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

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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 the yeast-phase in ML-Gema medium was achieved in all isolates. MAT1-1 idiomorph was identified in CE0311, CE0411 and CE2813 isolates; 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 mixed infections caused by *H. capsulatum* isolates with different mating types in Brazilian AIDS-patients for the first time in the literature.

KEYWORDS: Mixed infection. *Histoplasma capsulatum.* Mating types. Multilocus sequence typing. Histoplasmosis. HIV coinfections.

INTRODUCTION

Histoplasma capsulatum is a dimorphic fungus found in the form of mold in the environment and *in vitro* at 25–28 ºC. The yeast form is observed during parasitism conditions or in cultures at 34–37 ºC 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,* resulting from 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

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some fungi as *Cryptococcus neoformans* and *Aspergillus fumigatus*, the mating type is associated with the virulence[8,9]. In addition, mixed infections with different mating types have been rarely described in pathogenic fungi[10].

A high genetic diversity of *H. capsulatum* has been reported worldwide[11-13]. 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[11]. 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 clades; an Australian clade[11]. In addition, diverse new lineages have also been identified in different geographic regions of the world[11,12]. 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[14]. The LAm A clade was regrouped in LAm A1, LAm A2 and LAm B clade was reclassified in LAm B1 and LAm B2[14]. In addition, two new phylogenetic clades (RJ from Southeastern Brazil and BCA1 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 cases of histoplasmosis in AIDS-patients were evaluated by morphological criteria, mating type determination and phylogenetic classification by MLST analysis.

MATERIAL AND METHODS

Medical records

Clinical data of patients with histoplasmosis and AIDS from Sao Jose Hospital, Fortaleza city, Ceara State, Brazil, were retrospectively retrieved from medical records. Only patients that had positive cultures for *H. capsulatum* in two or more different samples or in different hospitalization time, between 2011 to 2014 period were included. A total of 13 biological samples of six patients were studied by mating type- PCR. Only two patients were selected because they presented more than one *H. capsulatum* isolate with different mating types 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 State, 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 25 °C during 21 days. The macromorfology of filamentous fungal cultures were visually examined and recorded. Colonies were described according to pigmentation (albino and scale beige - light beige, dark beige, and beige) and texture (cottony or powdery). Their micromorphologies were observed in 10 different fields by optical microscopy at a 40 X magnification of *H. capsulatum* colonies, stained with Lactophenol Cotton Blue (Fluka Analyted, France). Dimorphism was demonstrated by conversion to the yeast-like form on ML- Gema agar medium[15], for 7 to 14 days at 37 °C.

Mating type determination

Yeast cells were submitted to limiting dilution and a single colony was used for DNA extraction as previously reported[16]. The *MAT1* locus of *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[17]. Briefly, PCR was performed in a 25 µL reaction mixture, containing 200 µM of 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. G-217B from USA (*MAT1-1*) and G-186AR from Panama (*MAT1-2*) are references strains and 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 and 30 s at 72 °C; and (c) 10 min at 72 °C. Amplicons were visualized on 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 obtained sequences were deposited in GenBank database (http://www.ncbi.nlm.nih.gov). They were edited and aligned for BLASTn analysis[18], using as reference the sequence of the strains G-217B from USA (MAT1-1, GenBank accession

Nº EF433757) and G-186AR from Panama (MAT1-2, GenBank accession Nº EF433756).

Phylogenetic relationship among the studied isolates

The genetic reconstruction analysis was performed by MLST using PCR amplification of partial DNA sequences from four nuclear genes (*arf, H-anti, ole1,* and *tub1*) according to the protocol described by Kasuga *et al.*[11] with some modifications. PCR was performed in 25 µL of reaction mixture, containing 200 µM of 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 65 °C in the first cycle, which was subsequently reduced by 0.7 °C/cycle for the next 12 cycles, and 1 min at 72 °C. In the remaining 20 cycles, the annealing temperature was kept at 56 °C; (c) a final extension cycle of 5 min at 72 °C – touchdown PCR[19].

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 Nº 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[20]. This matrix was constructed with 260 sequences, considering four genes per isolate, out of 65 analyzed *H. capsulatum* isolates: 57 isolates from TreeBASE (http://treebase.org, study ID S1063) reported by Kasuga *et al.*[11]; 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 are in Tables 1 and 2.

