-3} that recovered the CD4+ count and suppressed the HIV viraemia by HAART use.[47–49]

Brilhante *et al.* [9] demonstrated that *H. capsulatum* has a high rate of sensitivity to the primary antifungal agents (amphotericin B and itraconazole) used in the treatment of DH in patients from Ceará. In this study, 53.6% of the patients used EFZ, which is recommended by the Brazilian guidelines, together with 2 NRTI, to compose original HAART.[50] It is known that EFZ can decrease itraconazole levels in serum and cause treatment failure,[51–53] consequently resulting in increased relapse and death rates. In this study, EFZ in the HAART regimen was considered a risk factor for the overall mortality in the multivariate analysis, although it had no impact on relapse. A subsequent study involving more relapses needs to be conducted to be sure that the absence of significance was not due to the sample size.

Nevirapine, when coadministered with 200 mg of itraconazole daily, can decrease the itraconazole area under the curve (AUC) by 61%.[54] Otherwise, protease inhibitors may increase itraconazole concentrations, thus prolonging its half-life and leading to its accumulation.[55] No trials of higher dose itraconazole coadministered with non-nucleoside analogue reverse transcriptase inhibitors (NNRTIs) have been performed, so no recommendations in the guidelines for the dose adjustments were found.[56] However, the interaction between these drugs should always be evaluated and this substitution is advised. The 2007 IDSA guidelines for the *Histoplasma* treatment recommend itraconazole blood level measurements to ensure adequate drug exposure[12]; still, this parameter is not available in the hospitals studied. In the state of Ceará, changes in the HAART regimen occur mainly due to adverse reactions of ARV drugs and their interaction with antituberculosis drugs.[57] A routine practice change in the HAART regimen is necessary to reduce the risk of death in these patients because the substitution of itraconazole for amphotericin B has poor applicability due to the duration of the DH treatment.

Laboratory parameters, such as low levels of haemoglobin, leucocytes, platelets and high AST and LDH, as demonstrated here, could indicate which patients are at risk for relapses. In the U.S., the DH patient follow-up is conducted through *Histoplasma* antigenuria and antigenemia; however, these laboratory methods are not available outside that country.[58,59] Lower levels of haemoglobin have been shown to be associated with an 88–91% increase in the risk of progression of the disease or death [60] and haemoglobin <10.0 g dl^{-1} has been considered a predictive factor for death in AIDS patients.[61] De Franchesco Daher *et al.* [7] identified a haemoglobin level <8.0 g dl^{-1} and an AST level = 2.5 × NUL as independent risk factors for death in AIDS patients with DH during admission in the HSJ. Despite these results, in the current study, no laboratory changes were associated with death by histoplasmosis during the follow-up.

There are some limitations present in this study. Because the study involved a retrospective cohort, missing information and a loss of follow-up could have interfered with the data collection and statistical analysis. In addition, the relapse definition used in the methods section was obtained from multiple data sources because no standardisation exists in the literature.

Finally, we emphasise an urgent need to identify difficulties in adherence to HAART, especially in patients with AIDS/DH coinfection in this highly endemic part of Brazil. Beyond access to health services and the universal distribution of ARV drugs by the Brazilian government, it is necessary for the adoption of intervention strategies to improve the adhesion to HAART

through the appropriate follow-up, rapid diagnosis of relapses, adequacy of the HAART regimen and prophylaxis to prevent opportunistic illness.

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Conflict of interest

We declare that there are no conflicts of interest.

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Artigo 2

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Note

Multiple opportunistic fungal infections in an individual with severe HIV disease: A case report

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ABSTRACT

Background: Fungal infections have been commonly diagnosed in individuals with advanced HIV disease. Cryptococcosis, pneumocystosis, and histoplasmosis are the most frequent systemic mycoses in people suffering from HIV/AIDS.
Case report: We report a case of multiple fungal infections in an advanced AIDS-patient. A 33-year-old HIV-positive man from Brazil was hospitalized due to diarrhea, dyspnea, emaciation, hypoxemia, extensive oral thrush, and a CD4+ T lymphocyte count of 20 cells/mm^3. Honeycombed-structures consistent with *Pneumocystis jirovecii* were observed by direct immunofluorescence in induced sputum. *Cryptococcus neoformans* was recovered from respiratory secretion and cerebrospinal fluid cultures. Histopathology of the bone marrow also revealed the presence of *Histoplasma capsulatum*. Molecular assays were performed in a sputum sample. Nested-PCR confirmed the presence of *P. jirovecii* and *H. capsulatum*; qPCR multiplex was positive for *C. neoformans* and *H. capsulatum*. With the treatment of antifungal drugs the patient progressed satisfactorily.
Conclusions: The diagnosis of several systemic mycoses demonstrates the vulnerability of advanced AIDS-patients. Thus, the detection of AIDS cases in the early stages of infection is necessary for a prompt and adequate introduction of HAART therapy, and the use of prophylaxis to control opportunistic infections.

Múltiples infecciones fúngicas oportunistas en un individuo con enfermedad grave por VIH. Informe de un caso

Palabras clave:
Neumocistosis
Criptococosis
Histoplasmosis
Sida
Síndrome inflamatorio de reconstitución inmune

RESUMEN

Antecedentes: Las infecciones fúngicas se han diagnosticado comúnmente en individuos con enfermedad avanzada por VIH. La criptococosis, la neumocistosis y la histoplasmosis son las micosis sistémicas más frecuentes en personas con VIH/sida.
Caso clínico: En este trabajo se describe un caso de múltiples infecciones fúngicas en un paciente con sida avanzado. Un hombre de 33 años, brasileño, con serología positiva para VIH, fue hospitalizado con pérdida de peso, diarrea, disnea, caquexia, hipoxemia, extensa candidiasis oral y recuento de linfocitos CD4+ de 20 cél./mm^3. La inmunofluorescencia directa puso de manifiesto estructuras típicas compatibles con *Pneumocystis jirovecii*. En el cultivo de las muestras de secreción respiratoria y de líquido cefalorraquídeo creció *Cryptococcus neoformans*. En el análisis histopatológico de una muestra de médula ósea se observó *Histoplasma capsulatum*. Se llevaron a cabo ensayos de marcadores moleculares en una muestra de esputo. Se realizó un ensayo de PCR anidada que fue positivo para *P. jirovecii* y *H. capsulatum*, y una qPCR multiplex que fue positiva para *C. neoformans* e *H. capsulatum*. Con un tratamiento con antimicóticos el paciente evolucionó satisfactoriamente.

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Conclusiones: El diagnóstico de las micosis sistémicas demuestra la vulnerabilidad de los pacientes con sida avanzado. El diagnóstico de la infección por VIH en sus etapas iniciales es fundamental para la introducción precoz y adecuada de la terapia antirretroviral altamente activa y la profilaxis de las infecciones oportunistas.

The highly active antiretroviral therapy (HAART) has considerably changed the characteristics of the HIV epidemic. However, many patients still present to health services with advanced HIV-related immunosuppression, particularly in developing countries.[3] Although pulmonary tuberculosis remains as the main opportunistic infection in HIV-patients, systemic mycoses have been commonly diagnosed in this population, particularly in individuals who live in endemic areas of these mycoses.[6,16]

In the early 1980s, *Pneumocystis jirovecii* pneumonia was the most common pulmonary infection associated with advanced HIV disease. However, cryptococcal meningitis (CM) accounts nowadays for the majority of worldwide deaths from HIV-related fungal infection.[3,16] In the sub-Saharan Africa and Latin America, cryptococcosis, pneumocystosis, and histoplasmosis are the most frequent systemic mycosis in people with HIV/AIDS (PHA).[7,8] These infections are acquired by inhalation of propagules, and can occur as an acute pulmonary infection or as an endogenous reactivation of a latent infection in immunocompromised individuals.[12]

P. jirovecii infection is usually characterized by interstitial pneumonia leading to respiratory failure.[3,12] *Cryptococcus* and *Histoplasma capsulatum* infections can affect only the lungs. However, systemic dissemination to the central nervous system and lymphomonocytic system can also occur, leading to several and fatal cases of CM or disseminated histoplasmosis, respectively.[8,16] Here we report a case of multiple fungal infections in an individual with advanced HIV disease.

