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HISTOPATHOLOGY, SEROLOGY AND CULTURES IN THE DIAGNOSIS OF CRYPTOCOCCOSIS

Alexandra Flávia GAZZONI(1), Cecília Bittencourt SEVERO(2,3), Emily Ferreira SALLES(2,4) & Luiz Carlos SEVERO(3,5,6)

SUMMARY

Cryptococcosis is one of the most common opportunistic fungal infections in patients with acquired immunodeficiency syndrome (AIDS). We report 13 cases of cryptococcal infection based on histopathology, serology and cultures. Epidemiological analysis, histochemical techniques of hematoxylin and eosin (HE) and Grocott's silver (GMS), as well special histochemical techniques such as Mayer's mucicarmine (MM) and Fontana-Masson (FM), cryptococcal antigen test (CrAg) and isolation on fungal media: Sabouraud's (SAB), brain-heart infusion agar (BHI) and canavanine-glycine-bromothymol blue (CGB) agar were analyzed. Unsatisfactory staining results by MM stain associated to negative titers by CrAg test, which FM stain confirmed that capsule-deficient *Cryptococcus* infections were observed in four cases. Eight isolated cases were identified as follows: six cases were infection with *Cryptococcus neoformans* and two cases were *Cryptococcus gattii*.

KEYWORDS: Budding index; Carminophilic index; Mayer's mucicarmine stain; Fontana-Masson stain; Cryptococcal antigen test; Cultures.

INTRODUCTION

Cryptococcosis is a systemic infection caused by naturally encapsulated basidiomycetous of genus *Cryptococcus*^{7,13}. This yeast causes human infection ranging from asymptomatic pulmonary colonization to meningitis and disseminated disease⁶. Cryptococcosis is caused by two species: *Cryptococcus gattii* is typically found in tropical and subtropical climates zones, and usually causes disease in apparently normal hosts^{5,8,23,25}, whereas the *C. neoformans* present in urban pigeon droppings, has a worldwide distribution and is a common opportunistic infection^{5,6,23,25}. Specifically, conditions predisposing to a change in cellular immunity have been associated with a significant increase in risk to obtain the cryptococcosis, and these include lymphoproliferative disorders, organ transplantation, and receiving immunosuppressive therapy²³. Cryptococcosis is also one of the most common opportunistic fungal infections in acquired immunodeficiency syndrome (AIDS) patients, and, in that group, it is associated with a high mortality rate²³.

Microscopically, *Cryptococcus* has spherical to oval yeast cells, 5-10 µm in diameter that are surrounded by a polysaccharide capsule^{2,7}, which is a major virulence factor and the substrate detected by cryptococcal antigen tests (CrAg)^{4,7}. However, capsule-deficient *Cryptococcus* infections may result in false-negative results using the CrAg test^{13,15,29}.

Cryptococcosis laboratory diagnosis includes conventional methods (direct microscopic examination and histopathology)^{5,11,12,13}, usually associated to serology^{3,4,20} and to isolation of the organism in

culture^{3,5,23}. Histopathologic identification in biopsy specimens is based on the micromorphological characteristics of *Cryptococcus*, and include histochemical techniques of hematoxylin and eosin (HE) and Grocott's silver (GMS), as well special histochemical techniques such as Mayer's mucicarmine (MM), which stains the capsule magenta, and Fontana-Masson (FM), which stains fungal melanin reddish-brown^{1,12,13}.

In the Schwartz classification, infection is divided into two major histological categories, based upon reactions of the tissues²⁴. The reactive pattern presents intense granulomatous inflammatory response, composed of macrophages, multinucleated giant cells, and lymphocytes^{5,13,24}; the yeasts are found in an intracellular location (phagocytosed)^{15,23}. In the paucireactive pattern, yeasts are found extracellularly, associated to a minimal or absent inflammatory response, and tissue destruction results from compression necrosis by masses of cryptococcal tecdial^{6,23,24,26,27}.

To evaluate the inflammatory response of *Cryptococcus* infections, two morphologic parameters are analyzed^{6,24}. The viability of the organisms is estimated using the Carminophilic Index (CI), which indicates capsular synthesis. The divisional or mitotic activity of yeast is estimated by the Budding Index (BI), and indicates replication *in vivo*²⁴.