Table 1 - Major data of *H. capsulatum* strains/isolates whose sequences were acquired from databases of the MLST analysis of the present study. (continues on the next page)

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

Table 1 - Major data of *H. capsulatum* strains/isolates whose sequences were acquired from databases of the MLST analysis of the present study. (cont.)

Strain/isolate	Phylogenetic clade	Source	Origin	Year of isolation
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

The sequences of all *H. capsulatum* strains/isolates in this table were obtained from *TreeBase and †GenBank. Numbers in parenthesis are acronyms.

Table 2 - Characteristics of *H. capsulatum* isolates associated with mixed infections.

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

The generated combined matrix containing 1539-nt was analyzed through two methods: a) Maximum likelihood (ML) in RaxMLGUI ver. 1.31[21] through the General Time Reversible substitution model gamma distribution; and b) Bayesian inference (BI) by MrBayes ver. 3.2[22] 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[23], 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[24,25]. An unrooted tree was constructed using the combined matrix.

RESULTS

During the study period, 40 AIDS-patients were hospitalized in the Sao Jose Hospital with histoplasmosis diagnosis confirmed by culture in different biologic samples. For this study, six patientswere recruited and 13 biologic samples of these six patients, which were available for mating type – PCR. Four patients presented the same mating type, two patients with MAT 1-1 and two patients with MAT 1-2. However, for this report we selected only the two patients presenting with *H. capsulatum* isolates with different mating types.

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 Baturite, Ceara State, Brazil. Physical examination revealed cervical adenomegaly and hepatosplenomegaly. Pulmonary and cardiac auscultation as well as vital signs were normal. He used antiretroviral drugs irregularly (estavudine, lamivudine, and efavirenz). Laboratory evaluations revealed hemoglobin level of 9.3g/dL, white blood cells count 1,200/mm^3 (neutrophils = 80%; lymphocytes = 10.8%; monocytes = 4.2%; eosinophils = 5%), and platelets count 65,000/mm^3. Renal function was normal. The level of lactate dehydrogenase (LDH) was high (2,086 U/L). Aspartate aminotransferase (AST) level was 274 U/L, alanine aminotransferase (ALT) level was 67 U/L and alkaline phosphate (AP) was 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 (1 mg/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 Maracanau, Ceara State, Brazil. His vital signs were as follow: temperature 37.2 °C; pulse rate 106/min, respiratory frequency 30/min, and blood pressure 70/30 mm Hg. 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 antiretroviral drugs irregularly (zydovudine, lamivudine, atazanavir, and ritonavir). Laboratory

evaluations revealed hemoglobin level of 4.3 g/dL, white blood cells count 3,860/mm^3 (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, γGT = 197 U/L). The patient had CD4+ lymphocytes count of 80 cells/mm^3. A chest X-ray 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 patients' 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 on day 3 (CE2813) and day 12 (CE2513) of hospitalization. *H. capsulatum* mycelial cultures of patient 1 presented a 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 in 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 *H. capsulatum* isolates, with typical budding yeast cells. Figure 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[18]. The CE0311 and CE0411 isolates showed 99% similarity and CE2813 isolate