Case report

The 33-year-old, HIV-positive male patient from Brazil was hospitalized in August 2013 due to diarrhea and dyspnea. At the moment of hospitalization, the patient presented emaciation, tachypnea, hypoxemia, extensive oral thrush, and a CD4+ T lymphocyte count of 20 cells/mm^3. Chest tomography showed ground-glass opacity, particularly in the lung apices. The induced sputum sample was submitted to microbiological tests. Microscopical examination of a cerebrospinal fluid sample was made in order to diagnose or rule out CM. India ink preparation confirmed the presence of yeast-like *Cryptococcus* (Fig. 1A). Direct immunofluorescence showed the presence of typical honeycombed-structures compatible with *P. jirovecii* (Fig. 1B). Culture of respiratory secretion on agar Sabouraud and modified Middlebrook 7H9 broth base (MGIT – Mycobacteria Growth Indicator Tube) showed the growth of *Cryptococcus* sp., and *Mycobaterium tuberculosis* complex, respectively. The species *Cryptococcus neoformans* was identified by *Cryptococcus* inoculum on canavanine-glycine-bromothymol blue (CGB) medium. The patient received a treatment with trimethoprim-sulfametoxazole, amphotericin B, fluconazole, and tuberculostatic drugs. After 20 days of hospitalization, HAART was started with tenophovir, lamivudine, lopinavir and ritonavir. Immunological improvement (CD4+ T lymphocytes count = 291 cells/mm^3) was observed in October 2013. However, the patient developed intermittent fever, pancytopenia, hepatomegaly and splenomegaly. In the examination of induced sputum sample, yeast cells with single budding, compatible with *H. capsulatum*, were observed (Fig. 1C). Bone-marrow histopathology showed fungal structures compatible with *H. capsulatum* and *C. neoformans* stained by Gomori-Grocott silver stain (Fig. 1D). Molecular assays were also performed in the sputum for diagnostic confirmation. Nested-PCR confirmed the presence of *P. jirovecii*[19] and *H. capsulatum*[5] (Fig. 2). A multiplex real-time PCR assay (qPCR Multiplex)[9] for detecting simultaneously *P. jirovecii*, *C. neoformans* and *H. capsulatum* was performed, and a positive result for *C. neoformans* and *H. capsulatum* was obtained. After four months of hospitalization, the patient improved satisfactorily and was discharged.

Discussion

It is estimated that a late diagnosis of HIV infection can occur in up to 60% of all patients.[2] In Brazil, although the health public policies have considerably contributed to a most extensive access of the population to serological tests and HAART, with remarkable impact on the mortality rate, it is estimated that approximately 40,000 new cases of HIV infection still occur annually.[14] Therefore, the late diagnosis is still a reality, which favors the identification of AIDS cases in an advanced stage (CD4+ T lymphocyte count below 200 cells/mm^3 or AIDS clinical symptoms).[10]

It is estimated that CM affects almost one million PHA with approximately 650,000 deaths per year.[16] Pneumocystosis and histoplasmosis affect to more than 300,000 PHA annually.[6,7] In Brazil, the systemic mycoses are not subjected for epidemiologic surveillance. The real prevalence and incidence of these diseases are unknown. However, the main fungal infections associated with AIDS mortality in Brazil are cryptococcosis, candidiasis and histoplasmosis.[17] To date, only a seroprevalence study has been performed in a histoplasmosis endemic area of northeastern Brazil in PHA with CD4+ count >350 cells/mm^3, by means of a skin test with histoplasmin. The results showed a histoplasmosis prevalence of 12% in this region.[4]

Fungal infections also are favored by immune response inflammatory syndrome (IRIS), which is due to an intense and exacerbated inflammatory response associated with the immune reconstitution caused by HAART.[18] A CD4+ T lymphocyte count below 100 cells/mm^3, a rapid decrease in viral load and the institution of HAART during the first month of treatment of opportunistic infections are the risk factors strongly associated with IRIS.[13] It is characterized by paradoxical clinical deterioration, when the patient develops a worsening of the infection under treatment or develops new opportunistic infections. Fungi most commonly related to IRIS are *C. neoformans* and *H. capsulatum*.[11,15] In the case herein reported, disseminated histoplasmosis was diagnosed after the start of HAART and, thus, we believe this mycosis occurred due to IRIS.

The diagnosis of systemic mycosis is time consuming and laborious because usually requires the identification of the fungus in biological samples by culture, histopathological or direct examination with proper stainings.[12] The lack of proper facilities and medical supplies to perform the diagnosis of these opportunistic fungal infections such as specific culture media, specialized professionals to perform invasive procedures (lumbar puncture and bone marrow biopsy) or immunofluorescence techniques, may delay the

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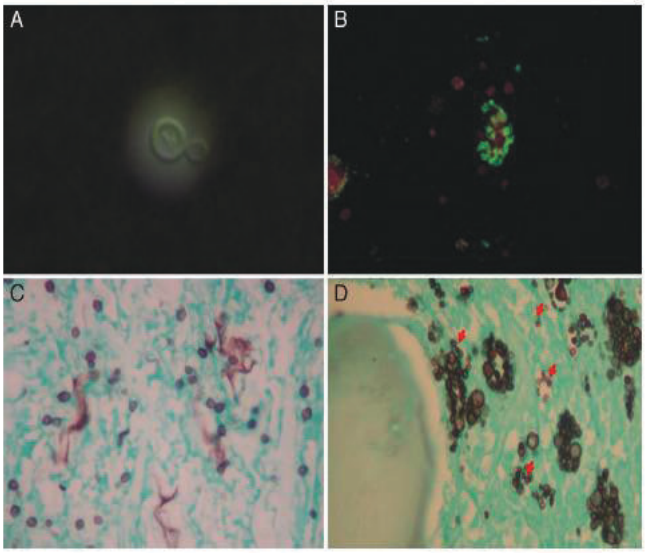

Fig. 1. Fungal agents. (A) India ink preparation of cerebrospinal fluid showing an encapsulated yeast-like *Cryptococcus neoformans* (1000x). (B) Direct immunofluorescence in induced sputum showing the honeycombed-structures consistent with *Pneumocystis jirovecii* (400x). (C) Gomori-Grocott silver stain in induced sputum showing small fungal cells consistent with *Histoplasma capsulatum* (400x). (D) Bone marrow histopathology showing yeasts consistent with *Histoplasma capsulatum* (arrows) and *Cryptococcus neoformans* (Gomori-Grocott silver stain, 400x).

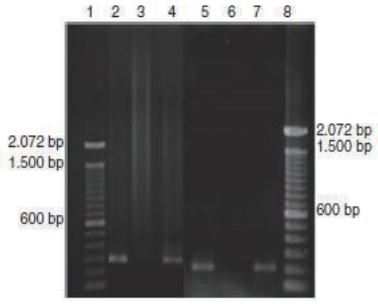

Fig. 2. Representative nested PCR. Lanes 1 and 8: Molecular marker DNA ladder, 100 bp (Invitrogen); Lanes 2 and 4: *P. jirovecii*; Lanes 5 and 7: *H. capsulatum;* Lanes 3 and 6: Negative controls.

diagnosis of these infections and, therefore, avoid the appropriate treatment. Molecular techniques show improved sensitivity and specificity when compared to conventional diagnostic methods; nevertheless, these tests are only standardized in some research institutions and are not commercially available.[1]

In conclusion, the detection of several systemic mycoses demonstrates the vulnerability of advanced AIDS-patients. The introduction of HAART and primary prophylaxis to opportunistic infections has promoted better quality of life in these individuals. Thus, it is necessary to improve the detection of HIV infection in the early stages. In addition, it is indispensable a major investment in health services, laboratories and reference hospitals for the care of PHA to improve the time of diagnosis, allowing a correct and adequate treatment of these patients.

Conflict of interest

The authors declare that there is no conflict of interest among them or with any financial organization regarding the material discussed in the present manuscript.

