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Major Article

Clinical and microbiological features of cryptococcal meningitis

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ABSTRACT

Introduction: In this study, the clinical features, underlying diseases and clinical outcomes of patients with cryptococcosis were investigated. In addition, a molecular analysis of the *Cryptococcus neoformans* species complex isolated from these patients was performed. **Methods**: A prospective study of 62 cases of patients with cryptococcal infection was conducted at the Hospital de Doenças Tropicais de Goiás Dr. Anuar Auad from 2009-2010. Cryptococcal meningitis cases were diagnosed by direct examination and cerebrospinal fluid (CSF) sample culture. The profiling of these patients was assessed. The CSF samples were submitted to India ink preparation and cultured on Sabouraud dextrose agar, and *C. neoformans* was identified by the production of urease, a positive phenoloxidase test and assimilation of carbohydrates. *C. neoformans* and *C. gattii* isolates were distinguished by growth on L-canavanine-glycine-bromothymol blue medium, and molecular analysis was conducted via PCR fingerprinting reactions using M13 and $(GACA)_4$ primers. **Results**: From the 62 patients with cryptococcosis, 71 isolates of CSF were obtained; 67 (94.4%) isolates were identified as *C. neoformans* var. *grubii*/VNI, and 4 (5.6%) were identified as *C. gattii*/VGII. Of these patients, 53 had an HIV diagnosis. The incidence of cryptococcosis was higher among patients 20-40 years of age, with 74.2% of the cases reported in males. *Cryptococcus*-related mortality was noted in 48.4% of the patients, and the symptoms were altered sensorium, headache, fever and stiff neck. **Conclusions**: The high morbidity and mortality observed among patients with cryptococcosis demonstrate the importance of obtaining information regarding the epidemiological profile and clinical course of the disease in the State of Goiás, Brazil.

Keywords: Cryptococcosis. HIV. Epidemiological and clinical features.

INTRODUCTION

Cryptococcosis is a fungal infection resulting from the inhalation of *Cryptococcus neoformans* species complex present in the environment. This yeast has a strong tropism for the central nervous system (CNS) and causes meningoencephalitis with symptoms that include headache, fever, visual problems and an altered mental state[1]. Acquired immunodeficiency syndrome (AIDS) and an expanding population with immunosuppressive therapies have contributed to an increase in cryptococcal disease, which is one of the most important opportunistic infections related to AIDS, particularly in the developing world[2,3]. Among AIDS patients, the rate of infection ranges from 23% to 48.6%, and approximately 70% to 90% of cryptococcal patients have signs and symptoms of subacute meningitis or meningoencephalitis with high mortality rates[4,5]. AIDS-associated cryptococcosis is primarily caused by *C. neoformans* var. *grubii* (serotype A). *C. neoformans* var. *neoformans* (serotype D) and AD hybrids have been recovered from approximately 98% of these patients[4,6]. A smaller proportion of disease is caused by *C. gattii* (serotypes B and C), which predominantly affects immunocompetent individuals, and *C. gattii* is considered to be endemic in the tropical and subtropical regions of the world[7].

The aims of this study were to investigate the clinical features, underlying the diseases and clinical outcomes of patients with cryptococcosis and to perform a molecular analysis of *Cryptococcus neoformans* species complex isolates collected from patients with meningitis in a tertiary hospital in Goiânia-GO, Brazil, from 2009 to 2010.

