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Refractory and/or Relapsing Cryptococcosis Associated with Acquired Immune Deficiency Syndrome: Clinical Features, Genotype, and Virulence Factors of *Cryptococcus* spp. Isolates

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Abstract. Refractory and relapsing crytococcoccosis in acquired immune deficiency syndrome (AIDS) patients have a poor prognosis. The risk factors for this complicated infection course were evaluated by comparing refractory and/or relapsing cryptococcosis in human immunodeficiency virus–coinfected patients (cohort 1) with another group of AIDS patients who adequately responded to antifungals (cohort 2). Except for one isolate of *Cryptococcus gattii* from a cohort 2 case, all other isolates were identified as *Cryptococcus neoformans* var. *grubii*, sex type α, genotype VNI, including *Cryptococcus* reisolated from the relapse or in the refractory state. No differences were observed with respect to *Cryptococcus* capsule size and in the melanin and phospholipase production. The cohort 1 patients presented higher prevalence of cryptococcemia, cerebral dissemination, chronic liver disease, and leucopenia, and have increased death rate. Apparently, the refractory and/or relapsing cryptococcosis in the AIDS patients were more related to the host and the extent of the infection than to the fungal characteristics.

INTRODUCTION

Opportunistic *Cryptococcus* spp. infection in patients with acquired immune deficiency syndrome (AIDS) is considerably prevalent in some world regions, and is associated with high mortality and neurological sequelae.[1,2] Although *Cryptococcus* species are usually sensitive to amphotericin B, flucytosine, and fluconazole,[3,4] some patients treated with these drugs do not have a favorable outcome. In addition to early death due to the complications of cryptococcosis, there are cases in which the infection is controlled late or not controlled at all despite the continuous administration of antifungal agents.[5] These are the cases of cryptococcosis refractory to treatment, which were already known before the AIDS epidemic and have a poor prognosis.[6] Relapsing cryptococcal infection is another form of therapeutic failure, which occurs in up to 13% of patients despite fluconazole secondary prophylaxis.[5] Treatment failure in cryptococcosis is a critical medical condition for which few therapeutic resources are available. Risk factors for a poor prognosis of cryptococcosis, usually defined as death within up to 90 days, include altered mental state, wasting syndrome, bloodstream infection or of another site outside the central nervous system (CNS), and cerebrospinal fluid (CSF) with elevated opening pressure, low leucocyte count, a large number of yeast cells, and high titers of cryptococcal antigens.[7,8] The risk factors for cryptococcosis refractory to treatment and/or relapsing have been less precisely determined, but include infection at sites outside the CNS, a low number of leucocytes in the CSF, and the use of corticosteroids during and after antifungal therapy.[6] In addition to the clinical conditions of the patients, certain characteristics of *Cryptococcus* spp. can result in more severe infection. *Cryptococcus gattii* has been related to greater neurological morbidity and to a slower response to treatment,[9] and different *Cryptococcus* spp. genotypes may have different susceptibility to antifungal agents.[4] The virulence of this microorganism has also been associated with the polysaccharide capsule size and a greater capacity for melanization and phospholipase production.[3,10] Previous studies with human immunodeficiency virus (HIV)–infected patients have assessed the general characteristics and outcome of cryptococcosis cases. The objective of the present study was to characterize refractory and/or relapsing cryptococcosis in patients with AIDS based on clinical aspects and on a phenotypic and molecular study of the causal agent. The data were compared with those for another group of patients with AIDS with nonrefractory and nonrelapsing cryptococcosis.

MATERIALS AND METHODS

The study was conducted on patients with cryptococcosis–HIV coinfection who were diagnosed and treated at the University Hospital, Medical School of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil, from 2000 to 2011. The patients were divided into two cohorts: 1) 19 patients with refractory or relapsing cryptococcosis (cohort 1). Refractory cryptococcosis was defined as persistent infection confirmed by the isolation of *Cryptococcus* spp. from the CSF after 2 months of antifungal therapy. Relapse was defined as reactivation of infection confirmed by the isolation of *Cryptococcus* spp. from the CSF after a period of improvement with negative cultures. 2) Thirty patients selected at random each year among those coinfected with HIV who had cryptococcosis during the same period as the patients of the previous cohort and who had shown culture negativity for *Cryptococcus* spp. within up to 2 months after the beginning of antifungal therapy (cohort 2—control).

Clinical and laboratory data. The clinical study was conducted retrospectively by analysis of the medical records and by collection of demographic and clinical data and of associated conditions at the beginning of and during treatment. Cryptococcal meningitis was defined by the isolation of *Cryptococcus* spp. from the CSF. Cryptococcal infection of brain parenchyma was inferred on the basis of brain computed tomography and nuclear magnetic resonance findings.

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Brain granulomas were attributed to cryptococcosis only when the patient did not show current or past evidence of CNS infection by another microorganism. The presence of pseudocysts in imaging examinations was always attributed to cerebral cryptococcosis. Cryptococcemia was detected using standard BacT/ALERT blood culture flasks (Biomérieux Brasil AS, Rio de Janeiro, Brazil) or BD flasks (Becton Dickinson and Company, Franklin Lakes, NJ). The detection of *Cryptococcus* spp. in sample from the respiratory tract or in the urine was an occasional finding in some patients and no systematic search was performed at these sites.