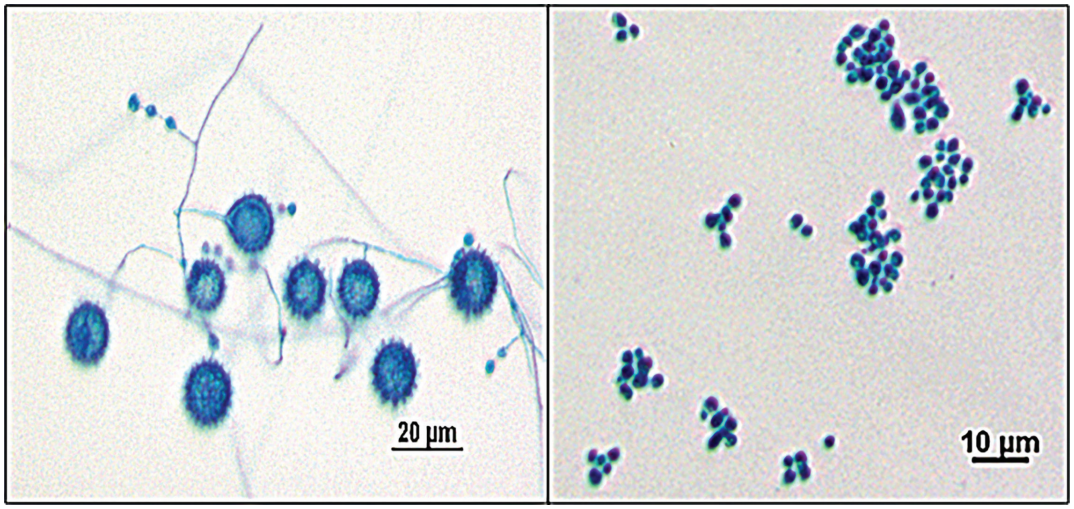

Figure 1 - Representative micromorphology of *H. capsulatum* mycelium and yeast phases of CE0411 isolate from an AIDS-patient. Both fungal phases were stained by Lactophenol Cotton Blue. Magnification of 40 X.

Table 3 - GenBank accession numbers of the sequences from each gene used to characterize the five *H. capsulatum* isolates in 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

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 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 (Figure 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, Figure 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) (Figure 2). In regard to the sequences of other isolates used to develop the MLST analyses, Figure 2 shows that they clustered according to Kasuga *et al.*[11] criterion, representing the different clades reported in Table 1.