METHODS

Cases series study

A prospective study of 62 patients with cryptococcal infection was conducted in the Hospital de Doenças Tropicais de Goiás Dr. Anuar Auad (a reference hospital for tropical

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disease) from January 2009 to December 2010. Patients were enrolled if they had a qualitative cerebrospinal fluid (CSF) culture that yielded *C. neoformans*. Subjects were excluded if they refused to participate in the study. Patient HIV infection status was evaluated according to the standards established by the *Ministério da Saúde*[8]. Cases of cryptococcal meningitis were diagnosed by direct examination and culture of CSF samples. Patients were considered to have suffered relapse if the symptoms occurred >6 months after the previous episode[9]. The epidemiological profile of these patients, including gender, age, underlying diseases, $CD4^+$ T cell count (determined using a FACScan Flow Cytometer - Becton Dickinson, San Jose, CA, USA), recurrence, clinical outcome and related symptoms, were reviewed in the relevant medical and laboratory records. The molecular type of the *Cryptococcus neoformans* species complex was identified using PCR fingerprinting**.**

Microbiology

The 71 CSF samples that originated from 62 patients were submitted to India ink preparation and cultured on Sabouraud dextrose agar (SDA). *C. neoformans* was identified by urease production, a positive phenoloxidase test and assimilation of carbohydrates. Species identification was determined using L-canavanine-glycine-bromothymol blue (CGB) medium to distinguish between *C. neoformans* and *C. gattii* isolates[10]. Molecular analysis was conducted using oligonucleotides of the minisatellite-specific sequence of phage M13 and of the microsatellite-specific sequence $(GACA)_4$ as single primers in the PCR-fingerprinting reactions. The following standard strains representing each molecular type were included in the analysis: *C. neoformans* WM 148 (VNI-serotype A), WM 626 (VNII-serotype A), WM 628 (VNIII-serotype A-D), WM 629 (VNIV-serotype D), WM 179 (VGI-serotype B), WM 178 (VGII-serotype B), WM 161 (VGIII-serotype C) and WM 779 (VGIV-serotype C). These strains were kindly provided by the Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul[11,12]. Written informed consent was obtained from each patient. This study was approved by the ethics committee of the Hospital de Doenças Tropicais.

Statistical analysis

Statistical analysis was performed using Epi-Info 6.0 software. The proportions were compared using the chi-square test or Fisher´s Exact test of probability for small number analyses. All statistical tests were two tailed. P values below 0.05 were considered statistically significant.

RESULTS

Among the 71 isolates, 67 (94.4%) were identified as *C. neoformans* var. *grubii*/VNI, and 4 (5.6%) were identified as *C. gattii*/VGII. Of the 62 patients with cryptococcosis, 53 had an HIV diagnosis. Infections with *C. gattii* and *C. neoformans* var. *grubii* occurred in 2 and 51 patients, respectively. Among the 53 HIV-infected patients, 81.1% (43/53) had a $CD4^+$ T cell count of <100 cells/mm^3, and 18.9% (10/53) had a $CD4^+$ cell count between 200 and 300 cells/mm^3. The incidence of cryptococcosis was higher among persons 20-40 years of age ($p<0.0001$), and 74.2% of the cases were reported in males ($p<0.0001$). Infection or illness associated with cryptococcosis was observed in 37 patients when they were hospitalized. Oral candidiasis was the most frequent ($p=0.0001$) illness in 19 patients, followed by toxoplasmosis, tuberculosis (3 HIV+ and 2 HIV- patients) and hanseniasis (4 HIV- patients). Out of 62 patients, *Cryptococcus*-related mortality was observed in 30 (48.4%) patients with *C. gattii* as the causative agent in 2. Relapse of cryptococcosis was observed in 9 patients. No significant differences were found in patient characteristics between the HIV-negative and HIV-positive patients. The characteristics of the patients are shown in **Table 1**.

At the time of cryptococcosis diagnosis, symptoms were reported in all patients. Of the 62 patients studied, headache (75.8%) was the most common symptom ($p=0.0004$) followed by fever (61.3%) and stiff neck (58.1%). There were no differences in symptoms between the surviving patients and those who died. Headache was also the most common symptom, but only altered sensorium was significantly ($p=0.03$) associated with a poor outcome in patients with cryptococcosis. The clinical findings are summarized in **Table 2**.