Chronic alcoholism and malnutrition were considered to be risk factors for cryptococcosis when these clinical diagnoses were registered in the medical records of the patient. Liver disease was defined as chronic liver damage by hepatitis B virus and/or hepatitis C virus, also including cirrhosis of the liver due to alcohol consumption or due to an undefined reason. Chronic renal failure was characterized by serum creatinine ≥ 3 mg/dL during the previous 2 months and at baseline. The use of corticosteroids was defined as the use of dexamethasone or prednisone at doses meant to obtain an anti-inflammatory effect for a period of at least 5 days, since 60 days before the diagnosis of cryptococcosis and after start of antifungal therapy. In some cases, classified as delayed antifungal therapy, the use of an antifungal agent was started only 30–84 days after the onset of symptoms due to a delayed diagnosis of cryptococcosis owing to confusion with other diseases. Problems in the administration of amphotericin B were characterized by the need to reduce the intended dose (1 mg/kg weight/day, maximum daily dose = 50 mg) to 20–40 mg/day and even to interrupt its administration for 24–48 hours because of immediate adverse effects, hematologic toxicity, and especially nephrotoxicity. As a consequence, there was a prolongation of the phase of therapeutic induction. Some patients also showed poor adherence to treatment with amphotericin B, which impaired the regular daily administration of this antifungal agent, and eventually interrupted its use for 1–7 days.

Leucopenia was defined at least one episode of leucocyte counts of < 2,000/μL blood during the course of treatment. Blood CD_4^+T-cell and CD_8^+T-cell counts were performed by flow cytometry during a period immediately after the diagnosis of cryptococcosis. The cryptococcal antigen was detected at baseline in the CSF using the Latex-*Cryptococcus* antigen kit (Immuno-Mycologics, Inc., Norman, OK), and the corresponding titers were determined using a reagent prepared in-house (polystyrene coated with a polyclonal anticryptococcal antigen obtained from immunized rabbits).

Amphotericin B deoxycholate was administered to all but two patients in the phase of therapeutic induction to obtain *Cryptococcus*-negative cultures in two consecutive CSF samples. Liposomal amphotericin B (2–3 mg/kg weight/day) was administered to one patient in each cohort at the beginning of therapy. However, because of its toxicity, the deoxycholate formulation was replaced by liposomal amphotericin B in 4/19 (21%) and 2/30 (7%) patients of the refractory and/or relapsing and control cohorts, respectively. Deoxycholate and/or liposomal amphotericin B were administered for a mean period of 118 ± 68 days (> 60 days in 79% of the patients) and 45 ± 27 days (≤ 60 days in 75% of the patients), respectively, to patients of the refractory and/or relapsing and control cohorts. The mean cumulative dose of amphotericin B was 3,575 ± 1,981 mg for the refractory and/or relapsing cohort (in the relapsing cryptococcosis cases, only the first episode of infection was computed) and 1,492 ± 977 mg for the control cohort patients. The mean cumulative amphotericin B dose for relapsing cryptococcosis patients was 2,208 ± 746 mg (N = 8) in the first episode of infection and 4,744 ± 3,354 mg (N = 7) in the relapse. Flucytosine was used for 7–21 days during the course of therapy with amphotericin B in 4/19 (21%) and 2/30 (7%) patients of the refractory and/or relapsing and control cohorts, respectively. One patient with relapsing infection was treated with posaconazole after a previous course of amphotericin B. Fluconazole was used for surviving patients in the consolidation phase after therapeutic induction. The doses for refractory and/or relapsing patients were the following: 400 mg/day (N = 6, 46%), 600 mg/day (N = 3, 23%), and 800 mg/day (N = 4, 31%); the duration of the consolidation phase was 2–6 weeks (N = 4, 31%) and ≥ 8 weeks (N = 9, 69%). The doses of fluconazole for control patients were 200 mg/day (N = 6, 29%), 400 mg/ day (N = 9, 43%), and 600 mg/day (N = 6, 29%); for control patients with a daily dosage ≥ 400 mg, the duration of the consolidation phase ranged from 2 to 6 weeks (N = 8, 53%) and ≥ 8 weeks (N = 7, 47%). Fluconazole of 200 mg/day was used during the maintenance phase of the antifungal therapy. The patients of both groups also received antiretroviral therapy with a combination of three drugs, which was usually introduced 15–30 days after the diagnosis of cryptococcosis for patients who were not yet using these drugs.

Outcome was determined for patients who had received a minimum cumulative dose of 500 mg amphotericin B and two patients of cohort 2 who had not reached this dose were excluded. The mortality rate for each group was calculated by considering the deaths attributed to cryptococcosis alone or with associated bacterial infection. The late death of a patient with persistent cryptococcal infection (cohort 1) was also computed, as also were the deaths that occurred during relapse episodes. A cure was defined as the control of cryptococcosis with no relapse after a follow-up period of 2–11 years. The patients who showed a significant clinical improvement but were lost to follow-up were classified together with the cured cases.

