UNUSUAL MORPHOLOGIES OF Cryptococcus spp. IN TISSUE SPECIMENS: REPORT OF 10 CASES

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SUMMARY

Ten cases of cryptococcosis due to unusual microscopic forms of Cryptococcus sp. observed over a twenty-eight year period (1981-2009) are presented. The most important clinicopathological and laboratory data are tabulated. The uncommon forms of cryptococcal cells given are: structures resembling germ tube (one case), chains of budding yeasts (one case), pseudohyphae (two cases) and nonencapsulated yeast-like organisms (eight cases). The diagnosis was based on the histopathological findings. The causative organism was isolated and identified in seven cases; five were due to C. neoformans, and two to C. gattii. In addition, the importance of using staining histochemical techniques - Grocott’s silver stain (GMS), Mayer’s mucicarmine stain (MM) and Fontana-Masson stain (FM) - in the diagnosis of cryptococcosis is argued.

KEYWORDS: Cryptococcosis; Pseudohyphae; Chains of budding yeasts; Germ tube-like structures; Nonencapsulated yeast-like organisms.

INTRODUCTION

In clinical specimens, Cryptococcus species are usually identified as spherical-to-oval yeast cells, range from 4-20 μm in diameter, and are surrounded by a mucopolysaccharide capsule, which is a major virulence factor.

Histopathological identification of the cryptococcosis is based on the micromorphological and staining features of the cryptococcal cells, and include histochemical techniques of hematoxylin and eosin (HE), and Grocott’s silver stain (GMS), as well as special histochemical techniques such as Mayer’s mucicarmine method (MM), which stains the capsule magenta, and Fontana-Masson procedure (FM), which stains fungal melanin reddish-brown.

In this study, we highlight the unusual micromorphological forms of the Cryptococcus species in tissue specimens. It also emphasizes the use of histochemical techniques in the diagnosis of cryptococcosis.

MATERIAL AND METHODS

Through database analysis, we retrospectively reviewed all cases of cryptococcal infections diagnosed between January 1981 and May 2009 at the Mycology Laboratory of Santa Casa Complexo Hospitalar (Porto Alegre, RS), in Southern Brazil.

Clinical-epidemiological and laboratory records of the cases diagnosed by histopathological examination were reviewed, and we were primarily interested in collecting data such as: sex, age, underlying diseases, titers of cryptococcal antigens (CrAg) in sera, urine and cerebrospinal fluid (CSF) and species of Cryptococcus recovered in cultures.

Cultures: The identification was confirmed by: (a) colony morphology - by isolation of yeast colonies with white mucoid aspect (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; (b) microscopy morphology - by demonstration of spherical-to-oval encapsulated yeast cells and budding on a narrow base. After identity of an isolate had been established, such as Cryptococcus, we proceeded to determine its species status. Canavanine-glycine-brothymol blue (CGB) agar was successfully used for this purpose. In one to five days, isolates of C. gattii cause the CGB medium to turn blue, whereas those of C. neoformans do not.

CrAg detection: For serological diagnosis, latex agglutination for cryptococcal polysaccharide antigens was performed a specific and sensitive alternative for rapid diagnosis. In this study, the commercial kit IMMY test was used, which has a vital component in Detacher Enzyme (DE), Pronase®. DE eliminates the rheumatoid factor, which can produce false positives.

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Histopathological studies: Fragments obtained through biopsy or surgical excision were fixed in formalin, embedded in paraffin and, after sectioned, stained for evaluation. The histopathological examination was made on routine HE preparations to determine organ involvement by fungal pathogen and to determine details of tissue response. In all cases, tissue sections stained with selective staining such as GMS, MM and FM were examined.

RESULTS

Between 1981 and 2009, 925 patients with cryptococcosis were diagnosed in our mycology laboratory, with 33 cases being established through histopathological diagnosis.

In a total of 10 out of these 33 cases unusual microscopic forms of Cryptococcus were observed. Gender distribution showed a predominance of males (70% - 7/10), and the mean age was 42.4 years old (range, 23 - 60 years). Table 1 summarizes the clinical and pathological data for the 10 patients. With the exception of three immunocompetent patients, the remaining patients had the following underlying diseases: AIDS (three cases), kidney and lung transplantation (two cases and one case, respectively), and lymphoma (one case).

Cryptococcal antigen detection: CrAg test of patients showed titres ranging from 1:5112 to 1:1048576. In six cases (60%), CrAg test was negative.

Identification of cultures: Cryptococcus isolated from these patients was identified as species neoformans in five cases (50%). Cryptococcus gattii was recovered from an immunocompetent patient and from a patient with AIDS. In three cases, the identification from cultures - on fungal media, namely SAB, BHI and CGB - was not performed due to biopsy specimens being in formalin fixation.