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Artigo 3

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BMC Infectious Diseases

RESEARCH ARTICLE Open Access

Validation of western blot for *Histoplasma capsulatum* antibody detection assay

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Abstract

Background: Histoplasmosis is worldwide systemic mycoses caused by the dimorphic fungus *Histoplasma capsulatum*. The isolation and identification of *H. capsulatum* in culture is the reference test for histoplasmosis diagnosis confirmation. However, in the absence of it, serology has been used as a presumptive diagnosis through antibody and antigen detection. The purpose of the present study was to validate an immunoassay method (western blot) for antibodies detection in the diagnosis of histoplasmosis.

Methods: To validate the western blot (WB) a study was conducted using 118 serum samples from patients with histoplasmosis and 118 serum controls collected from January 2000 to December 2013 in residents of the Rio de Janeiro State, Brazil. Diagnostic validation parameters were calculated based on the categorization of results obtained in a 2 × 2 table and subjected to statistical analysis. In addition, the viability of deglycosylated histoplasmin antigen (ptHMIN) onto nitrocellulose membranes previously sensitized was evaluated during the same period.

Results: The WB test showed sensitivity of 94.9 %, specificity of 94.1 %, positive predictive value of 94.1 %, negative predictive value of 94.9 %, accuracy of 94.5 %, and almost perfect precision. Besides, the strips have proved to be viable for using at least 5 years after ptHMIN antigen sensitization.

Conclusion: Western blot test using ptHMIN provides sensitive, specific, and faster results. Therefore, could be considered a useful tool in the diagnosis of histoplasmosis being used by public health system, even in situations where laboratory facilities are relatively limited.

Keywords: Histoplasmosis, Immunodiagnostic, Western blot, Sensitivity, Specificity

Background

Histoplasmosis is a systemic disease caused by the dimorphic fungus *Histoplasma capsulatum*. This disease has a worldwide distribution and is one of the most common respiratory mycosis, presenting endemic areas in certain regions of the United States and Latin America [1, 2]. In Brazil, these regions are located throughout the country, especially in the Midwest, Northeastern and Southeast regions [3, 4]. The infection is acquired by inhalation of fungal infectious propagules present in organic matter rich soil, mainly with excreta of birds and bats [1].

The clinical spectrum of this illness ranges from asymptomatic, self-limited illness to a progressive disseminated disease. Although the clinical manifestations of histoplasmosis are well described, there is significant overlapping of symptoms with other diseases, and the diagnosis cannot be achieved based on clinical information alone. [5]. Microbiological diagnosis is based on isolation of the fungus in cultures, and microscopic examination of fluids and tissues using specific staining techniques [5]. Nonetheless, these methods have limitations. Culture examination is slow, taking up to 2–4

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weeks, and requires level 3 biosafety facilities [6]. The sensitivity and specificity of histopathological examination vary depending on the patient's clinical status, exhibiting reduced sensitivity in the subacute and chronic forms of the pulmonary histoplasmosis [6, 7]. In addition, the specific role of each test fluctuates according to the clinical form, since variations in sensitivity have been associated to different clinical presentations.

Serological methods usually have a rapid turnaround time and detection of either antibodies or antigens can provide information indicative of current disease. Historically the identification of antibody responses in patients with histoplasmosis has proved useful in the diagnosis of disease since serological evidence is the prime diagnostic indicator of histoplasmosis [8].

Serological diagnosis for histoplasmosis focuses on the identification of antibodies to the H [9] and/or M antigens [10]. The two routine antibody detection methodologies are complement fixation (CF) and immunodiffusion (ID), because of convenience, availability, and accuracy of these assays. In the past, CF was a popular test to diagnose histoplasmosis but ID has been found to be more specific [3]. The ID test qualitatively measures precipitating antibodies, and is specific for the detection of antibodies to M and H antigens, ranging from 70 to 100 % [11]. However, it has low sensitivity, mainly in acute, disseminated and opportunistic manifestations of the disease. In general, ID is useful for detecting antibodies 4–6 weeks after infection with *H. capsulatum*. Detection of precipitins by immunodiffusion is one of the most widely available techniques for diagnosis [3], and although the presence of immunodiffusion bands are less reliable than culture, anti-Histoplasma antibodies may be detected in the serum of 90 % of patients with histoplasmosis [12]. The presence of M and H bands is highly suggestive of active Histoplasma infection [8]. Several enzyme-linked immunosorbent assays (ELISA) protocols have been described for antibody detection in histoplasmosis using diverse antigenic preparations showing sensitivity among 75 to 100 % [13–16].

The reference protocols for the evaluation of the diagnosis of infectious diseases show that the diagnostic target must first be identified, followed by the optimization of reagents and test used. Then, the performance of the method should be evaluated [17]. In the past, a WB test was developed for detection of antibodies against native glycosylated and chemically deglycosylated M and H antigens of *H. capsulatum* in serum obtained from patients during the acute phase of pulmonary histoplasmosis showing 90 and 100 % sensitivity, for the acute and convalescent-phase respectively, and 100 % specificity [18]. This test met the requirements of a good diagnostic test for the acute and convalescent-phase of histoplasmosis. The advantage of the WB test in relation to routine serology is the identification of some cases early in infection, before seroconversion can be detected by CF and ID, showing a high degree of sensitivity and specificity, Also, this methodology is faster and easier to realize than those tests used in diagnostic routine. However, these immunoassays were used only in acute and proven histoplasmosis cases [16, 18], and it would be very important validate this methodology for diagnosis of several forms of histoplasmosis in a large number of cases, since it could be used in conjunction with culture to improve the diagnosis of *H. capsulatum* infection particularly in cases when microorganism isolation procedure is negative, and also guide for the specific therapy [5].

Our aim in this study was to validate this western blot immunoassay to detect antibodies in the serodiagnosis of histoplamosis following the reference protocols for the evaluation of diagnostic tests for infectious diseases in order to determine the diagnostic accuracy of this test. In addition, the antigenic reactivity of deglycosylated histoplasmin antigen (ptHMIN) onto nitrocellulose membranes previously sensitized was evaluated during the same period as a secondary objective of this study.

Methods

Target population and histoplasmosis case definition

A study was conducted in a total of 236 serum samples collected from January 2000 to December 2013 at the Instituto Nacional de Infectologia Evandro Chagas (INI), Fiocruz from residents of Rio de Janeiro State without prior treatment for histoplasmosis. The target population was separated into two groups, histoplasmosis group and a control group. The histoplasmosis group ($n = 118$) was comprised of patients with epidemiological and clinical history compatible with histoplasmosis. The diagnosis criteria were based on a positive result in culture, or *Histoplasma* antibody detection demonstrating H or M precipitin bands by immunodiffusion [19]. The control group was obtained based on the proportion 1:1 case–control, a total of 118 individuals of the same population. The serum samples of control group were randomly obtained from 40 patients with clinical suspicion of fungal infection, but excluded of the diagnosis criteria selected, 28 patients suspected of other pulmonary mycosis (paracoccidioidomycosis, $n = 10$; aspergillosis, $n = 9$; coccidioidomycosis, $n = 6$; and cryptococcosis, $n = 3$); 10 patients with tuberculosis; and 40 healthy blood donors (Fig. 1).

The definition of histoplasmosis was based on the consensus EORT/MSG [20], and the clinical forms followed recommendations [7, 21]. Proven histoplasmosis included individuals with identification of *H. capsulatum* yeasts in cultures or histopathological analyzes. Positive ID combined with clinical and radiologic findings were required for classification of probable disease. All individuals included in the study were probed to