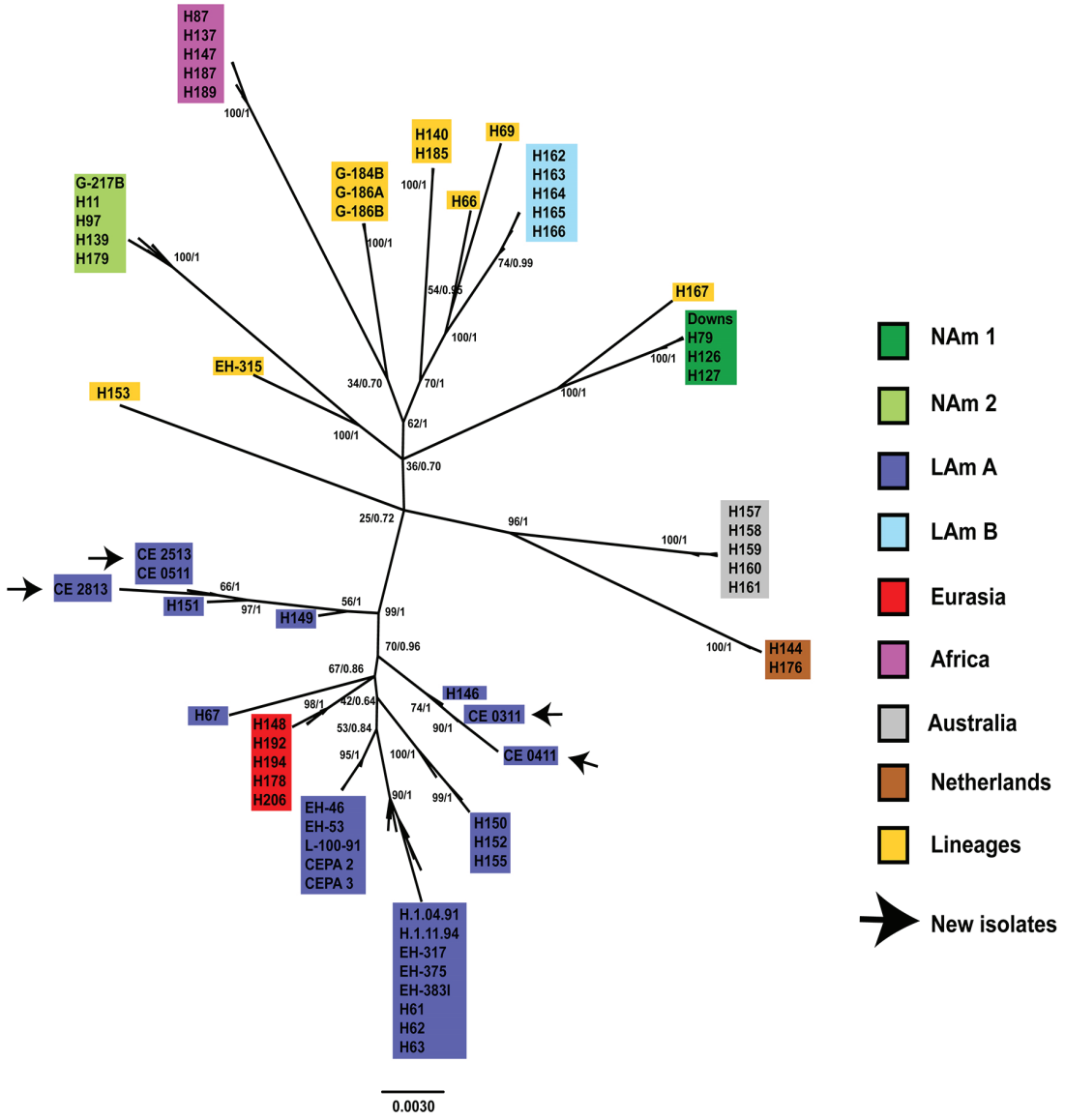

Figure 2 - Unrooted phylogenetic tree of *H. capsulatum* isolates. The tree was constructed with a concatenated matrix of 1539-nt using four gene fragments (*arf*, *H-anti*, *ole1*, and *tub1*). It was generated by BI and is representative of both ML and BI analyses. The values of bt/pp are indicated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

DISCUSSION

This study reports for the first time in theliterature , two cases of mixed infection caused by *H. capsulatum* isolates with different mating types in AIDS-patients, highlighting the genetic diversity among the five fungal isolates recovered from clinical samples. The *H. capsulatum* isolates associated with mixed infections were from the Ceara 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%) among them[2,26].

Mixed infections caused by microorganisms with different genetic profiles have been described mainly with pathogenic bacteria[27,28]. Fungal mixed infection with different mating types of the same species was described in individuals colonized or infected by *Aspergillus fumigatus*[10]. 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 coinfection by recently acquired strains, and superinfection by a new strain different from preexisting clones[29,30]. Here, mixed infections caused by different isolates were supported by the finding of different *H. capsulatum* mating type's idiomorphs in 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 with the severity of infections[8,9,31]. *MAT1*-1 has been associated with invasive aspergillosis[32] and in *C. neoformans* infections, the α-mating type has been described as more virulent than the a-mating type[8,31]. However, more recently, experimental studies revealed that there is not association between mating type and virulence of *C. neoformans* and *A. fumigatus*[33,34].

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 increased resistance to antifungal drugs[7,30].

Few studies have investigated the mating type of *H. capsulatum* isolates and its impact on the virulence of the pathogen[5,35]. 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[35]. 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 transplanted 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[17]. 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[17]. As new data, it was reported here two *MAT1-2 H. capsulatum* isolates 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 strainand melanin production. Even though the melanization of *H. capsulatum* protects the fungus against antifungal drugs such as amphotericin B[36], experimental studies did not find any difference in virulence between albino (non-melanized) and brown (melanized) *H. capsulatum* strains[37].

All *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 to the new phylogenetic clusters proposed by Teixeira *et al.*[14], 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 (Figure 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 coinfection with a new isolate that diverged and coexists in the same area, or by superinfection with 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[11-14,38-40]. Therefore, coinfection and

superinfection are events that can occur in mixed infections and cannot be discarded in *H. capsulatum* infections. In spite of all this, more studies aiming at evaluating the consequences of mixed infections with *H. capsulatum* harboring different genetic and morphologic characteristics are necessary to better understand the pathogenesis of histoplasmosis.

CONCLUSIONS

This research indicates that mixed infection caused by *H. capsulatum* isolates with different mating types can occur due to different mechanisms as microevolution, coinfection or superinfection. More studies are necessary to evaluate the results this coinfection in the virulence of pathogen and in the pathogenesis of histoplasmosis.

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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 manuscript.

AUTHORS' CONTRIBUTIONS

LSD, TVG, JAR, GRA, MAA, JRLM carried out the laboratory work of the study; LSD, TVG, MMM, MLT, RMZO evaluated and interpreted data; LSD, TMJSL, MLT, RMZO drafted the manuscript; All authors participated in the design of the study and revised manuscript. All authors have contributed intellectually during the writing process and have read and approved the final manuscript.

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