Histopathological findings: Histologically, Cryptococcus was found in multiple organs (lungs, brain, liver, cervical and axillary lymph nodes and left axillary region). The lungs were the organs most often involved (cases 1-3, 5 and 8) (50% - 5/10).

In cases 4, 6, 9 and 10, HE staining followed by microscopic examination showed usual rounded refractile bodies with a surrounding halo, accompanied by minimal, predominantly lymphatic infiltration. These paucireactive patterns were associated with complete replacement of host tissue by extracellular yeasts and mucin (Fig. 1A).

In cases 4 and 6, staining with GMS revealed black organisms on a light green background - numerous spherical-to-oval yeasts were surrounded by a narrow, circumferential, refractile zone of negative staining (halos) (Fig. 2A).

Several uncommon micromorphologies of Cryptococcus were identified. These included germ tube-like structures (one case), chains of budding yeasts (one case), pseudohyphae (two cases) and nonencapsulated yeast-like organisms (eight cases).

Nonencapsulated yeast-like organisms were observed in cases 1-3, 5 and 7-10. The numerous fungal cells were stained strongly with GMS, although encapsulation was not evident (pericellular clear zone absent) (Fig. 2B).

Germ tube-like structures were seen in case 6 (Fig. 2C). However, these possessed a constriction at their base at the point of attachment to the adjacent yeasts. Chains of budding yeasts were also observed in this case (Fig. 2D). In spite of having a pseudohypha-like appearance, the individual cells did not possess the characteristic elongation of typical pseudohyphae.

In cases 4 and 6, the MM staining strongly stained the capsules with a deep magenta color, consistent with the presence of mucopolysaccharides. Although many of the yeasts were arranged singly, some demonstrated narrow-based budding, and a few clusters of yeasts with multiple budding were seen. In these cases, pseudohyphae were seen that varied in size. In several instances, these elongated structures, consistent with pseudohyphae, were seen in continuity with a chain of budding yeasts.

Table 1
Clinicopathologic summary of cases which presented unusual morphologies of Cryptococcus species in tissue specimens at Mycology Laboratory, Complexo Hospitalar/Santa Casa

<table>
<thead>
<tr>
<th>Cases Ref.</th>
<th>Sex, Age</th>
<th>Underlying diseases</th>
<th>Tissue site (type of specimens)</th>
<th>Histologic pattern</th>
<th>Grocott’s silver stain</th>
<th>Mayer’s mucicarmine stain</th>
<th>Fontana-Masson stain</th>
<th>Cr Ag titers</th>
<th>Identification of culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 [8]</td>
<td>F, 42</td>
<td>Immunocompetent patient</td>
<td>Lung</td>
<td>Reactive</td>
<td>Nonencapsulated yeast-like within multinucleated giant cells</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>F, 40</td>
<td>Immunocompetent patient</td>
<td>Lung</td>
<td>Reactive</td>
<td>Nonencapsulated yeast-like</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>M, 59</td>
<td>Lung transplant</td>
<td>Lung</td>
<td>Reactive</td>
<td>Nonencapsulated yeast-like within multinucleated giant cells</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>M, 56</td>
<td>Immunocompetent patient</td>
<td>Left axillary region</td>
<td>Paucireactive</td>
<td>Spherical-to-oval yeasts-like with pericellular clear halos Pseudohyphae</td>
<td>Fungal cell with magenta capsule Chains of budding yeasts</td>
<td>Positive</td>
<td>Classical aspects of Cryptococcus</td>
<td>1:5112 a</td>
</tr>
<tr>
<td>5</td>
<td>M, 60</td>
<td>Lymphoma</td>
<td>Lung</td>
<td>Reactive</td>
<td>Nonencapsulated yeast-like</td>
<td>Negative</td>
<td>Positive</td>
<td>NA</td>
<td>C. neoformans d</td>
</tr>
<tr>
<td>7</td>
<td>M, 40</td>
<td>Kidney transplant</td>
<td>Skin</td>
<td>Reactive</td>
<td>Nonencapsulated yeast-like</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>C. neoformans d</td>
</tr>
<tr>
<td>8</td>
<td>M, 42</td>
<td>Kidney transplant</td>
<td>Lung</td>
<td>Reactive</td>
<td>Nonencapsulated yeast-like Pseudohyphae</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>C. neoformans d</td>
</tr>
<tr>
<td>9</td>
<td>M, 23</td>
<td>AIDS</td>
<td>Liver</td>
<td>Paucireactive</td>
<td>Nonencapsulated yeast-like</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>C. neoformans d</td>
</tr>
<tr>
<td>10</td>
<td>M, 10</td>
<td>AIDS</td>
<td>Axillary lymph node</td>
<td>Paucireactive</td>
<td>Nonencapsulated yeast-like in the intercellular spaces</td>
<td>Negative</td>
<td>Positive</td>
<td>1: 4096 a C. gattii c</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Brain</td>
<td>Paucireactive</td>
<td>Nonencapsulated yeast-like</td>
<td>Negative</td>
<td>Positive</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*: serum; AIDS: acquired immunodeficiency syndrome; #: urine; in 1 to 5 days, Cryptococcus gattii c isolates turn the CGB medium blue, whereas those of Cryptococcus neoformans d do not; C: Cryptococcus; Cr Ag: Cryptococcal antigen; NA: not available.

