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Report of filamentous forms in *a* mating type VNI clinical sequential isolates of *Cryptococcus neoformans* from an HIV virus-infected patient ☆

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ABSTRACT

We reported a cryptococcal meningitis Aids-patient infected with *a* mating type VNI isolate showing filamentous cells in direct examination of cerebrospinal fluid. Clinical data, outcome, treatment features and microbiological findings were discussed.

1. Introduction

Cryptococcosis is one of the most life-threatening mycoses affecting human hosts. It is estimated that about 957,000 cases presenting the serious meningeal form of disease occur annually worldwide associated with the human immune deficiency virus infection (HIV), leading to 600 thousand deaths [1]. In Brazil, the disease represents the primary cause of opportunistic meningitis and the second most frequent neurologic opportunistic infection in HIV virus-infected patients [2]. The main etiological agents present MAT*a* and MATα sexual states and at least 8 molecular types: VNI, VNII, VNIII and VNIV for *Cryptococcus neoformans* and VGI, VGII, VGIII and VGIV for *Cryptococcus gattii*. Various studies have reported that the two species differ in many aspects including epidemiological, virulence, and antifungal resistance differences resulting in distinct clinical presentation and clinical outcome [3]. Similar to the majority of regions where it manifests worldwide, overall in Brazil the main causative agent is *C. neoformans* VNI alpha mating type [4]. All molecular subtypes enclosing the two species are morphologically identical with typical capsulated, rounded to elongated budding cells, measuring 5–7 μm in diameter. Nevertheless, previous studies have reported the occurrence of giant cells (titan cells) and microforms or even hyphal forms besides normal cells [5,6,7]. We report a case of cryptococcal meningitis caused by *a* mating-type VNI *C. neoformans* showing filamentous cells in Indian ink preparation of cerebrospinal fluid (CSF) examination.

2. Case

A male patient 43-old, native of La Paz city in Bolivia and living in São Paulo City, Brazil for the last 15 years, was admitted to Emilio Ribas Institute of Infectious Diseases, a tertiary public hospital in São Paulo, Brazil. The patient presented headache, nausea, vomiting and mental confusion. He presented umbilicated papule skin lesions on the trunk and limbs, besides pulmonary and meningeal symptoms. Thorax computed tomography (CT) for screening of fungal lesions indicated pneumonitis. Laboratory results showed both positive India ink preparation and qualitative latex agglutination in CSF. The leukocytes counting revealed 7 cells/mm^3 with a predominance of lymphocytes (87%), protein level of 78 mg/dL and glucose level of 34 mg/dL. The serology for HIV virus was positive, viral burden was 925 viral copies/mL, and CD4 cell count was 43 cells/mm^3. Table 1 shows the principal features from the case reported.

The blood culture, CSF and skin tissue cultures yielded the recovery of typical cryptococci encapsulated yeasts. CFS samples

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from day 0, 7, 14 and 21 were sent to Adolfo Lutz Institute, a Public Health Reference Laboratory for quantitative culture and identification by morphological and biochemical characteristics including CGB screening test to differentiate the species *C. neoformans* and *C. gattii*, molecular type testing, and antifungal susceptibility pattern.

Treatment began on the first day of hospitalization with deoxycholate amphotericin B (1 mg/kg/day) in combination with fluconazole (400 mg) twice a day for four weeks. Relief punctures were performed daily in the first seven days of treatment. The patient was discharged on the 30th day of hospitalization after two negative culture results, receiving fluconazole (consolidation phase with 800 mg/day). Two months later the individual's condition relapsed evolving to convulsions and the patient was re-hospitalized requiring combined antifungal therapy initially with amphotericin B and fluconazole for a month. Due to worsening of the neurological status, the patient was admitted to the intensive care unit. The results showed high fluconazole MIC (minimal inhibitory concentration). Cerebral MRI (Magnetic Resonance Imaging) resulted in findings compatible with parenchyma involvement. Fluconazole was switched to voriconazole (400 mg/day) twice a day. The strategy yielded CSF sterilization and the patient was discharged in a few weeks under a voriconazole therapy regimen. A second episode of clinical recurrence was verified four months later with clinical presentation of convulsions, headache, and trembling. Amphotericin B and fluconazole was re-introduced, but renal failure led to monotherapy with fluconazole. Clinical and laboratory cure was observed after 3 weeks.