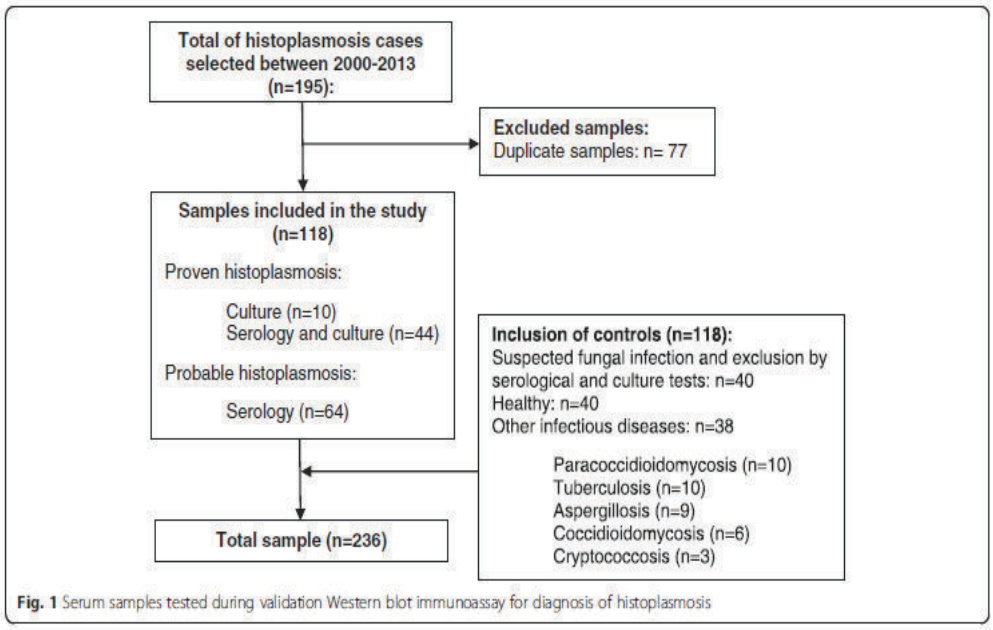

Fig. 1 Serum samples tested during validation Western blot immunoassay for diagnosis of histoplasmosis

detect antibodies against *H. capsulatum* by WB and ID tests.

Study design

This is a retrospective study based on standard clinical, laboratorial and epidemiological dates collected in the medical records of INI/Fiocruz.

The variable analyzed were age, gender, comorbidity (AIDS, tuberculosis), specific laboratory tests as serology by ID, mycological tests and clinical form of illness.

Clinical and laboratory data were collected by an independent investigator, blinded to clinical information.

Ethical statement

This study was approved by the Research Ethics Committee of the Instituto Nacional de Infectologia Evandro Chagas, Fiocruz, accession number 19109913.0.0000.5262.

Serologic tests and antigens

ID tests to detect antibodies to histoplasmin (HMIN), paracoccidioidin and *Aspergillus fumigatus* antigen were performed on serum specimens obtained from all individuals enrolled in this study [22]. HMIN was produced from mycelium-form cultures of *H. capsulatum* IGS 4/5 (INCQS 70308) as described previously [23], and H and M antigens were chromatographically purified [24]. Chemical deglycosylation was achieved according to previously studies [25, 26]. Briefly, sodium *meta*-periodate ($NaIO_4$) was added to 2-ml aliquots of purified antigens to a final concentration of 100 mM. After incubation for 18 h at 4 °C in the dark, residual periodate was consumed by incubation for 15 min with an equimolar amount of glycerol, followed by addition of 1 M sodium borohydride. After incubation for 2 h at 4 °C, the reaction mixture was dialyzed against cold deionized water.

Western blot (WB) immunoassay

Native and deglycosylated antigens were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), on 10 % polyacrylamide resolving gels with a 4 % polyacrylamide staking gel. The gels were then processed for western blot performed according to the protocol previously established [18]. Briefly, after electro transfer of proteins (16.75 ug) to nitrocellulose membranes in a Mini Trans Blot cell (Bio-Rad), membranes were sliced vertically, and free binding sites in the membranes were blocked by incubation for 60 min in 5 % (wt/vol) nonfat milk in 20 mM Tris–HCl–500 mM NaCl–0.2 % Tween 20 (pH 7.5) (TTBS). Next, strips were incubated for 60 min at room temperature with serum specimens diluted 1:100 in TTBS containing 5 % nonfat milk. Strips were washed in TTBS three times for five minutes each; and then goat anti-human immunoglobulin G (IgG)-alkaline phosphatase conjugates (Jackson ImmunoResearch, EUA) diluted in TTBS (1:3.000) were added and incubated as described above. Following incubation, blot strips were washed and incubated with substrate solution consisting of 5-bromo-4-chloro-3-indolylphosphate (BCIP; 15 mg/ml in dimethylformamide [DMF]) and nitroblue tetrazolium (NBT; 30 mg/ml in 70 % aqueous DMF). Substrate stock solutions were diluted 1:100 before use in Tris/NaCl buffer (100 mM

Tris–HCl [pH 9.5], 100 mM NaCl, 50 mM $MgCl_2$). After color development strips were rinsed exhaustively in deionized water.

Two experienced investigators with longstanding experience in the WB performed the analysis in the anonymous sera, blindly with regard to the clinical and laboratorial findings. The samples were identified with sequential numbering by a third investigator avoiding the identification of cases and controls by the executors of the analysis. The immunoassays were then carried out separately for each of the two investigators, including all steps of the methodology. The test was repeated three times with readings performed by two investigators in order to evaluate the intra and interobserver agreement index.

Western blot performance characteristics

To calculate the performance characteristics of the test, the true-positive (TP), false-positive (FP), false-negative (FN), and true-negative (TN) results were analyzed [27].

Analysis of the membranes

Nitrocellulose membranes previously sensitized with the antigen ptHMIN, lot # Hc05 were maintained at room temperature and tested progressively for the antigenic reactivity over the time periods of 1 day, 1 month, 6 months, 1, 2, 3, 4 and 5 years, by WB with serum sample (#20965) obtained from a proven histoplasmosis case to assess the viability of the membranes containing ptHMIN, respecting the conditions listed above.

Statistical analysis

The epidemiological and laboratorial data were evaluated by bivariate analysis using Chi-squared test or Fisher exact test if any value in the cells of the contingency table was less than five. Results were considered statistically significant when $p < 0.05$. The diagnostic accuracy of the WB was evaluated by sensitivity, specificity, predictive values and likelihood ratios, with ranges of 95 % confidence intervals (CI). The reproducibility of the test was calculated and classified using agreement index by Kappa coefficient according to Landis & Koch (1977) [28]. In addition, we used odds ratio (OR) to measure the presence of H and M bands simultaneously in the laboratorial tests applied in this study. All data were subjected to statistical analysis by using of Statistical Package for Social Sciences SPSS software, version 16.0.

Results

Baseline characteristics of patients

Between January 2000 and December 2013, 118 cases of histoplasmosis were diagnosed in the Instituto Nacional de Infectologia Evandro Chagas, and could be filled on the criteria of inclusion for this study (Fig. 1). The mean age of all patients (cases and controls) was 41.5 years (10-81 years), 75.4 % of the patients were male and 24.6 % female. Among this population, 37.3 % were HIV-patients (Table 1).

The clinical form most commonly found in all patients with histoplasmosis was pulmonary (50.8 %), followed by disseminated (36.5 %) and mediastinal histoplasmosis (4.2 %). Fifty-four patients had proven histoplasmosis. In the proven cases of histoplasmosis the pathogen was obtained by culture isolation in 60 clinical samples such as bone marrow ($n = 15$), respiratory secretion ($n = 14$), skin lesions ($n = 9$), blood ($n = 8$), mucosal lesions ($n = 8$) and lymph node aspirate ($n = 6$). It must be emphasized that six patients had the fungus isolated from two different clinical samples. In addition, sixty-four were classified as probable histoplasmosis (40 acute pulmonary, 6 chronic pulmonary, 5 disseminated, 3 mediastinal, and 10 non-defined clinical and serological suspected cases who could not be classified in none of the preceding forms) [7, 21] based on the presence of M and/or H bands in immunodiffusion in conjunction with clinical and radiologic findings.

Immunodiffusion test

All the samples from patients with histoplasmosis were re-tested by ID for the detection of anti-*H. capsulatum* antibodies. Of 118 sera from patients with histoplasmosis, 103 (87.3 %) had reactivity, characterized by the presence of at least one line of precipitation. Fifteen samples (12.7 %) were negative. The results were compared with those performed immediately after blood collection, demonstrating excellent agreement (Kappa = 0.96). None of

Table 1 Baseline characteristics histoplasmosis cases ($n = 118$) and control group ($n = 118$)

Variables	Cases n° (%)	Controls n° (%)	*p* value**
Sex			
Men	89 (75.4)	90 (76.3)	1.000
Woman	29 (24.6)	28 (23.7)	
Age (years)			
<40	57 (48.3)	51 (43.2)	0.513
≥40	61 (12.7)	67 (11.0)	
HIV status			
Positive	44 (37.3)	22 (18.6)	0.002
Negative	21 (17.8)	59 (50.0)	
Non-tested	53 (44.9)	37 (31.4)	
Tuberculosis[a]			
Present	20 (16.9)	17 (14.4)	0.720
Absent	43 (36.4)	30 (25.4)	
Missing	55 (46.6)	71 (60.2)	

[a]Diagnosis by isolation of *Mycobacterium tuberculosis* in sample of various clinical specimens; **p value < 0.05

the 118 control sera that were probed for histoplasmosis, paracoccidioidomycosis, and aspergillosis, were prior positive by this method.