yeasts (Fig. 3A). In other instances, several contiguous pseudohyphae demonstrated prominent branching. In cases 1-3, 5 and 7-10, the usual magenta color of the capsule after MM staining was absent (Fig. 3B).

When stained by FM, the black color in the cell wall of the organisms was disclosed. All the cases were positive - patients 4 and 6 presented classical micromorphological aspects in this yeast (Fig. 3C), while patients 1-3, 5 and 7-10 revealed a capsule-deficient form, confirming infection by Cryptococcus species (Fig. 3D).

DISCUSSION
As observed by others, male patients predominated in our study. In the AIDS era, the relationship of cryptococcosis with gender distribution...
In the current series, it is worthy of note that the cryptococcal cells had characteristic yeast-like forms, pseudohyphae or germ tube-like structures, because polysaccharide capsules are known to be a major cryptococcal virulence factor. The loss of capsule material elicits an intense inflammatory response that includes early suppuration, phagocytosis and granuloma formation. Previous reports described cryptococcal cell wall and selectively stains these yeasts in tissue specimens..

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The lungs are invariably the portal of entry, by inhalation from an environmental source, and also the initial site of infection for Cryptococcus spp. The infection can then spread via the bloodstream. In these conditions, all areas of the body can be infected, including liver, lymph nodes, kidneys, adrenal glands and most notably the central nervous system (CNS).

Definitive diagnosis of cryptococcosis requires specific histopathological examination, detection of the cryptococcal antigen in body fluids by latex agglutination and identification of the yeast from cultures. Characteristic cryptococcal morphology does not pose any difficulty in recognizing the fungus. However, unusual forms can give rise to a diagnostic dilemma. Recognition of variants by morphology examination is important in the laboratory confirmation of the disease. In the current series, it is worthy of note that the cryptococcal cells had aberrant morphologies in about 1/3 of our patients whose diagnosis was made histologically. Here, we have also highlighted that formalin fixation is important in the laboratory confirmation of the disease.

As previously noted, the appearance of Cryptococcus sp., a budding yeast cell, and the absence of pseudohyphae have been used as a point of differential diagnosis between cryptococcosis and candidiasis in several recent reviews that have discussed the histopathology of cryptococcal infection. Other reports describe the identification of variant forms having pseudohyphae, germ-tube like structures and hyphae-forming structures, all identified in an Indian ink preparation of CSF. In a review of prior necropsy specimens, a case was discovered in which germ tubes and hyphae were observed in brain tissues. Rare germinating forms of Cryptococcus spp. have also been reported in a subcutaneous, erythematous nodule. In 1971, a murine model study demonstrated hyphae-formation in 11 strains of this yeast. Interestingly, BAVA et al. concluded that the pseudohyphae should be considered a virulence factor and that great immunological deficit and further clinical evolution will better enable the fungal cells to produce atypical forms instead of the conventional yeast forms. In retrospect, KWON-CHUNG reported that the hyphal forms represented attempts of Cryptococcus to produce its sexual teleomorph, F. neoformans. However, some authors believe that although Cryptococcus rarely occurs in short chains resembling pseudohyphae, neither pseudohyphae nor true hyphae are presented.

Our current series demonstrates the existence of the uncommon features such as pseudohyphae formation, chains of budding yeasts and structures resembling germ tubes during cryptococcal infection in humans. Awareness that Cryptococcus can form these features highlights the importance of special stains, such as mucicarmine. In cases 4 and 5, mucicarmine strongly stained the capsules of the individual yeasts, there were chains of budding yeasts, pseudohyphae and germ tube-like structures; all of which proved critical in histopathological diagnosis of the organisms as Cryptococcus rather than as a Candida-type species. In addition, these histopathological findings, together with cryptococcal antigen detection by latex agglutination in sera and urine, and identification from the cultures, confirmed the Cryptococcus species infection in these two cases.

In summary, the current series emphasizes the importance of recognizing that cryptococcal cells do not always show typical encapsulated morphology, but may also be present as nonencapsulated yeast-like forms, pseudohyphae or germ tube-like structures, because
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 criptocoácil (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 títulários 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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REFERENCES