Mycological study. *Cryptococcus* spp. isolated from the patients was identified by standard laboratory mycology methods and also using the automated system Vitek® 1 or Vitek® 2 (Biomérieux, Marcy-l'Étoile, France). The yeast cells were maintained in the laboratory by periodic culture in Sabouraud medium at 25°C.

Capsule diameter and phospholipase and melanin production were determined in *Cryptococcus* spp. isolated before the beginning of antifungal therapy. In cohort 1, these virulence factors were also assessed in *Cryptococcus* spp. reisolated from the patients during the refractory period or during relapse. Capsule diameter was determined as described by Zaragoza and others[11] and yeast cells were incubated in RPMI-1640 medium (Roswell Park Memorial Institute Media no. 1640) with the addition of 3-(*N*-morpholino)propanesulfonic acid, HCO_3, pH 7.3, and 5% CO_2 at 37°C for 48 hours. Yeast-like cells were contrasted with India ink, observed, and photographed with a Zeiss microscope (Zeiss, Jena, Germany). The capsule diameter was determined as the difference between the whole cell with the capsule minus the diameter of the

yeast cell alone using the Image J software, version 1.43 (National Institutes of Health, Bethesda, MD).

Melanin production was quantitated by determining laccase activity as described by Ngamskulrungroj and Meyer.[12] Yeast cells were incubated in yeast nitrogen base melanin-inducing medium (1% glucose with no amino acids and ammonium sulfate) containing 10 mM dopamine and incubated at 37°C with shaking at 200 rpm. The culture supernatant was collected within 5 days and absorbance at 450 nm was measured with a Multiskan MS spectrophotometer (Labsystems, Vantaa, Finland).

Extracellular phospholipase production was determined by the method of Souza and others.[13] A yeast cell suspension was deposited at equidistant points on plates containing Sabouraud dextrose agar supplemented with 1 M NaCl, 5 mM $CaCl_2$, and 8% sterile egg yolk and incubated at 37°C for 7 days. Enzyme activity (Pz) was determined by the ratio of colony diameter (cd) to the precipitation zone (cdp), as follows Pz = cd/cdp.

Genotyping. Genotyping was carried out by the polymerase chain reaction (PCR) using seven pairs of specific primers to identify the species, serotype, and sexual type, that is, *Cryptococcus neoformans* var. grubii/Aα and Aa, *C. neoformans* var. neoformans/Dα and Da, and *C. gattii*/B/Cα and B/Ca.[14–18] Molecular typing was performed by PCR fingerprinting using primers for the specific sequence of the minisatellite $(GACA)_4$.[19]

Internal transcribed spacer sequence analysis for the identification of clinical isolates. The clinical isolates were identified molecularly by the sequencing of the internal transcribed spacer (ITS) regions of ribosomal DNA. All PCR procedures were performed using Phusion® High Fidelity DNA Polymerase (New England BioLabs Inc., Ipswich, MA) with the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′).[20] PCR products were sequenced using the same primers with the ABI3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Each sequence was analyzed with ChromasPro® Software (ChromasPro 1.7.6; Technelysium Pty Ltd., Tewantin, Queensland, Australia), and the DNA sequences were compared with the DNA sequence database of the Basic Local Alignment Search Tool.[21]

Statistical analysis. Data were analyzed statistically with the aid of the GraphPad Prism 6 software (San Diego, CA). Mean values were analyzed by analysis of variance, and proportions were compared by the chi-squared test or the Fisher's exact test, with the level of significance set at $P < 0.05$. HIV viral load, $CD4^+$ cells, $CD8^+$ cells, CSF leucocyte number, and CSF cryptococcal antigen titer were analyzed by the Mann–Whitney test, with the level of significance set at $P < 0.05$.

Ethics. The study was approved by the Research Ethics Committee of the University Hospital, Medical School of Ribeirão Preto, University of São Paulo (Protocol HCRP no. 12247/2010).

RESULTS

Among the 19 patients with refractory or relapsing cryptococcosis (cohort 1), 11 had refractory cryptococcosis with no sterilization of CSF cultures and eight had relapse of CSF culture–positive infection after initially sterilizing the CSF. Three patients had a single relapse and two of them obtained control of the infection. Five other patients had a relapse associated with refractory cryptococcosis in the second episode ($N = 1$) or in both episodes of infection ($N = 4$), the outcome being death for four of them. In eight patients, relapse occurred 6 weeks to 6 years (median = 13 weeks) after a negative CSF culture in the first episode of infection. For five patients, the time of death after relapse was 1–42 months (median = 14 months). In patients with refractory cryptococcosis, *C. neoformans* was isolated from consecutive CSF samples over periods of 10–83 weeks (median = 16 weeks), with death occurring in 8/11 cases during treatment. Among the 30 patients with negative CSF cultures for *Cryptococcus* spp. within up to 2 months after the beginning of amphotericin B (cohort 2), 18/28 (60%) obtained control of cryptococcosis.