The initial quantitative CSF culture, according to Robinson and co-authors (1999) showed high initial fungal burden (8.8×10^5 CFU/mL) dropping to 2.7×10^2 and 1.8×10^2 CFU/mL at day 7 and 14, respectively, followed by negative results on day 21. Pseudohyphal forms of fungal cells were observed in Indian ink examination only in the first CSF sample (Fig. 1). Otherwise primary and sequential cultures showed only normal budding cells. Multiple colonies *(n = 19)* from quantitative cultures related to sequential CSF samples were collected to speciation. Genotypes were characterized through the use of URA5-restriction fragment length polymorphism analysis (URA5-RFLP) [3]. The genetic variability was determined following Ulrich and co-authors (2009) by using PCR-fingerprinting with the minisatellite-specific primer M13 (M13 PCR fingerprinting) [4] and the mating type was established by PCR as recommended by Chaturvedi and co-authors [8].

The following standard strains representing each molecular type were included in the analysis: WM 148 (serotype A, VNI/ AFLP1), WM 626 (serotype A, VNII/AFLP1A), WM 628 (serotype AD, NIII/AFLP2), WM 629 (serotype D, VNIV/AFLP3), WM 179 (serotype B, VGI/AFLP4), WM 178 (serotype B, VGII/AFLP6), WM 175 (serotype B, VGIII/AFLP5), and WM 779 (serotype C, VGIV/AFLP7). *Filobasidiella neoformans* ATCC 28957 and ATCC 28958 were used as standard strains.

All multiple colonies were *C. neoformans* genotype VNI (Fig. 2), mating-type α and the M13 PCR fingerprinting (Fig. 3) of the 19 distinct colonies showed the same band patterns. Antifungal susceptibility test through broth micro dilution reference method (European Committee EUCAST AFST.EUCAST) was used to determine the minimal

Table 1
CSF findings during period of patient hospitalization.

Day	Results				
	Protein (mg/dL)	Glucose (mg/dL)	Yeasts (mm^3)	Culture	Quantitative culture (cfu/mL)
0	78	34	618	(pos)	440,000
1	81	21	1420	(pos)	NP
4	395	10	329	(pos)	NP
7	207	7	128	(pos)	135
12	81	18	NR	(pos)	NP
14	61	26	10	(pos)	90
17	44	21	16	(pos)	NP
18	30	72	NR	(pos)	NP
20	49	36	50	(neg)	NP
22	56	40	NR	(neg)	0

pos: positive; neg: negative; NP: Not Performed; CFU/mL: colony-forming units per milliliter of CSF.

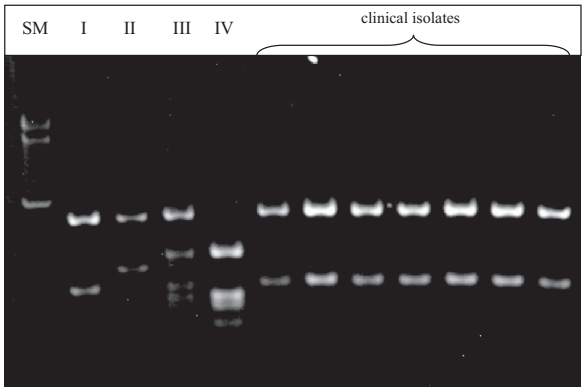

Fig. 2. Electrophoresis of PCR-RFLP products to determine molecular genotyping. SM: size marker 100bp; I, II, III and IV refer to genotypes VNI,VNII,VNIII and VNIV, respectively (W. Meyer strain-types).