Western blot

Any well-defined band, with molecular weight of 115 and 88 kDa represent the specific antibodies against H and M antigen, respectively (Fig. 2). H and M bands were identified in sera of 47 patients with histoplasmosis (39.8 %). In 65 samples (55.1 %) it was verified just the M band and in six patients (5.1 %) no band was identified.

Diagnostic Accuracy of the WB for anti-*H. capsulatum* antibodies detection

The analysis of 118 serum samples from proven and "probable histoplasmosis" showed that 112 were correctly classified as true-positive samples and only six samples had false-negative results. These samples were from HIV-patients with disseminated histoplasmosis. Of the 118 control samples, false-positive results were observed in seven samples. Cross-reactivity has seen in sera from patients with paracoccidioidomycosis (5), aspergillosis (1) and tuberculosis (1).

The results of the diagnostic accuracy and their confidence intervals are shown in Table 2. The WB had a sensitivity of 94.9 % (95 % CI: 92.1 to 97.7 %), a specificity of 94.1 % (95 % CI: 91.1 to 97.1 %), an accuracy of 94.5 % (95 % CI: 91.6 to 97.4 %), a positive predictive value (PPV) of 94.1 % (95 % CI: 91.1 to 97.1 %), and a negative predictive value (NPV) of 94.9 % (95 % CI: 92.1 to 97.7 %).

The reproducibility of the test has shown that the intraobserver and interobserver agreement (Kappa = 0.99 and 0.96, respectively) were classified as almost perfect.

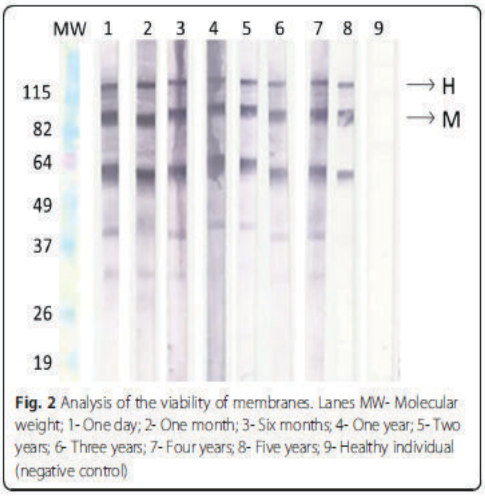

Fig. 2 Analysis of the viability of membranes. Lanes MW- Molecular weight; 1- One day; 2- One month; 3- Six months; 4- One year; 5- Two years; 6- Three years; 7- Four years; 8- Five years; 9- Healthy individual (negative control)

Table 2 Serological parameters for the Western blot using ptHMIN

Parameter	Values (%)	CI[a] 95 %
Sensitivity	94.9	92.1–97.7
Specificity	94.1	91.1–97.1
Accuracy	94.5	91.6–97.4
PPV[b]	94.1	91.1–97.1
NPV[c]	94.9	92.1–97.7
Positive likelihood ratios	16.0	-
Negative likelihood ratios	0.1	-

[a](*CI*) confidence interval; [b](*PPV*) positive predictive value; [c](*NPV*), negative predictive value

Differently from ID; where both H and M precipitin bands were observed just in 8.9 % of the positive samples tested, in WB the H and M were detected simultaneously in 42.0 % of samples, showing a gain in sensitivity (Table 3).

Analysis of the membranes

The evaluation of the viability of previously sensitized nitrocellulose membranes with the antigen ptHMIN was performed by WB probed against the serum sample # 20965 from proven histoplasmosis patient. It was demonstrated reactivity on the membranes up to 5 years (Fig. 2).

Discussion

The definitive diagnosis of histoplasmosis is usually based on the isolation and identification of the *H. capsulatum* in cultures or by identification of fungi in biological samples with special staining [7, 29]. However, this process is time-consuming and has limitations in sensitivity [30], mainly in acute and disseminated histoplasmosis that need be rapidly diagnosed for the prompt initiation of therapy [31].

Presently, there are additional diagnostic tools available for diagnosis of histoplasmosis to supplement culture and microscopic examination. These laboratory tests have a rapid turnaround time and reasonable specificity and sensitivity. For instance, serological techniques involving antibody and antigen detection have been developed using different methodologies. Immunoassays for antibody detection using ptHMIN, has played an important role in the diagnosis of histoplasmosis showing high sensitivity and specificity for diagnosis of histoplasmosis. Nevertheless, these immunoassays were used only in acute forms and proven histoplasmosis cases [16, 18].

In the past, a WB test was developed for detection of antibodies against native glycosylated and chemically deglycosylated M and H antigens of *H. capsulatum* in serum obtained from patients during the acute phase of pulmonary histoplasmosis showing 90 and 100 %

Table 3 Performance results of serological tests for detection of antibodies against *Histoplasma capsulatum*

Histoplasmosis	Immunodifusion (ID)[a]		Western blot (WB)[a]		*p*-value**	OR
	H and M bands	M band	H and M bands	M band		
Proven ($n = 54$)	8	36	21	27	0.009	3.88
Provable ($n = 64$)	2	62	26	38	0.000	21.21
Total ($n = 118$)	10	98	47	65	0.000	7.08

[a]Cases non reagent: ID – 10 cases; WB – 6 cases; **p-value < 0.05 (Chi-square Test and Fisher Exact Test)

sensitivity, for the acute and convalescent-phase respectively, and 100 % specificity [18]. These results can be compared with the values of sensitivity (94.4 %) and specificity (94.1 %) obtained in this study. However, in the latter it was evaluated serum samples from other clinical forms of histoplasmosis as well from individuals with other fungal infections, with tuberculosis besides healthy individuals, which could explain the small differences found in the sensitivity and specificity parameters. In addition, the results presented here demonstrate the utility of this methodology in the diagnosis of histoplasmosis through more robust evaluation in different populations being faster than those test used in diagnostic routine, and should be applied in microbiology laboratories since has almost perfect reproducibility, producing repeated and consistent results.

The WB evaluated here showed to be an excellent diagnostic test for histoplasmosis since it fits all criteria suggested by international guides for accuracy in testing such as high sensitivity and specificity; ease to use and storage [17, 32]. Then, this is the first description about WB validation using ptHMIN for diagnosis of several forms of histoplasmosis in a large number of cases. Serological histoplasmosis diagnosis is focused on the identification of anti-H and anti-M antibodies by ID and the presence of both the H and M precipitins in a serum sample is considered to be conclusive for the diagnosis of this mycoses [3]. However, the M band is mostly found in acute and chronic forms of histoplasmosis. This band persists for months to years after the infection has resolved [30], and the H band is present only in 7.0 % of sera from patients with acute histoplasmosis, and rarely can be found without M band. The presence of H band is indicative of chronic or severe acute forms of histoplasmosis [33]. In this study, the presence of both anti-M and anti-H antibodies was more frequently observed in WB than ID, in both proven and probable cases of histoplasmosis due the higher sensitivity of primary binding assays, such as ELISAs and western blot than precipitin tests, and mainly for the inactivation of carbohydrate epitopes present in our antigenic preparation that led to increased WB test sensitivity [18, 26]. WB could be useful as conclusive method of laboratorial diagnosis of this mycosis.

Although, the WB showed a high sensitivity, false negative results were found in six HIV-patients with confirmed disseminated histoplasmosis. This mycosis usually occurs in HIV-patients that have advanced immunosuppression [34, 35]. Probably, the absence of detectable antibodies may be associated with this event. The time of serum collection is also an important variable in the detection of antibodies in histoplasmosis since anti-M and anti-H antibodies are detected between 2 and 6 weeks, and posteriorly formation of immune complexes [30].