The two patient cohorts were similar in terms of age range, predominance of men, HIV viral load, $CD4^+$ and $CD8^+$ cell counts, number of leucocytes, and cryptococcal antigen titer in the CSF (Table 1). The patients with refractory and/or relapsing cryptococcosis (cohort 1) presented a more extensive infection than the control group (cohort 2), as determined by significantly higher percentages of positive blood cultures (cryptococcemia) (73.7% versus 43.3%), brain pseudocysts (36.0% versus 3.3%), and brain granulomas (47.4% versus 10.0%) (Table 1). The death rate caused by direct or indirect complications of cryptococcosis was 68.4% (13/19) for refractory and/or relapsing cases and 33.3% (10/28) for control cases (Table 1). Five of the six patients who obtained control of the refractory and/or relapsing cryptococcal infection showed neurological sequelae, an event that was not observed

TABLE 1

Demographic and baseline clinical laboratory data and outcome of cryptococcosis in HIV-coinfected patients according to classification of the infection as refractory and/or relapsing (cohort 1) or not (cohort 2)

	Cohort 1	Cohort 2	
	Refractory and/or relapsing ($N = 19$)	Control ($N = 30$)	P value
Gender/age			
Men, n (%)	16 (84)	21 (70)	0.322
Age, mean ± SD	33.0 ± 8.3	33.5 ± 6.8	0.615
HIV infection: median/range			
Viral load (log copies/mL) ($N = 38$)	5.1/ND–6.8	4.9/ND–6.2	0.375
$TCD4^+$ cells/μL ($N = 45$)	49.5/3–271	42.0/1–248	0.573
$TCD8^+$ cells/μL ($N = 45$)	531/155–1,051	413/50–1,486	0.830
Cryptococcosis: CSF median/range			
Leucocytes/μL ($N = 49$)	16.0/0.6–1,950	32.5/0.0–2,400	0.962
Cryptococcal Ag titer* ($N = 48$)	256/8.0–4,096	384/0.0–4,096	0.417
Cryptococcosis: clinical (n [%])			
Meningitis	19 (100.0)	30 (100.0)	1.000
Brain granuloma	9 (47.4)	3 (10.0)	0.005
Brain pseudocyst	7 (36.8)	1 (3.3)	0.003
Cryptococcemia†	14 (73.7)	13 (43.3)	0.045
Pulmonary infection	1 (5.3)	1 (3.3)	1.000
Skin infection	1 (5.3)	1 (3.3)	1.000
Cryptococcuria	0 (0)	1 (3.3)	1.000
Patient outcome,‡ n (%)			
Cure/improvement	6 (31.6)	18 (60.0)	0.039
Death	13 (68.4)	10 (35.7)	

Ag = antigen; CSF = cerebrospinal fluid; HIV = human immunodeficiency virus; ND = nondetectable; SD = standard deviation.
*Titer is the inverse of CSF dilution.
†*Cryptococcus* isolated from blood culture.
‡Two patients of cohort 2 were excluded because the cumulative dose of amphotericin B they received was < 500 mg.

TABLE 2

Underlying diseases and risk factors for a complicated progression of HIV-related cryptococcosis according to classification as refractory and/or relapsing infection (cohort 1) or not (cohort 2)

	Cohort 1	Cohort 2	
	Refractory and/or relapsing (N = 19)	Control (N = 30)	
	n (%)	n (%)	P value
Alcoholism	9 (47.4%)	13 (43.3%)	1.000
Chronic liver disease*	8 (42.1%)	1 (3.3%)	0.001
HCV and/or HBV infection†	3 (15.8%)	6 (20.0%)	1.000
Diabetes mellitus	1 (5.3%)	0 (0%)	0.387
Chronic renal failure	0 (0%)	1 (3.3%)	1.000
Malnutrition	0 (0%)	2 (6.7%)	0.515
Use of corticosteroids	9 (47.4%)	8 (26.7%)	0.217
Delayed antifungal therapy‡	5 (26.3%)	9 (30.0%)	1.000
Problems in using amphotericin B§	12 (63.2%)	12 (40.0%)	0.148
Leucopenia	12 (63.2%)	7 (23.3%)	0.007
Poor adherence to treatment¶	5 (26.3%)	2 (6.7%)	0.092

HBV = hepatitis B virus; HCV = hepatitis C virus; HIV = human immunodeficiency virus; SD = standard deviation.
*Caused by HCV and/or HBV and/or alcoholism or of unknown cause.
†Apparently with no associated liver disease.
‡Amphotericin B started > 30 days after the onset of symptoms.
§Daily dose below the recommended quantity (1 mg/kg weight/day, maximum dose = 50 mg) and temporary interruption because of amphotericin B deoxycholate toxicity.
¶Daily dose below the recommended quantity (1 mg/kg weight/day, maximum dose = 50 mg) and temporary interruption because of patient refusal.

in control patients. Among the underlying diseases and risk factors evaluated, chronic liver disease (P = 0.001) and leucopenia (P = 0.007) were more common in refractory and/or relapsing patients (Table 2).

All isolates from patients with persistent and/or refractory infection were identified as *C. neoformans* var. *grubii* and sex type α, characteristics that also predominated in control cases (Supplemental Figures 1 and 2A and B). Eight isolates not identified by PCR were analyzed by the comparative sequence of the ITS region of ribosomal DNA and identified as *C. neoformans* var. *grubii* (Table 3 and Supplemental Table 1). Only one isolate from a control patient was identified as *C. gattii* and sex type α and genotype VGI (Supplemental Figure 2C). All isolates (cohorts 1 and 2) showed the same genotype VNI and VGI (Figure not shown).