DISCUSSION

Capsular polysaccharide characteristics allow the differentiation of *C. neoformans* into serotypes A, D and the AD hybrid and of *C. gattii* into serotypes B and C.

Cryptococcus neoformans var. *grubii* (serotype A) is an opportunistic fungus that affects primarily AIDS patients, whereas *C. gattii* is a primary pathogen rarely associated with AIDS[7,13]. Differences in the strain prevalence have been observed in different geographic regions, but according to Lin, *C. neoformans* var. *grubii* occurs in 95% of cryptococcal infections in AIDS patients[7]. Our study supports previous studies[14,15] performed in Brazil that found that *C. neoformans* var. *grubii* is the predominant etiologic agent of cryptococcosis in AIDS patients along with a small percentage of *C. gattii* isolates. Souza et al. found 80 *C. neoformans* var. *grubii* (serotype A) and 4 *C. gattii* (serotype B) isolates in clinical samples from AIDS patients[16]. The low *C. gattii* AIDS coinfection rate obtained in our study may have occurred because this species is not endemic in our geographic region. In the 290 environmental samples collected from pigeon droppings and material associated with the eucalyptus trees of Goiânia-GO, Brazil, Kobayashi et al. observed 41 *C. neoformans* isolates, which all were identified as *C. neoformans* var. *grubii* (serotype A)[17].

The majority of the patients in our study (85.5%) had AIDS, and 93.5% (58/62) of isolates were classified as *C. neoformans* var. *grubii.* In regards to the molecular types observed in our study, we identified VNI and VGII. These two types are the most common in Brazil[18,19]. Molecular studies of infective agents may contribute to answering questions about epidemiology and the evaluation of interventions and treatments. Molecular methods suggest that different patients may be infected

TABLE 1 - Main characteristics associated with cryptococcal meningitis cases in HIV-positive and HIV-negative patients.

	HIV positive (n=53)		HIV negative (n=9)		Total (n=62)	
Characteristics	n	%	n	%	n	%
Age distribution						
< 20 years	0	0.0	1	11.1	1	1.6
20-40 years	44	83.0	3	33.3	47	75.8*
41-60 years	9	17.0	4	44.5	13	21.0
> 60 years	0	0.0	1	11.1	1	1.6
Gender						
male	39	73.6	7	66.7	46	74.2*
female	14	26.4	2	22.3	16	25.8
Underlying diseases						
candidiasis	19	35.8	0	0.0	19	30.6*
hanseniasis	1	1.9	4	44.4	5	8.1
toxoplasmosis	8	15.1	0	0.0	8	12.9
tuberculosis	3	5.7	2	3.8	5	8.1
Clinical outcome						
survived	28	52.8	4	44.0	32	51.6
expired	25	47.2	5	55.6	30	48.4
recurrence	9	28.1	0	0.0	9	14.5
Species/serotyping						
Cryptococcus neoformans/VNI	51	96.2	7	77.8	58	93.5*
Cryptococcus gattii/VGII	2	3.8	2	22.2	4	6.5

*p<0.05; HIV: human immunodeficiency virus; VNI: serotype A; VGII: serotypes B and C.

TABLE 2 - Clinical manifestations in 62 cryptococcal meningitis patients.

	Survived n=32		Expired n=30		Total n=62	
Signs/symptoms	n	%	n	%	n	%
Headache	24	75	23	76.7	47	75.8
Vomiting	12	37.5	16	53.3	28	45.2
Fever	17	53.1	21	70.0	38	61.3
Stiff neck	18	56.2	18	60.0	36	58.1
Altered sensorium*	7	21.8	15	50.0	22	35.5

*p=0.03.

with a *C. neoformans* strain that presents the same genetic profile[20]. In addition, among sequential isolates from the same patient, the response to antifungal therapy may be different[21]. According to Yee-Chun et al., the rate of mutation is high in VGII isolates, and this could be responsible for a poorer response to antifungal drugs[22].