Reactivity against the M band (88 kDa), was also observed in 7 false-positive serum samples; five sera from patients with paracoccidiodomycosis, one with aspergillosis, and one with tuberculosis, similar to previously results [18]. However, antibodies against M band can persist for long time after disease resolution [3] without clinical manifestations compatible with histoplasmosis. For instance, in individuals from endemic areas, such as Rio de Janeiro state, the presence of these antibodies can represent only previous exposition to *H. capsulatum*. In these cases, the results should be carefully interpreted, and in this way, does not lead to a mistaken diagnosis of histoplasmosis in patients affected with other infectious diseases [33]. However, the association of clinical and epidemiological data with the laboratory evidences address in favor of the reliability of the test. In addition, co-infections with other fungal infections or granulomatous lung diseases such as tuberculosis may coexist in these patients [36]. This event also may usually occur in individuals with severe immunosuppression or in those that suffer of other pulmonary disease [37, 38].

The accuracy is an essential parameter for the validation of a diagnostic test, and it is characterized by reproducibility measurements of the test. In this study we observed intra- and interobserver agreement in the WB test, which classify the test as almost perfect, making it possible to be applied in multicenter studies.

It has been demonstrated that nitrocellulose membrane is optimal for transfer and subsequent binding of specific proteins. We previously demonstrated that western blot using nitrocellulose membranes probed with ptHMIN was an excellent method to detect antibodies in patients infected with *H. capsulatum* [18, 25, 26]. However, up to date, there is not information about the

time that the nitrocellulose membranes, sensitized with ptHMIN antigen, can remain viable and without structural damage for the use in WB [18, 25, 26]. Our study demonstrated that WB sensitized strips may be stored at room temperature up to 5 years, presenting reactivity in WB without compromising test quality. With this, there is a potential for pre-manufactured blot strips to simplify test performance, permitting the distribution to other laboratories in order to promote a worldwide multicenter evaluation.

Future studies of WB validation will be conducted in different Brazilian regions in order to evaluate a large number of samples. Furthermore, additional evaluation of this assay is needed to better characterize its clinical sensitivity in untreated and treated patients, the kinetics of antibody clearance as measured by this test, and the ability of antibody tests in general to predict treatment success or failure, mainly in the acute form of histoplasmosis. Importantly, the validated test is simple to perform, requiring only a basic lab infrastructure for its realization. This method can be performed by any professional, if properly trained and special attention should be given to reading the test result.

Conclusion

The WB for diagnosis of histoplasmosis, using ptHMIN antigen, showed that this method can be considered a useful tool in the diagnosis of histoplasmosis being used by public health system, even in situations where laboratory facilities are relatively limited. In addition, WB is faster than those test used in diagnostic routine, and should be applied in microbiology laboratories since has almost perfect reproducibility, producing repeated and consistent results. Cost evaluations are necessary to completely define the role of this technique on large scale. As future perspective, multicenter trials should be held involving laboratories from other Brazilian regions or even other countries engaged in the diagnosis of this mycosis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MAA, CVP, MMM, RAP, RHSP, carried out the laboratory work of the study; AGV obtained, donated the control serum samples and carried out the evaluation and interpretation of data; MAA, LSD, drafted the manuscript; CLTA, RMZO, participated in the design of the study; MAA, LSD, RVCO, CLTA, evaluation and interpretation of data; JMP, RMZO, revised manuscript. All authors have contributed intellectually during the writing process and have read and approved the final manuscript.

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Artigo 4

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Short Communication

Spatial distribution of disseminated histoplasmosis and AIDS co-infection in an endemic area of Northeastern Brazil

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ABSTRACT

Introduction: The spatial distribution of disseminated histoplasmosis (DH) and acquired immunodeficiency syndrome (AIDS) co-infection in adult residents of Fortaleza, Ceará, Brazil was evaluated. **Methods:** Socio-demographic data for the DH/AIDS cases were obtained from a reference hospital, and socio-environmental indicators were obtained from an official Brazilian institute. Kernel analysis and local indicators of spatial autocorrelation (LISA) cluster maps were used to estimate the case density within the city. **Results:** DH/AIDS cases were concentrated in the Northwestern and Southwestern peripheral areas of the city, related with low human development indices, but different from AIDS cases distribution. **Conclusion:** Risk factors other than AIDS infection must affect histoplasmosis development in this area.

Keywords: Disseminated histoplasmosis. AIDS. Spatial analysis.

Histoplasma capsulatum, considered the most common endemic mycosis in humans[1], has emerged as an important opportunistic pathogen in individuals with impaired cellular immunity such as those undergoing prolonged corticosteroid and other immunosuppressive therapy, transplant recipients, those with advanced acquired immunodeficiency syndrome (AIDS), and more recently, those undergoing tumor necrosis factor antagonist treatment[2]. In Brazil, the State of Ceará had the highest number of deaths with histoplasmosis reported as the primary cause[3] [4] [5] [6] than other endemic areas. Brilhante et al.[5] conducted research with 208 patients with disseminated histoplasmosis and acquired immunodeficiency syndrome (DH/AIDS) over a 5-year period in Ceará, resulting in an overall mortality rate of 42.3%[5]. A histoplasmin survey conducted with 161 HIV-positive patients with CD4 T-lymphocyte (CD4 cell) counts >350 cells/mm^3 who resided in Fortaleza, the capital of Ceará, found a 11.8% prevalence of reactivity suggesting important *Histoplasma* transmission in that area[7]. We aimed to investigate the role of the district-environmental and demographic factors in the spatial distribution of DH/AIDS cases in Fortaleza.

The study population consisted of patients diagnosed with AIDS according to the Brazilian Ministry of Health criteria[8]; with or without antiretroviral therapy (ARV); >18 years old; of either sex; residing in Fortaleza, the capital of Ceará (Northeastern Brazil); and diagnosed with DH during hospital admission at São José Hospital (SJH), which is the reference hospital for infectious diseases in the State of Ceará, during the 1999-2007 period. DH was considered when *H. capsulatum* was identified by microscopy (histology or stained smears) and/or culture of the affected tissues or fluids outside the lungs and hilar lymph nodes.

Cases were identified using a databank of previous studies performed with patients with AIDS and DH at São José Hospital[4] [9]. Socio-demographic data (age, sex, address, education level, birthplace, and income) were obtained from medical records; missing records were excluded from the analysis. Data relating to HIV infection comprised the timing of the AIDS diagnosis (if already diagnosed before hospital admission), CD4 cell count, previous admissions due to DH, and use of antiretroviral therapy or illicit drugs. Data regarding clinical outcomes (cure or death) were also collected. The following socio-environmental indicators were provided by the Brazilian Institute of Geography and Statistics [*Instituto Brasileiro de Geografia e Estatística* (IBGE)][10]: resident population, which represents the number of persons residing in the neighborhoods in the macro-administrative regions of Fortaleza (regional), excluding the floating population; human development index (HDI), reflecting regional development that included aspects of the educational services (literacy rate, school enrollment), longevity of the population (life expectancy), and income; resident population by type of permanent private household (PPH), representing the number of persons residing in dwellings, subdivided into house, apartment, room, and improvised, by the resident population of a neighborhood; water supply index (WSI), representing the amount of private

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households supplied by water from the general network, by the total number of households in a neighborhood; regional WSI, which was obtained by averaging all of the WSIs from the neighborhoods; sewage index (SI), indicating the number of households covered by the general network of sewage collection divided by the total number of households in a certain neighborhood; and waste collection index (WCI), representing the number of households served by public cleaning services (garbage collected), by the total number of households in a neighborhood. These data were compiled with georeferenced maps to evaluate the spatial distribution of DH. According to the IBGE, Fortaleza is the fifth most populated Brazilian city, with an estimated 2,551,805 inhabitants and a population density of 8,102,77 inhabitants/km^2, corresponding to approximately 30% of the state population[10].