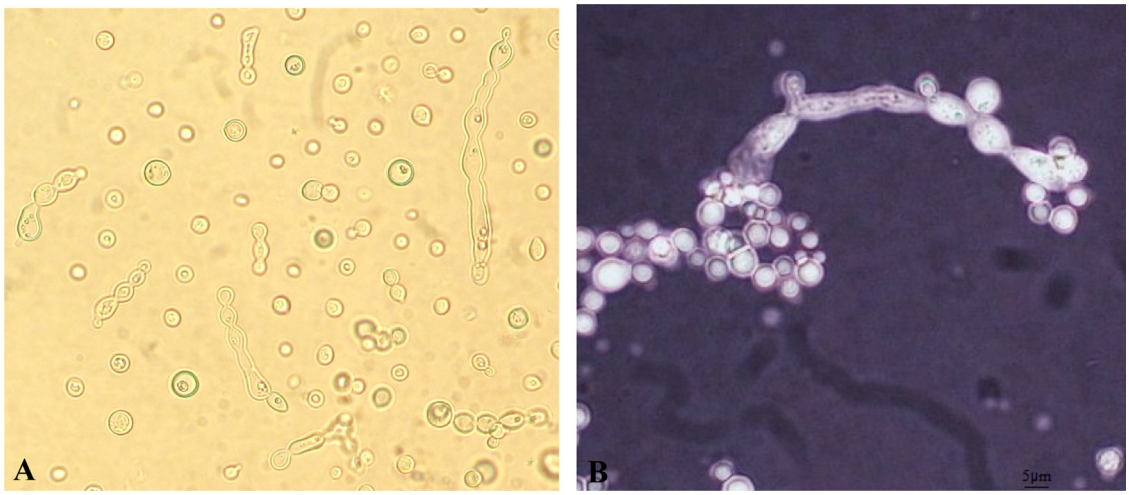

Fig. 1. Pseudohyphae observed in direct microscopy (A) and India Ink examination (B) of cerebral spinal fluid of Cryptococcal meningitis case.

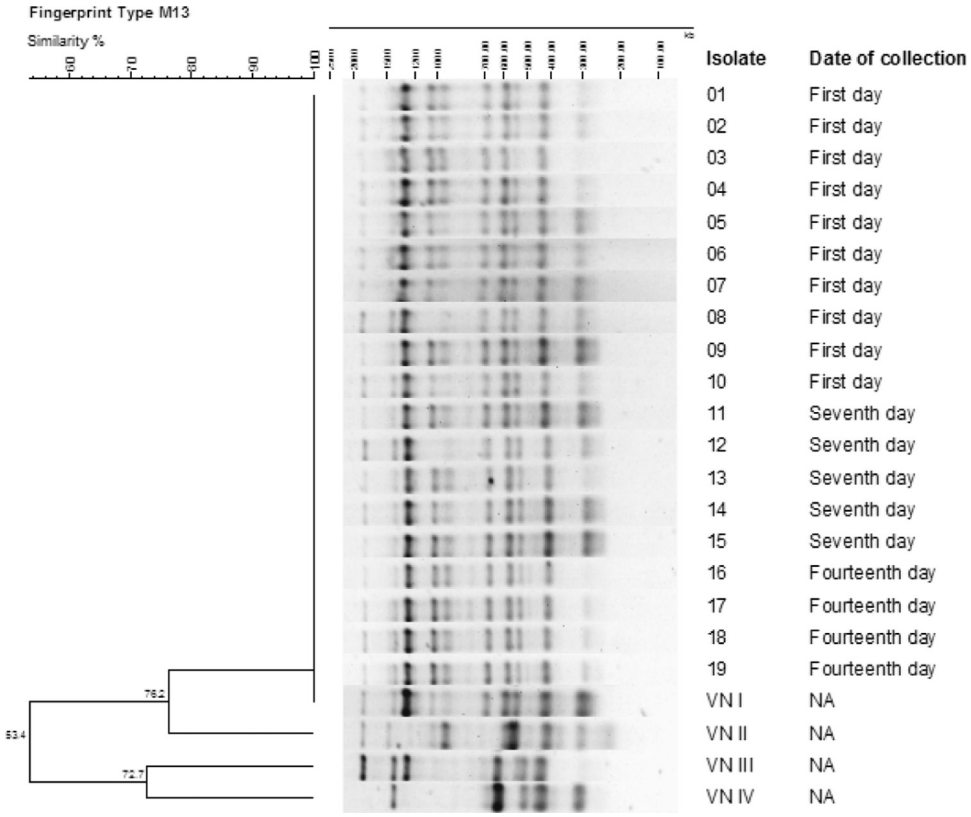

Fig. 3. Dendrogram of PCR-fingerprint profiles with primer M13, of 19 clinical isolates from a Cryptococcal meningitis case (BioNumerics, Dice coefficient and UPGMA).

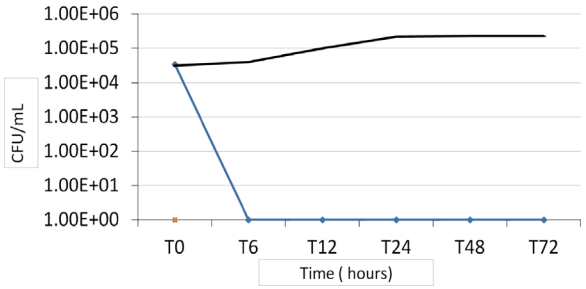

Fig. 4. Time–kill curve results of amphotericin B for 19 isolates of Cryptococcosis patient. Black line, positive control.

inhibition concentration (MIC) of fluconazole and voriconazole. The commercial method of diffusion using the drug-containing strip Etest® was employed for testing amphotericin B. Homogeneous high MIC-values of fluconazole (MIC 16 mg/L) and voriconazole (MIC 0.25 mg/L), and low amphotericin B-MIC (0.064 mg/L) were found for all multiple colonies tested. The fungicidal activity of amphotericin B (1 mg/L) was assessed by time-kill curve tests demonstrating complete colony killing in the first 6 h drug-exposure for all multiple isolates (Fig. 4).