This histopathological study has two main purposes: (a) to characterize the inflammatory response of *Cryptococcus*; (b) to show the relationship between CI and the titer of the antigen test, since capsule-deficient strains may cause false-negative CrAg results.

(1) Doutora em Ciências Pneumológicas, Unidade de Microbiologia, Universitat Rovira i Virgili, Reus, Espanha.

(2) PPG, Ciências Pneumológicas, Mestrado e Doutorado, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brasil.

(3) Laboratório de Micologia/Hospital Santa Rita/Santa Casa-Complexo Hospitalar, Porto Alegre, RS, Brasil.

(4) Departamento de Patologia/Santa Casa-Complexo Hospitalar, Porto Alegre, RS, Brazil.

(5) Departamento de Medicina Interna, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.

(6) Pesquisador nível 1B CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico).

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Correspondence to: Dr. Luiz Carlos Severo. Laboratório de Micologia/Hospital Santa Rita, Santa Casa-Complexo Hospitalar, Anne Dias 295, 90020-090 Porto Alegre, RS, Brasil, Phone: +55 51 32148409; Fax: +55 51 32148435. E-mail: severo@santacasa.tche.br

MATERIAL AND METHODS

A retrospective study was conducted by reviewing the medical records of patients who were diagnosed with cryptococcal infection based on histopathological identification, serological and cultures at the Micology Laboratory of Santa Casa Complexo Hospitalar (Porto Alegre, RS), in Southern Brazil. The data reviewed included age, gender, predisposing factors, host immune status, sites of infections, CrAg titer, and clinical presentation (localized or disseminated).

Culture: The identification of *Cryptococcus* was confirmed by the isolation of yeasts with white mucoid colonies (depending on the capsule thickness) after cultivation on fungal media (within 48-72 h), namely Sabouraud's (SAB) at 25 °C, and brain-heart infusion (BHI) agar at 35 °C; and by microscope by demonstrating the presence of spherical to oval yeast cells, budding on a narrow base, with a surrounding capsular structure. The species were discriminated by a color reaction when grown on canavanine-glycine-bromothymol blue (CGB) agar.

Analysis of microscopic pathology: Biopsy specimens were submitted to standard histological processing¹. Tissue sections were stained by histochemical techniques of HE¹⁸ and GMS^{14,18}, sections stained by special histochemical techniques of MM^{13,19} were also included. The histological reactions to HE^{2,24}, the measurement of BI to GMS²⁴,

and the presence of magenta capsular structure^{12,13,19} and measurement of CI to MM stain²⁴ were determined. Special histochemical techniques of FM stain^{13,17,18,19} were applied in those cases that showed negative results by the MM stain, and negative titers by the CrAg test.

CrAg detection: The IMMY test - the commercial kit used in this study - has a vital component in Detacher Enzyme (DE), Pronase®. DE digests antibodies in immune complexes (the antigen is not affected), which can mask the detection of antigens. DE also eliminates the rheumatoid factor, that can cause false positives^{3,4}.

RESULTS

Patients characteristics: During the 28-year period between 1980 and 2008, there were 13 patients diagnosed with cryptococcosis based on histopathology, serology and cultures. Out of those, nine (70%) patients were men and four (30%) women. Their age ranged from 10 - 56 years old (average of 37.5 years). The median age was 36 years for men and 41 for women.

The majority of patients (77%) were considered to be immunocompromised with at least one identifiable risk factor, including infection with human immunodeficiency virus (HIV) in five (39%) patients, solid organ transplantation in four (31%) patients - renal (three

Table 1
Histologic pattern, measurements of BI and CI, CrAg titers and identification of *Cryptococcus* species complex in 13 patients