For the spatial analysis, we adopted the districts as the unit of analysis. Fortaleza is divided into 116 districts, grouped in six macro-administrative regions that are denominated *Secretarias Executivas Regionais* (SER). Descriptive spatial analysis was modeled by applying the Kernel estimator and empirical Bayes smoothing techniques. The spatial analysis methods used to evaluate the spatial dependency of the cases were the Global Moran's I index, which auto-correlated variables spatially to indicate a similarity, or lack of similarity, between the neighbors, and the local Moran Index (LISA), which allowed the identification of significantly high values and positive clusters of both high and low values[11]. This analysis was performed using the program Terraview (version 4.0.0) and the LISA cluster map, which shows only neighborhoods with statistical significance ($p < 0.05$) classified into four possibilities: high-high and low-low, representing spatial units with a high/low frequency of cases and neighboring units also with a high or low frequency, and high-low and low-high, representing units with high and low frequencies surrounded by units with low and high frequencies[12]. The research protocol 041/2011 was reviewed and approved by the Ethical Committee of the São José Hospital. No variables allowed identification of individuals.

We identified 264 patients with progressive (or acute) DH/AIDS co-infection for the period 1999-2007. Of these, 152 patients were residents of Fortaleza, which 43.4% were born in the city; after excluding 17 patients with missing records, 135 patients (82.2% men) were included in the analysis. The patients had a mean age of 40.3 years, 4-7 years of schooling (34.8%), and a monthly income (51%) of <3 minimum wages of approximately 1140.00 USD in 2007 (51%). AIDS was diagnosed because of illness with DH for 62 patients (45.9%); of these patients, 59 (43.7%) were undergoing highly active anti-retroviral therapy (HAART). The average CD4 count at discharge in patients with DH was 103.4 cells/mm^3 ([standard deviation (SD)], 161.2); 126 (93.3%) patients had not been admitted previously for DH. Illicit drugs were used by 62 (45.9%) patients, and 30% of the patients died during the study period. The average CD4 count of the patients who died was 117.7 cell/mm^3 (SD, 124.2).

The analysis included 75 (64.7%) districts of Fortaleza and all 6 SERs. The cumulative proportion identified districts with high numbers of DH cases, with the largest sample located in the Southwestern (21.8% of cases) and Northeastern outskirts of the city **(Figure 1)**. The socio-environmental indicators showed low sewage coverage in SERs IV (22%) and V (28.9%). Only SER II had a medium-level HDI (0.693); all other SERs had low HDIs (<0.499). The WSI and WCI were considered high (>90%) in all regions **(Table 1)**.

The spatial distribution of DH/AIDS cases using the Kernel estimator map showed districts with a high frequency corresponding to areas that composed SERs I, III, and IV. The point cloud extended throughout town, showing hotspots in the Northwest of the city **(Figure 1)**. LISA cluster maps of the DH/AIDS case distribution showed geographical confluences in the reported results, with high rates concentrated especially along the peripheral western edge of the town. Thematic maps, which deal with point cases and environmental factors, showed that the northwestern areas had a high number of cases and medium-level SI and HDI. A high density of houses, rather than apartments (PPH >67%), was observed with these analyses **(Figure 2)**.

Since the beginning of the AIDS epidemic, DH has been detected with increasing frequency in patients attending the reference hospital for HIV in Ceará[6]. This probably reflects the increasing incidence of AIDS cases in Fortaleza[12], instead of increased fungal dissemination in that area.

The socio-demographic profile of the patients is similar to that in the AIDS epidemiological bulletin of the local health department, which showed that the cumulative number of AIDS cases was present primarily in men (73.3%), with 4-7 years of education (23.7%) during 1983-2009 period[12]. The proportion of patients who were diagnosed with HIV/AIDS due to illness with DH (>54%) is also similar to that reported previously[4].

The Northwestern (SER I) and Southwestern (SER V) peripheral areas of the city were the regions with higher frequencies of DH/AIDS cases. The spatial distribution of AIDS cases in Fortaleza between 2000 and 2008 showed that AIDS patients were primarily in the mideastern part of the city (SER III)[12], which does not reflect the distribution of DH/AIDS cases in the present study, indicating that other risk factors beyond AIDS affect the spatial distribution of DH in the city.

Secretaria Executiva Regional V, the most populous area (452,875 inhabitants), included the districts with low sewage coverage (28.9%) and lower HDI, while SER I had higher SI (57.9%) and WCI (96.9%). In SER I, there are probable specific environmental factors for the spread of

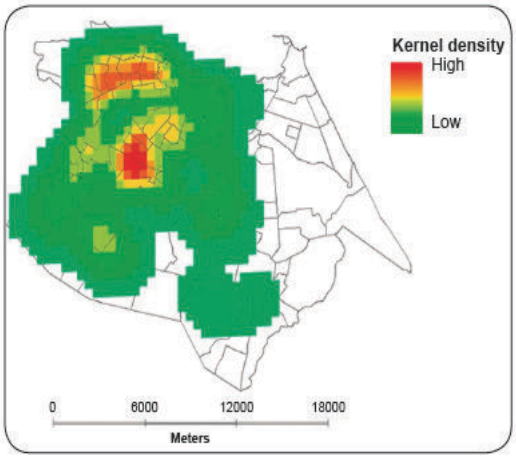

FIGURE 1 - Kernel estimator map of the cumulative frequency of disseminated histoplasmosis and acquired immunodeficiency syndrome co-infection in Fortaleza, Ceará, Brazil, 1999-2007.

histoplasmosis, that are not involved with sanitation which should be investigated, such as the presence of bat or bird droppings. SER I had some characteristics that can favor the presence of *Histoplasma;* it is surrounded by the Ceará River and has an area of environmental preservation for ecotourism and fishing. It has also an area of mangroves with birds, which should be investigated for the presence of the fungus. Furthermore, the low sanitation coverage in SER I can encourage the presence of birds and bats, producing conditions for human infection.

Areas with traditional houses can maintain some common dissemination methods for fungus such as soil manipulation and breeding of birds and tree crops for landscaping or eating. The presence of mango trees *(Mangifera indic*a) and activity involving soil were significantly associated with histoplasmin reactivity in a study performed with 161 HIV patients residing in Fortaleza[7]. Mango trees can also be related with the presence of bats; these animals are attracted by its fruit and can live for years in the nearest places to roost such as the roofs of homes, promoting areas of accumulated organic matter and manure, an excellent place for *H. capsultaum* proliferation[2][7][13].

In another study by Cordeiro et al.[14], *H. capsulatum* was not isolated from 83 bats that were captured from the countryside looking for fungus, but *Coccidioides posadasii* was recovered from *Carollia perspicillata* in bat lungs; no information concerning bats captured from Fortaleza has been published[14].

Analyses using the LISA showed significant spatial clusters (high values surrounded by high values) in SERs I and V, demonstrating areas of a high number of cases exhibiting low and medium sanitation coverage and medium HDIs, reinforcing that other factors are involved in co-infection.

The results of the 2000 Census were highly reliable owing to the use of modern technologies such as digital mapping of the municipalities, scanning and optical reading of the questionnaires, and managerial and operational controls over the internet, among other technological innovations[15].

TABLE 1 - Distribution of cases of disseminated histoplasmosis/AIDS co-infection by year of occurrence and socio-environmental indicators by Secretarias Executivas Regionais code.

Year of occurrence	SER I	SER II	SER III	SER IV	SER V	SER VI	Total
1999	0	0	0	1	3	0	4
2000	1	0	0	1	0	1	3
2001	1	0	3	0	2	1	7
2002	5	2	3	1	2	2	15
2003	8	3	2	2	3	5	23
2004	5	2	4	3	0	2	16
2005	5	0	3	1	4	3	16
2006	4	3	4	2	6	9	28
2007	2	1	4	4	8	4	23
Socio-environmental indicators							
resident population	340.134	311.842	340.516	390.589	452.875	305.446	-
HDI(range, 0.0-1.0)	0.483	0.693	0.495	0.462	0.44	0.559	-
water supply index (%)	95.2	96.3	96.1	90.6	94.9	96.4	-
sewage index (%)	57.9	64.6	39.9	22	28.9	46.3	-
waste collection index (%)	96.98	96.74	96.72	91.36	92.79	98.19	-

AIDS: acquired immunodeficiency syndrome; **SER:** *Secretaria Executiva Regional;* **HDI:** human development index.