TABLE 3

Genotype, sexual type, and virulence factors of *Cryptococcus* spp. isolated from HIV-coinfected patients according to classification of the infection as refractory and/or relapsing (cohort 1) or not (cohort 2)

	Cohort 1		Cohort 2		
	Refractory and/or relapsing (N = 19)		Control (N = 30)		
Identification*	n	%	n	%	P value
Cryptococcus neoformans var. *grubii* α/VNI (PCR)*	19	100.0	29	96.7	1.000
C. gattii α/VGI	0	0	1	3.3	1.000
Virulence factors†	Mean ± SD		Mean ± SD		
Capsule diameter (μm)	2.75 ± 0.67		2.71 ± 0.83		0.338
Melanin (OD)‡	0.11 ± 0.05		0.10 ± 0.06		0.675
Phospholipase (Pz)§	0.76 ± 0.08		0.77 ± 0.05		0.430

HIV = human immunodeficiency virus; OD = optical density; PCR = polymerase chain reaction; SD = standard deviation.
*Molecular identification and sexual type.
†*Cryptococcus* spp. isolated before antifungal treatment.
‡Absorbance at 450 nm.
§Pz—index obtained from the diameters of the *Cryptococcus* spp. colony and of the precipitation zone in agar; *P*, statistic.

Cryptococcus spp. from the two cohorts, isolated before the patient treatment, did not show differences in the capsule diameter or in the production of both melanin and phospholipase (Table 3). In seven refractory and/or relapsing cases it was possible to evaluate two to five isolates from the same patient during different treatment phases or during relapse. All isolates were identified as *C. neoformans* var. *grubii* and no defined tendency to variation occurred in the expression of the virulence factors investigated (Table 4).

DISCUSSION

The main result of the comparative assessment of cases of refractory and/or relapsing cryptococcal meningoencephalitis was the association of failure to respond to treatment with disseminated fungal infection and a greater involvement of the CNS. In many cases of relapse, recurrence was found to be preceded or followed by infection refractory to treatment. Men predominated in both patient cohorts because of the demographic features of AIDS, similar to other case series in Brazil.[22] The age ranges as well as the viral HIV load and the $CD4^+$ and $CD8^+$ cell counts were similar. In other studies, a low $CD4^+$ cell count was one of the factors predisposing to recurrent cryptococcosis,[23] and death was related to a high HIV viral load.[24]

Clinically, refractory and/or relapsing cryptococcosis was characterized by a disseminated infection represented by a higher prevalence of cryptococcemia and of expansive injury in the brain parechyma, as well as high lethality. Both bloodstream infection and the presence of pseudocysts and cryptococcal granulomas have been related to treatment failure and to patient death.[8,25,26] A high number of *Cryptococcus* spp. cells in the CSF, which represents infection with high fungal load was also associated with a poor prognosis.[24] Expansive brain lesions are caused more frequently by *C. gattii*,[9] but this species was identified in only one patient of cohort 2. The great brain involvement of cohort 1 patients may be a consequence of more intense CNS invasion during the pretreatment phase. Possibly it was due to the lack of the immune and phagocytic response of the patients or was later due to poor penetration of amphotericin B in the brain, pseudocysts, and granulomatous lesions. High cryptococcal antigen titers and low number of leucocytes in the CSF have been reported as factors of a poor prognosis in cryptococcosis.[5,7] However, in this study, the cases of persistent and/or recurrent infection (cohort 1) were not distinguished by these CSF baseline parameters from less complicated cases (cohort 2). The lethality of refractory or recurrent cryptococcosis was approximately double than that observed in control cases and exceeded the lethality close to 50% reported in Brazilian case series.[2,27] It was observed in African and Asian patients that persistent infection is one of the factors related to death within 10 weeks of treatment.[28] Uncontrolled intracranial hypertension and secondary bacterial infection during antifungal treatment were the main causes of death of the 14 patients of the cohort studied (data not shown). The seriousness of refractory and/or relapsing cases also had an impact on the six patients who were cured, since 5/6 patients showed neurological sequelae. The analysis of lethality among the present patients was limited by the lack of use of flucytosine during the phase of treatment induction. This antifungal agent is not available in Brazil and was administered

TABLE 4

Phenotypic characteristics of different *Cryptococcus* spp. strains isolated from seven patients with refractory and/or relapsing cryptococcosis before the antifungal treatment and at relapse of cryptococcal infection

		Capsule (μm)†		Melanin (OD)‡		Phospholipase (*Pz*)§	
Cases	Time (months)*	A	B	A	B	A	B
1	87.0	2.50	3.56	0.08	0.12	0.53	0.76
2	26.0	1.81	2.5	0.18	0.25	0.71	0.77
3	4.3	1.98	1.97	0.12	0.12	0.77	0.80
4	3.4	2.37	2.63	0.18	0.15	0.66	0.71
5	7.0	2.64	3.24	0.12	0.10	0.80	0.60
6	7.0	3.51	1.88	0.08	0.06	0.80	0.80
7	24.0	3.37	3.20	0.10	0.07	0.81	0.81
Mean ± SD		2.60 ± 0.644	2.711 ± 0.649	0.122 ± 0.042	0.124 ± 0.063	0.725 ± 0.102	0.750 ± 0.074
P value		0.7460		0.9613		0.621	