Although cryptococcosis has been reported in patients of all ages, a high incidence was observed in patients between 20 and 40 years of age in this study. A similar age group among cryptococcosis cases has been reported by other investigators[15,23,24]. Leal et al. observed that 46.2% of patients were 30-39 years old[15], whereas in 2008, Leiman and Koifman observed that 84% of patients were 20-40 years old[23]. The predominance of the male gender (p=0.0001) observed in our study has been consistently reported in cases of cryptococcosis[5,15]. This mycosis is known to be associated with AIDS, and in these patients, the incidence is higher in men than in women. In 2000, Imwidthaya and Poungvarin reported that cryptococcosis occurred in a proportion of 4 males to 1 female[24].

Most patients with cryptococcosis described in our work were HIV infected, but we also identified nine HIV-negative cases. Underlying immunosuppression associated with cryptococcosis in HIV-negative patients has been reported. Hematologic malignancies, autoimmune diseases, sarcoidosis and other diseases that present some degree of immunodeficiency may be risk factors for cryptococcosis[25]. In our study, four cases of leprosy were associated with cryptococcosis, and we also observed two tuberculosis cases in HIV-negative patients. The association of leprosy or tuberculosis with cryptococcosis has been rarely described in patients not affected by HIV. Azulay et al. described one case of lepromatous leprosy associated with cryptococcosis, but these authors suggested that this occurred due to the prolonged and high doses of corticosteroids used to treat the leprosy reactions[26]. Van-Tongeren et al. reported one case of coinfection with cryptococcosis caused by *C. gattii* and tuberculosis[27]. In the AIDS patients with associated cryptococcal

meningitis studied in our work, oral candidiasis occurred in 19 (35.8%) cases. Similar to our results, Baradkar et al. reported that 52.6% of 19 patients who suffered from cryptococcal meningitis had lesions suggestive of oral candidiasis[28].

According to a 2011 study by Fidel, oral candidiasis is the first clinical sign of underlying HIV infection, and it occurs in 50% to 90% of all HIV-infected persons during their progression to AIDS[29]. Our results emphasize the importance of considering more than one infection, even in the absence of evident immunodeficiency.

Several symptoms, such as headache, fever, vomiting, altered sensorium and stiff neck, are observed in cryptococcosis patients. In most studies, headache is considered the predominant clinical manifestation[4,30]. Similar to the results of Aslan and Chandrasekhara, in the present study, headache was the most common symptom observed, and altered sensorium was determined to be the poorest prognostic factor[30]. According to Satishchandra et al., the development of altered sensorium denotes a poor prognosis[4]. Although several symptoms were detected in cryptococcal meningitis patients in the present study, only altered sensorium was independently associated with poor outcome **(**p=0.03**)**. The high mortality rate of cryptococcal meningitis in AIDS patients may also be associated with some risk factors such as low $CD4^+$ cell counts. These cells are considered a marker of the progression of HIV infection and AIDS that reflects advanced immunossupression[27]. Thus, the low $CD4^+$ cell count verified in our patients, as 81.1% of our patients had a cell count <100 cells/mm^3, may have contributed to the increased mortality. Similar results have been obtained by several investigators who observed patients with a mean $CD4^+$ count <100 cells/mm^3. Such clinical findings are associated with a high mortality rate in cryptococcosis patients[28,29]. Mortality in HIV-negative patients is difficult to explain; however, for four patients, the cause of mortality was acute respiratory failure due to pneumococcal pneumonia. Respiratory failure is one of the top causes of death in patients with pneumococcal pneumonia[33].

To the best of our knowledge, the epidemiological profile of the cryptococcal meningitis patients associated with the clinical outcomes described in our work represents the first study performed in State of Goiás, Brazil. These findings could play an important role in aiding the evaluation of cryptococcal meningitis trends in our state by highlighting the knowledge of the species, serotype, clinical outcome, presence of underlying diseases and symptoms associated with poor prognosis.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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