3. Discussion

Little is known about the occurrence of filamentation in *C. neoformans* cells although low virulence in filamentous forms have been previously reported in murine models [5 – 10]. One hypothesis is that pseudohyphal production is an adaptive mechanism to environmental pressures to yeast development. The occurrence of these morphological changes like increases in cell size or filamentation in isolates of *C. neoformans* may be related to virulence. Some authors reported the interaction of *C. neoformans* and amoebas, like *Acanthamoeba polyphaga*, one of its natural predators, observing that, in addition of pseudohyphae formation, these structures could not be engulfed by amoebas, showing a form developed by yeast to ensure its survival in the environment [9]. However, low virulence was observed when these cells were subjected to tests *in vivo*. Similar results were found in rats, leaving a clear need for more studies on these findings [10]. Likewise, strains producing pseudohyphal forms were observed to be less phagocyted by amoeba *Acanthamoeba castelanni* than the wild-type strain. However, mice inoculated with wild-type strain succumbed and were sacrificed 26 h after inoculation, whereas mice inoculated with pseudohyphal strains remained healthy during the same period. Later, some of these cases produced yeast cell forms causing disease in their hosts [11]. Another relevant morphologic alteration is the titan and microforms of *C. neoformans*. It has been demonstrated that titan cells are resistant to phagocytosis, they may evade immune cells in lungs of mice and could tolerate oxidative and nitrosative stresses [12].

All colonies showed identical band patterns by M13 PCR fingerprinting, suggesting that the patient was infected by only one strain and this strain persisted in all sequential CSF samples. The antifungal susceptibility patterns to amphotericin B, fluconazole, and voriconazole were identical for all the colonies. The success of the therapy was demonstrated by serial quantitative culture, which showed a considerable decrease in fungal load during treatment. Mortality is thought to be associated with a higher burden of infection, according one study that indicated the number of cryptococci cultured from the CSF as an independent predictor of early death [13]. The fungal burden by quantitative cultures in 14 day-therapy correlated with our patient's mycological response to treatment and has been confirmed to monitor the clinical outcome [14,15,16]. The combined therapy using amphotericin B and fluconazole resulted in clinical improvement. Interestingly, we observed this occurrence correlated with the good efficacy of amphotericin B *in vitro* demonstrated by

low MIC results as expected for this species [17]. Furthermore, the clear association between *in vivo* rapid fungal clearance and early *in vitro* killing effect provided a strong argument in favor of extending studies to evaluate the time-curve method as a tool to predict outcome. Despite that amphotericin B was introduced in the late 1950s, in addition to its dose-limiting toxicity, this agent remains the first-line induction treatment for cryptococcal meningitis [18]. It would be worthwhile to evaluate the potential of time kill curves in isolates with distinct susceptibility patterns and correlated with clinical outcome.

Our patient experienced two clinical relapse months after being discharged. Of note, the primary and sequential isolates of the etiologic agent showed high fluconazole-MIC and voriconazole-MIC values showing no correlation with the good outcome observed in our case. Globally, such high values are rarely observed for *C. neoformans* isolates, conversely to high fluconazole-MICs frequently found for *C. gattii* [16,17,19]. We found these findings have potential clinical implications, since this azole agent is the recommended standard to treat Cryptococcosis, particularly during the maintenance phase of antifungal treatment of cryptococcal in HIV virus-infected patients [3,18]. Although our patient had a good outcome before hospital discharge, he was re-admitted to the hospital a few months after the first episode. Infection relapse on commencement of fluconazole maintenance therapy associated with high fluconazole-MIC (>8 mg/L) was previously reported [20]. However, there is no conclusive information about the clinical relevance of susceptibility tests in the management of cryptococcal meningitis. In addition, several factors are responsible for poor responses to fluconazole therapy in patients with HIV virus associated cryptococcal meningitis, mainly profound immunodeficiency as demonstrated in our patient and inadequate fluconazole exposure due to poor drug adherence as likely in this case. It is unclear whether clinical relapse is attributable to low susceptibility resistance of the isolates to fluconazole. Patient non-compliance is a factor that could have contributed to the observed clinical relapse. Further studies are necessary to explain the clinical relevance of filamentous forms and molecular profiles of the etiologic Cryptococcosis agent and its relationship with antifungal susceptibility changes.

Conflict of interest

There are none.

Acknowledgments

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