A = isolate obtained before antifungal treatment; B = *Cryptococcus* spp. reisolated from the patient after the time indicated; OD = optical density; SD = standard deviation.
*Time interval between *Cryptococcus* spp. isolation during the first episode and at relapse of cryptococcosis.
†Capsule diameter.
‡Absorbance at 450 nm.
§Index obtained by dividing the diameters of *Cryptococcus* spp. colony by the zone of precipitation in agar.

to few patients in a later phase or when it became evident that the infection was refractory to amphotericin B. The combination of flucytosine with amphotericin B improves the prognosis of patients with cryptococcosis and failure to use flucytosine may contribute to the persistence of infection and to the higher lethality observed.[29] The causes of the persistence and relapse of cryptococcosis were investigated among the underlying diseases and also in the respective fungal isolates. Factors leading to an unfavorable outcome were observed in both patient groups such as alcoholism, late beginning of antifungal treatment, problems in using amphotericin B because of its toxicity, poor adherence to treatment, and the previous or concomitant use of corticosteroids. Corticosteroids have been used to reduce intracranial hypertension or for other reasons, but their use in cryptococcosis was associated with clinical failure and early death.[30] The adverse effects of the formulation of amphotericin B deoxycholate were common and required a reduction of the daily dose recommended, so that therapeutic levels could not be reached. The use of a low daily dose of amphotericin B motivated by toxicity or poor patient adherence possibly contributed to the poor outcome of cryptococcal infection in both patient cohorts. Chronic liver disease and leucopenia predominated in the cases of refractory and/or recurrent cryptococcosis. Cirrhosis was related to a greater lethality of cryptococcosis within 10 weeks[31] and may have increased the severity of infection by reducing the hepatic clearance of yeast cells and their antigen. Neutropenia can be a complication of the treatment of cryptococcosis, especially in patients with AIDS receiving flucytosine. In the present study, leucopenia was apparently caused by prolonged exposure to amphotericin B or to other drugs (data not shown). Leucopenia was possibly related to the poor outcome of patients with persistent and/or refractory infection due to the reduced phagocyte capacity to control the blood dissemination of *Cryptococcus* spp. and of other microorganisms.

Except for one patient from control cohort, all other patients including the cases of refractory and/or recurrent cryptococcosis were infected with *C. neoformans* var. *grubii*, genotype VNI, which has been predominantly identified in HIV-coinfected patients.[32] No evidence was obtained that persistent and/or relapsing infection may be associated with species, genotype, or sexual type of *Cryptococcus* spp. About 27% of the isolates from control patients (cohort 2) were identified only by sequencing. The lack of identification by PCR may suggest discrete genotypic changes, perhaps associated with variation in the virulence. A molecular subtype of *C. neoformans* var. *grubii* has been associated with higher patient mortalty.[33,34] These results further complicated the relationship of the host with the infecting fungus, because the possibility that certain strains may be more resistant to phagocytosis and to antifungal agents, a fact that facilitates their persistence in the patient.

The phenotypic expression of presumed virulence factors was comparable between the fungal isolates from the patient groups under study, suggesting that capsule size and melanin or phospholipase production do not have a causal relationship with persistent and/or recurrent infection. The virulence of *Cryptococcus* spp. isolated from patients with AIDS was variable in a murine model of infection and was not related to the capsule size of the microorganism or to the production of melanin and phospholipase.[35] However, virulence was related to capsule size in vivo in the murine model and to cryptococcal capsule size ex vivo in human infection.[36] No genotype or phenotype differences were detected between the *Cryptococcus* spp. isolated during the pretreatment phase and those isolated from the patients during the persistence of infection or on the occasion of a relapse. A previous study has shown that *Cryptococcus* spp. reisolated from persistent or recurrent infection had a genotypic profile identical to that of the initial isolate from the same patient despite the genotypic variability among fungal isolates of different patients.[32] Other studies have shown that *C. neoformans* var. *grubii* may undergo discrete genotypic modifications in the host, eventually accompanied by changes in the expression of virulence factors and of susceptibility to azole drugs.[37,38]

Multiple factors may be involved in the persistence and relapse of cryptococcal infection. In the present study, these complications were more related to the host and the extent of infection than to the causal agent. An earlier diagnosis and access to flucytosine to be used in the combined antifungal therapeutic scheme may perhaps reduce blood dissemination and brain injuries and consequently reduce the morbidity and mortality of patients with complicated cryptococcosis.