Cases	Sex, Age	Predisposing factors	Histologic pattern	BI	CI	CrAg titers	Specimen, CGB ^a
Immunocompetent							
01	M, 10	None	Reactive (Fibrotic nodules) (Cryptococcomas)	0	+++	1:128	Cerebrospinal fluid, <i>C. gattii</i>
02*	F, 42	None	Reactive	15	0	Negative	Axillary tumor biopsy, <i>C. gattii</i>
03	M, 56	None	Reactive	12	++++	1:5112	Not done
Immunocompromised							
04	M, 13	Tx pulmonary	Paucireactive (Pulmonary nodules)	2	++++	Negative	Lung tissue biopsy, <i>C. neoformans</i>
05	M, 29	AIDS	Paucireactive	4	+++	1: 1049.536	Lymph node biopsy, <i>C. neoformans</i>
06	F, 30	AIDS	Paucireactive	2	+	1:2	Not done
07	M, 31	AIDS	Paucireactive	10	++++	1:5112	Cerebrospinal fluid, <i>C. neoformans</i>
08	M, 40	Tx renal	Reactive	3	+	1:8	Cutaneous tissue biopsy, <i>C. neoformans</i>
09	M, 42	AIDS	Reactive	4	+	1:128	Not done
10*	F, 42	Tx renal	Reactive	5	0	Negative	Not done
11*	M, 50	AIDS	Reactive	8	0	Negative	Lung tissue biopsy, <i>C. neoformans</i>
12	F, 50	Tx renal	Paucireactive	3	+++	Negative	Not done
13*	M, 53	Immunosuppressive drugs	Reactive	2	0	Negative	Lung tissue biopsy, <i>C. neoformans</i>

*, cases of capsule-deficient *Cryptococcus* infections; †, in 1 to 5 days, *Cryptococcus gattii* isolates turn the CGB medium blue, whereas those of *Cryptococcus neoformans* do not; CGB, canavanine-glycine-bromothymol blue media; AIDS, acquired immunodeficiency syndrome; BI, budding index; CI, carminophilic index; CrAg: cryptococcal antigen; F, female; M, male; Tx, organ transplantation; Score employed for measurement of CI percentage 25% or less, +; 26% to 50%, ++; 51% to 75%, +++; 75%, +++++.

cases) and lung (one case) - and user of immunosuppressive drugs in one (8%) patient. Three patients were considered immunocompetent: one presented infection by a capsule-deficient strain, one exhibited an axillary tumoral form, and the other one was a 10 year-old child.

A total of 30% (n = 4) presented localized pulmonary infection; all were HIV-uninfected. Disseminated infection was found in 70% (n = 9); of these, 78% (n = 7) presented of the involvement central nervous system (CNS), these five were HIV-positive patients. Extranural, extrapulmonary manifestations were observed in seven (54%) cases, including mediastinal lymphadenopathy (four cases), concomitant to oral cavity involvement (one case), hepatic (one case) and cutaneous (one case).

It was possible to identify the *Cryptococcus* species complex in eight cases: six (46%) cases of *Cryptococcus neoformans* and two (15%) cases of *Cryptococcus gattii*. All *C. neoformans* isolated were immunocompromised hosts; two *C. gattii* isolated were in apparently immunocompetent patients. As shown in Table 1, the *Cryptococcus* species were isolated on fungal media, namely SAB, BHI and CGB.

Histological pattern

Reactive: Circumscribed granulomas were seen in seven (54%) patients. The lesions were composed of compactly aggregated histiocytes and multinucleated giant cells, including both Langerhans and foreign body type, with numerous intracytoplasmatic yeasts (phagocytosed) (Fig. 1A). Central area of necrosis associated to marginal fibrosis was observed (Fig. 1B). In one case, fibrotic nodules were identified.

Paucireactive: Six (46%) cases presented numerous extracellular organisms, associated with a minimal to absent inflammatory response. Typical cells were spherical, oval, or ellipsoid, and surrounded by optically clear, smoothly spherical zones, or halos that are unstained capsules. In three (23%) cases chronic lymphadenitis were identified showing destruction of the architecture by compact masses of

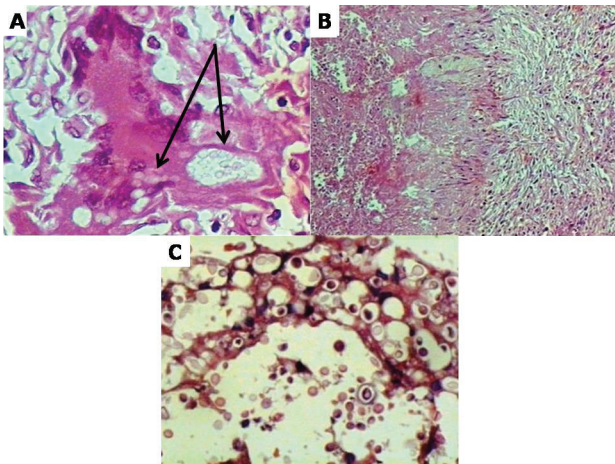


Fig. 1 - Section of the lung stained with HE. Histologic patterns of cryptococcosis. A, multinucleated giant cell of foreign body type (case 2) (x100). B, granulomatous inflammation consisting of necrosis, macrophages, lymphocytes and fibroblastic activity (case 2) (x10). C, section of the lymph node with complete effacement of lymphoid tissue by compact extracellular yeasts (case 5) (x100).

encapsulated yeasts (Fig. 1C). One case revealed organizing pneumonia. Table 1 summarizes the histologic patterns, measurements of CI and BI, and CrAg titers in 13 patients.