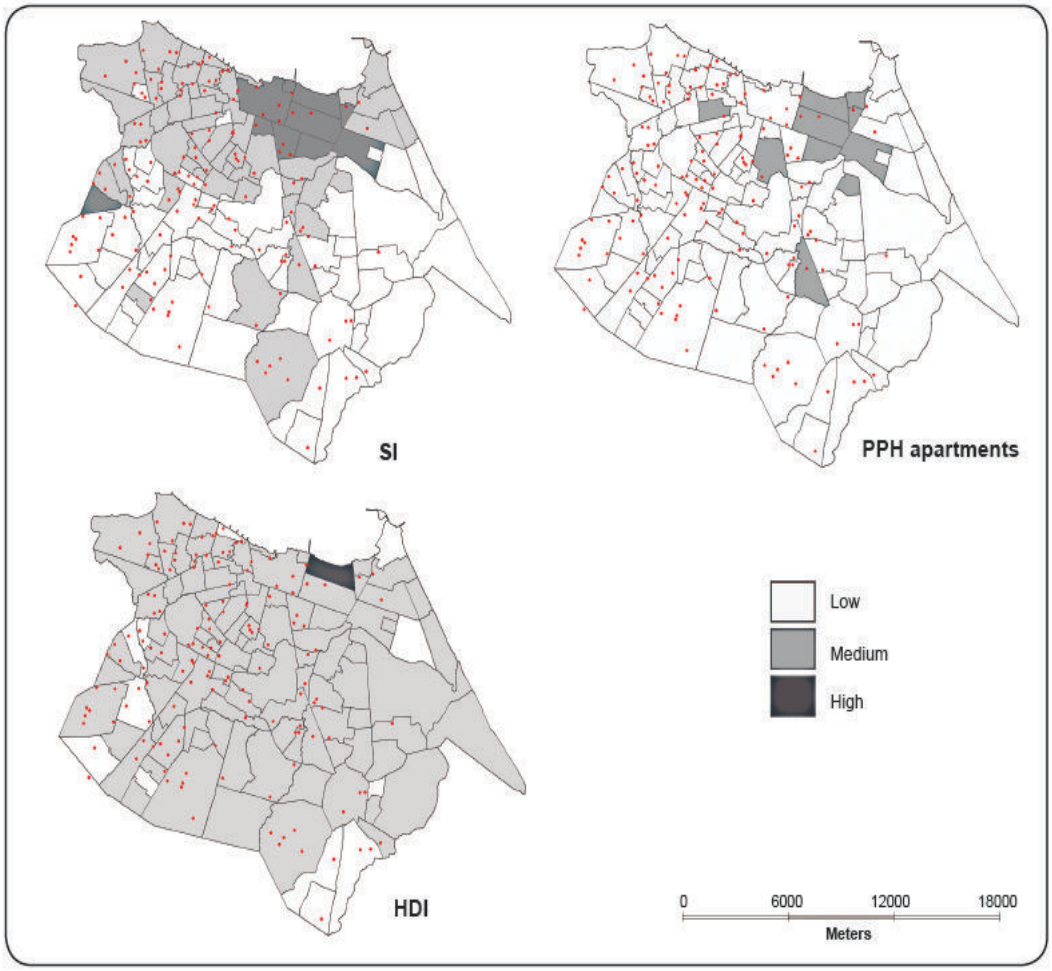

FIGURE 2 - Cases of disseminated histoplasmosis/AIDS co-infection and the sewage index (SI), human development index (HDI), and permanent private households (PPH) in the form of apartments, by district in Fortaleza, Ceará, Brazil, 1999-2007.

However, this study also had some limitations. The information was obtained from secondary data, which could limit the reliability of the results. Because the 135 patients are not likely representative of the estimated frequency of DH in Fortaleza, any extrapolation to the general population of patients with DH and AIDS should be conducted with caution. All patients diagnosed with DH were residents of Fortaleza; however, we cannot affirm that these individuals were infected in the city. Furthermore, a considerable number of cases were born in the countryside.

The need to study the relation between human beings and the environment resulted in several studies of medical geography, aimed at analyzing disease spatial distributions[15]. However, this is the first description of the spatial distribution of DH/AIDS co-infection and the relationship with environmental factors, allowing a different viewpoint of the morbidity related with this severe disease in an endemic area. The distribution of cases overlaying areas with low sanitation coverage and HDI indicated that other risk factors beyond AIDS must affect the development of histoplasmosis in these studied areas.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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APÊNDICES

APÊNDICE A – Formulário de pesquisa

AVALIAÇÃO FENOTÍPICA E GENOTÍPICA DE ISOLADOS CLÍNICOS DE *H. CAPSULATUM* NO ESTADO DO CEARÁ, E SUA RELAÇÃO COM OS ASPECTOS CLÍNICO-EPIDEMIOLÓGICOS		
Iniciais do paciente: Cepa:		Pront:
Idade:	Sexo: ( ) 1-masculino 2-feminino	
Profissão:	Escolaridade:	
Naturalidade:	Procedência:	
Atividade de risco: ( ) 1-visitação de gruta/caverna 2-limpeza de forro de casa 3-podagem de árvores 4-contato com galinhas/galinheiro 5-outros		
Comorbidades (0-não 1-sim)		
( ) Neoplasia	( ) Transplantado	( ) Tuberculose
( ) Diabetes Mellitus	( ) Uso de Imunossupressor/corticoide	( ) Outras
( ) Cirrose Hepática	( ) HIV/AIDS	
( ) DPOC	( ) Renal Crônico	
Sinais e Sintomas (0-não 1-sim)		
( ) febre	( ) hepatomegalia	( ) perda de peso _____
( ) tosse seca	( ) esplenomegalia	( ) tosse produtiva
( ) vômitos	( ) adenomegalias	( ) lesões de pele (descrever)
( ) dispneia	( ) IRA	
( ) dor abdominal	( ) Ins Resp	
( ) diarreia	( ) sangramento de mucosas	( ) outros
( ) astenia	( ) cefaleia	
Forma clínica (0-não 1-sim)		
( ) HPA	( ) Histoplasmose de SNC	( ) Outras
( ) HPC	( ) HD	
Evolução (0-não 1-sim)		
( ) 1º episódio de histoplasmose	( ) Alta ( ) Óbito	
( ) Reinfecção/recidiva	Se recidiva, data da 1ª infc:	
Material clínico – data do isolamento		
Medula óssea: ( )	LCR: ( )	Linfonodo: ( )
Creme leucocitário: ( )	Urina: ( )	Pele: ( )
Secreção traqueobrônquica: ( )	Sangue: ( )	Outro: ( )

IRA= insuficiência renal aguda; Ins Resp= insuficiência respiratória aguda;
Pesquisador: _____Data:___/____/_____

ANEXOS

ANEXO A - Parecer consubstanciado do CEP

INSTITUTO DE PESQUISA
CLÍNICA EVANDRO CHAGAS -
IPEC / FIOCRUZ

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: AVALIAÇÃO DO PERFIL FENOTÍPICO E GENOTÍPICO DE ISOLADOS CLÍNICOS DE Histoplasma capsulatum NO CEARÁ, E SUA RELAÇÃO COM OS ASPECTOS CLÍNICO-EPIDEMIOLÓGICOS

Pesquisador: Rosely Maria Zancopé Oliveira

Área Temática:

Versão: 3

CAAE: 19342513.2.0000.5262

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

Patrocinador Principal: Fundação Oswaldo Cruz

DADOS DO PARECER

Número do Parecer: 445.169

Data da Relatoria: 11/11/2013

Apresentação do Projeto:

Trata-se de um estudo observacional seccional no qual serão incluídos 50 isolados de H. capsulatum proveniente de pacientes assistidos no Hospital São José, Ceará, no período de agosto de 2013 a agosto de 2015. Posteriormente, estes isolados serão enviados para o IPEC/Fiocruz, aonde serão realizadas as técnicas de biolagia molecular descritas na metodologia.

Objetivo da Pesquisa:

Objetivo geral: Avaliar o perfil fenotípico e genotípico de isolados clínicos de H. capsulatum do Estado do Ceará.

Objetivos específicos: 1. Descrever o perfil fenotípico de isolados clínicos de H. capsulatum;2. Identificar a variabilidade genética de H. capsulatum;3. Comparar os genótipos encontrados de H. capsulatum com aqueles previamente observados em outros estudos no Brasil;4. Avaliar a relação de grupos ou

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