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REFERENCES

1. Park BJ, Wannemuechler KA, Marston BJ, Govender N, Pappas PG, Chiller TM, 2009. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *AIDS 23:* 525–530.
2. Mora DJ, da Cunha Colombo ER, Ferreira-Paim K, Andrade-Silva LE, Nascentes GA, Silva-Vergara ML, 2012. Clinical, epidemiological and outcome features of patients with cryptococcosis in Uberaba, Minas Gerais, Brazil. *Mycopathologia 173:* 321–327.
3. Liaw SJ, Wu HC, Hsueh PR, 2010. Microbiological characteristics of clinical isolates of *Cryptococcus neoformans* in Taiwan: serotypes, mating types, molecular types, virulence factors, and antifungal susceptibility. *Clin Microbiol Infect 16:* 696–703.
4. Trilles L, Meyer W, Wanke B, Guarro J, Lazéra M, 2012. Correlation of antifungal susceptibility and molecular type within the *Cryptococcus neoformans/C. gattii* species complex. *Med Mycol 50:* 328–332.
5. Antinori S, Galimberti L, Magni C, Casella A, Vago L, Mainini F, Piazza M, Nebuloni M, Fasan M, Bonaccorso C, Vigevani GM, Cargnel A, Moroni M, Ridolfo A, 2001. *Cryptococcus neoformans* infection in a cohort of Italian AIDS patients: natural history, early prognostic parameters, and autopsy findings. *Eur J Clin Microbiol Infect Dis 20:* 711–717.
6. Diamond RD, Bennett JE, 1974. Prognostic factors in cryptococcal meningitis. A study in 111 cases. *Ann Intern Med 80:* 176–181.
7. Anekthannon T, Manosuthi W, Chetchotisakd P, Kiertiburanakul S, Supparatpinyo K, Ratanasuwan W, Pappas PG, Filler SG, Kopetskie HA, Nolen TL, Kendrick AS, Larsen RA, BAMSG 3-01 Study Team, 2011. Predictors of poor clinical outcome of cryptococcal meningitis in HIV-infected patients. *Int J STD AIDS 22:* 665–670.
8. Brizendine KD, Baddley JW, Pappas PG, 2013. Predictors of mortality and differences in clinical features among patients with Cryptococcosis according to immune status. *PLoS One 8:* e60431.
9. Sorrell TC, 2011. *Cryptococcus neoformans* variety *gattii*. *Med Mycol 39:* 155–168.
10. Buchaman KL, Murphy JW, 1998. What makes *Cryptococcus neoformans* a pathogen? *Emerg Infect Dis 4:* 71–83.
11. Zaragoza O, Fries BC, Casadevall A, 2003. Induction of capsule growth in *Cryptococcus neoformans* by mammalian serum and CO_2. *Infect Immun 71:* 6155–6164.
12. Ngamskulrungroj P, Meyer W, 2009. Melanin production at 37°C is linked to the high virulent *Cryptococcus gattii* Vancouver Island outbreak genotype VGIIa. *Australas Mycologist 28:* 9–15.
13. Souza LK, Souza Junior AH, Costa CR, Faganello J, Vainstein MH, Chagas AL, Souza AC, Silva MR, 2010. Molecular typing and antifungal susceptibility of clinical and environmental *Cryptococcus neoformans* species complex isolates in Goiania, Brazil. *Mycoses 53:* 62–67.
14. Lin X, Heitman J, 2006. The biology of the *Cryptococcus neoformans* species complex. *Annu Rev Microbiol 60:* 69–105.
15. Okabayashi K, Kano R, Watanabe T, Hasegawa A, 2006. Serotypes and mating types of clinical isolates from feline cryptococcosis in Japan. *J Vet Med Sci 68:* 91–94.
16. D'Souza CA, Hagen F, Boekhout T, Cox GM, Heitman J, 2004. Investigation of the basis of virulence in serotype A strains of *Cryptococcus neoformans* from apparently immunocompetent individuals. *Curr Genet 46:* 92–102.
17. Chatuverdi S, Rodeghier B, Fan J, McClelland CM, Wickes BL, Chatuverdi V, 2000. Direct PCR of *Cryptococcus neoformans* MATalpha and MATa pheromones to determine mating type, ploidy, and variety: a tool for epidemiological and molecular pathogenesis studies. *J Clin Microbiol 38:* 2007–2009.
18. Escadón P, Quintero E, Granados D, Huérfano S, Ruiz A, Castañeda E, 2005. Aislamiento de *Cryptococcus gattii* serotipo B a partir de detritus de *Eucalyptus* spp. en Colombia. *Biomédica 25:* 390–397.
19. Meyer W, Castañeda A, Jackson S, Huynh M, Castañeda E, IberoAmerican Cryptococcal Study Group, 2003. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. *Emerg Infect Dis 9:* 189–195.
20. White TJ, Bruns TD, Lee S, Taylor JW, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Innis MA, Gelfand DH, Sininsky JJ, White TJ, eds. *PCR Protocols: A Guide to Method and Applications*. San Diego, CA: Academic Press, 315–322.
21. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ, 1990. Basic local alignment search tool. *J Mol Biol 215:* 403–410.
22. de Oliveira RB, Atobe JH, Souza SA, de Castro Lima Santos DW, 2014. Epidemiology of invasive fungal infections in patients with acquired immunodeficiency syndrome at a reference hospital for infectious diseases in Brazil. *Mycopathologia 178:* 71–78.
23. Lortholary O, Poizat G, Zeller V, Neuville S, Boibieux A, Alvarez M, Dellamonica P, Botterel F, Dromer F, Chêne G, 2006. Long-term outcome of AIDS-associated cryptococcosis in the era of combination antiretroviral therapy. *AIDS 20:* 2183–2191.
24. Vidal JE, Gerhardt J, Peixoto de Miranda EJ, Dauar RF, Oliveira Filho GS, Penalva de Oliveira AC, Boulware DR, 2012. Role of quantitative CSF microscopy to predict culture status and outcome in HIV-associated cryptococcal meningitis in Brazilian cohort. *Diagn Microbiol Infect Dis 73:* 68–73.
25. Dammert P, Bustamante B, Ticona E, Llanos-Cuentas A, Huaroto L, Chávez VM, Campos PE, 2008. Treatment of cryptococcal meningitis in Peruvian AIDS patients using amphotericin B and fluconazole. *J Infect 57:* 260–265.
26. Dromer F, Mathoulin-Péllissier S, Launay O, Lortholary O, French Cryptococcosis Study Group, 2007. Determinants of disease presentation and outcome during cryptococcosis: the CryptoA/D study. *PLoS Med 4:* e21.
27. Lindenberg Ade S, Chang MR, Paniago AM, Lazéra Mdos S, Moncada PM, Bonfim GF, Nogueira SA, Wanke B, 2008. Clinical and epidemiological features of 123 cases of cryptococcosis in Mato Grosso do Sul, Brazil. *Rev Inst Med Trop Sao Paulo 50:* 75–78.
28. Bicanic T, Muzoora C, Brouwer AE, Meintjes G, Longley N, Taseera K, Rebe K, Loyse A, Jarvis J, Bekker LG, Wood R, Limmathurotsakul D, Chierakul W, Stepniewska K, White NJ, Jaffar S, Harrison TS, 2009. Independent association between rate of clearance of infection and clinical outcome of HIV-associated cryptococcal meningitis: analysis of a combined cohort of 262 patients. *Clin Infect Dis 49:* 702–709.
29. Bratton EW, El Husseini N, Chastain CA, Lee MS, Poole C, Stürmer T, Weber DJ, Juliano JJ, Perfect JR, 2013. Approaches to antifungal therapies and their effectiveness among patients with cryptococcosis. *Antimicrob Agents Chemother 57:* 2485–2495.
30. Graybill JR, Sobel J, Saag M, van Der Horst C, Powderly W, Cloud G, Riser L, Hamill R, Dismukes W, 2000. Diagnosis and management of increased intracranial pressure in patients with AIDS and cryptococcal meningitis. *The NIAID Mycoses Study Group and AIDS Cooperative Treatment Groups. Clin Infect Dis 30:* 47–54.
31. Tseng HK, Lin CP, Ho MW, Lu PL, Lo HJ, Lin YH, Cho WL, Chen YC, Taiwan Infectious Diseases Study Network for Cryptococcosis, 2013. Microbiological, epidemiological, and clinical characteristics and outcomes of patients with cryptococcosis in Taiwan, 1997–2010. *PLoS One 8:* e61921.
32. Igreja RP, Lazéra Mdos S, Wanke B, Galhardo MC, Kidd SE, Meyer W, 2004. Molecular epidemiology of *Cryptococcus*