CI: Ranged from one-plus to four-plus (+ to ++++). Paucireactive pattern had the highest measurements of CI; the reactive pattern has shown marked variation among cases, ranging from one-plus to three-plus (+ to +++). A total of nine (70%) patients exhibited a magenta capsule stained by MM stain (cases 1, 3, 4, 5, 6, 7, 8, 9, 12) (Fig. 2A); four (30%) patients did not show a reactive carminophilic staining (cases 2, 10, 11, 13); all presented the reactive pattern of the infection.

BI: Both patterns showed great variation in this index (measurements 0 to 15) (Fig. 2B).

CrAg test: Seven (54%) out of 13 patients had a positive serology, including samples from serum, cerebrospinal fluid and urine. Among those patients, the cases 1, 3, 5, 7 presented major measurements of CI. Of the six (46%) patients that presented negative results (cases 2, 4, 10, 11, 12, 13), four (30%) did not show the presence of a carminophilic capsule after staining with MM thus preventing the measurements of CI (cases 2, 10, 11, 13). Two (15%) transplant recipients presented localized pulmonary infection and showed negative results to the CrAg test (cases 4, 12).

Fontana-Masson stain: Cases 2, 10, 11, 13 did not show the presence of a mucicarminophilic capsule after staining with MM stain and presented negative results by CrAg test; in all cases the FM stain detected fungal melanin in the cell wall (Fig. 2C).

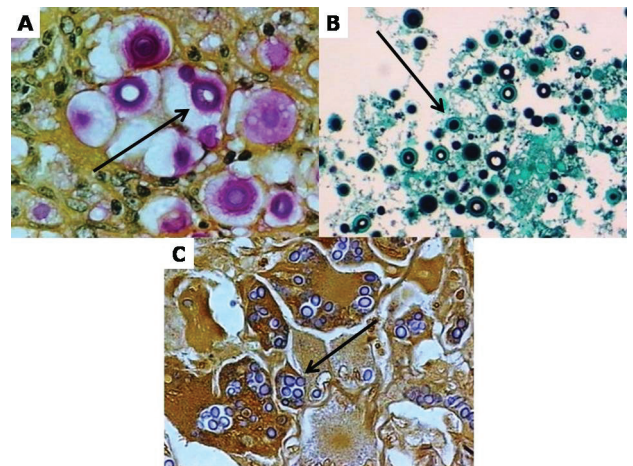


Fig. 2 - A, section of the mediastinal lymph node stained with MM (x100). Organisms presented CI of +++++ with magenta capsule (case 3). B, section of the axillary tumoral form stained with GMS (x100). Organisms showing budding yeasts with a BI of 2 (case 6). C, section of the lung stained with FM (x100). Yeasts exhibited react positively for melanin fungal (case 13).

DISCUSSION

Before the AIDS epidemic, cryptococcosis was considered an unusual infection with low incidence rate^{6,23}. After the recognition of AIDS, the diseases emerged as an important opportunistic infection^{16,20}. The association with organ transplantation (four cases in our group) and use of immunosuppressive drugs has been established^{6,9,23}.

Epidemiological analysis revealed a male predominance, with an average age similar to those in Colombia²¹ and France¹⁰. Among men and women, the average age showed differences in agreement with previous reports, suggesting that the average age is higher in women^{10,20}. Although rarely, cryptococcosis can affect children⁸. In our sample, it was present in two children (15%). One was immunocompetent and lived in a biogeoclimatic endemic zone for *Cryptococcus gattii* in Brazil (Pará)⁸, and the other was a lung transplant recipient.

Among organ systems, the respiratory tract is the most frequent site of entry involved^{6,16,22}. The organism is trophic to the CNS, and the majority of cases were related to meningitis^{9,10}, most commonly in HIV-positive patients. In contrast, pulmonary involvement was found to occur more frequently in HIV-negative patients. *Cryptococcus* may undergo hematogenous spread to other organ systems^{6,12}. Extrapulmonary and extraneural manifestations were observed in 54% of patients. The following body sites are considered to be manifestations of disseminated disease: lymph node (lymphadenopathy), head and neck (involvement of oral cavity), skin and gastrointestines (hepatic)^{12,24}.

C. gattii is likely to cause disease in healthy hosts and behaves as a primary pathogen, whereas *C. neoformans* affects predominantly immunocompromised individuals, especially those infected by HIV^{5,8,25}. It is emphasized that the biopsy specimens should be in saline (0.9% NaCl) solution for fungal isolation, as well identification of species, since formalin fixation causes death of fungal agent preventing its growth on culture media.

Paucireactive cryptococcosis results from the impaired capability to successfully mount an effective inflammatory response. Cell-mediated immunity has a role in the defense mechanism²⁴; in its absence, organisms proliferate extracellularly associated with destruction of tissue^{13,16,26}. Histological analysis of our cases is consistent with these features. In the paucireactive infection, the BI varied greatly, and most cases showed less than 5% budding forms. When compared with the reactive infection, organisms showed highest measurements of CI. There is apparently an inverse correlation between capsular production and intense inflammatory responses^{6,24}.

A reactive pattern results in hosts having an active cell-mediated immune response²⁴. The histology is characterized by a granulomatous response composed of histiocytes, giant cells, and lymphocytes associated with fibroblastic activity^{16,24,26,27,28}. Our cases demonstrated these histologic features in different degrees. When compared to paucireactive infection, the yeasts cells were less abundant, and both CI and BI showed a greater spectrum of variation.

The fibrotic nodules, better known as the cryptococcomas, represent an infection in immunocompetent persons²⁴. Similar lesions are presented in histoplasmosis²⁴ and tuberculosis^{24,28}. Case 1 contained scarce organisms having a CI of +++ and BI of zero.

The CrAg were first described by BLOOMFIELD *et al.*⁴ in the sera and cerebrospinal fluid (CSF) of seven from a total of nine patients. The CrAg test is approximately 95% sensitive and specific³. False-negative results reported were due to a poorly encapsulated strain^{6,23}. Although commercial kits differ, the CrAg test detects at least 10 ng of polysaccharide per mL of biological fluid⁶. These false-negatives

are due to capsular deficiency as this strain does not produce enough polysaccharides antigens to be detected by the CrAg test. In fact, the size of the polysaccharides of cells of the deficient-capsule strain is smaller than those of the typical strain. Patients with primary pulmonary cryptococcosis without dissemination may have false-negative serum tests, since yeasts have not yet disseminated from the lung. Once antigen is detected in the serum of a patient with cryptococcosis, this is a sign that infection has disseminated from its pulmonary location⁶. On the basis of these observations, in false-negative titers and unsatisfactory staining results by MM stain, the special techniques of the FM stain for fungal melanin confirm infections by capsule-deficient *Cryptococcus* strains.

RESUMO

Histopatologia, sorologia e cultivo no diagnóstico da criptococose

A criptococose é a mais comum infecção fúngica oportunística observada em pacientes com síndrome da imunodeficiência adquirida (AIDS). Relatamos 13 casos da infecção baseados no diagnóstico histopatológico, sorológico e cultivo. Foram analisadas: a epidemiologia, as técnicas histoquímicas básicas de hematoxilina-eosina (HE) e coloração pela prata (GMS), bem como as técnicas histoquímicas especiais de mucicarmim de Mayer (MM) e Fontana-Masson (FM), o teste do antígeno criptocócico (CrAg) e o isolamento em cultivos em ágar-Sabouraud (SAB), ágar infusão de cérebro-coração (BHI) e meio com canavanina azul de bromotimol (CGB). Em quatro casos, resultados tintoriais insatisfatórios pela coloração de MM associados a títulos negativos pelo teste do CrAg, a coloração de FM confirmou a infecção pelo *Cryptococcus* deficiente de cápsula. Oito isolados foram identificados: seis casos apresentaram a infecção por *Cryptococcus neoformans* e dois casos apresentaram a infecção por *Cryptococcus gattii*.

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