neoformans isolates from AIDS patients of the Brazilian city, Rio de Janeiro. *Med Mycol 42:* 229–238.

33. Alanio A, Desnos-Ollivier M, Dromer F, 2011. Dynamics of *Cryptococcus neoformans*–macrophage interactions reveal that fungal background influences outcome during cryptococcal meningoencephalitis in humans. *MBio 2:* pii: e00158-11.
34. Wiesner DL, Moskalenko O, Corcoran JM, McDonald T, Rolfes MA, Meya DB, Kajumbula H, Kambugu A, Bohjanen PR, Knight JF, Boulware DR, Nielsen K, 2012. Cryptococcal genotype influences immunologic response and human clinical outcome after meningitis. *MBio 3:* pii: e00196-12.
35. Clancy CJ, Nguyen MH, Allandoerffer R, Cheng S, Iczkowski K, Richardson M, Graybill JR, 2006. *Cryptococcus neoformans* var. *grubii* isolates recovered from persons with AIDS demonstrate a wide range of virulence during murine meningoencephalitis that correlates with the expression of certain virulence factors. *Microbiology 152:* 2247–2255.
36. Robertson EJ, Najjuca G, Rolfes MA, Akampurira A, Jain N, Anantharanjit J, von Hohenberg M, Tassieri M, Carlson A, Meya DB, Harrison TS, Fries BC, Boulware DR, Bicanic T, 2014. *Cryptococcus neoformans* ex vivo capsule size is associated with intracranial pressure and host immune response in the HIV-associated cryptococcal meningitis. *J Infect Dis 209:* 74–82.
37. Ormerod KL, Morrow CA, Chow EW, Lee IR, Arras SD, Schirra HJ, Cox GM, Fries BC, Fraser JA, 2013. Comparative genomics of serial isolates of *Cryptococcus neoformans* reveal gene associated with carbon utilization and virulence. *G3 (Bethesda) 3:* 675–686.
38. Illnait-Zaragozi MT, Martínez-Machin GF, Fernández-Andreu CM, Hagen F, Boekhout T, Klaassen CH, Meis JF, 2010. Microsatellite typing and susceptibilities of serial *Cryptococcus neoformans* isolates from Cuban patients with recurrent meningitis. *BMC Infect Dis 10:* 289.