Chapter 1 - Laboratory diagnosis of pulmonary mycoses*

Capítulo 1 - Diagnóstico laboratorial das micoles pulmonares

Melissa Orzechowski Xavier, Flávio de Mattos Oliveira, Luiz Carlos Severo

Abstract

In this era of immunosuppression and transplantation, it is imperative that laboratory scientists remain in close communication with physicians. In patients receiving immunosuppressive therapy, the diagnosis of mycoses must be rapid, which is complicated, requiring the cooperation and collaboration of a number of professionals from various fields of expertise. In this paper, the laboratory diagnosis of pulmonary fungal infection is reviewed. The following topics are included: host factors such as immunological response and predisposing anatomical features; collection, transport and storage of specimens; laboratory processing of samples; direct microscopy; staining techniques, culture and identification of fungi; laboratory biosafety; tissue tropism and reactions; serology; and antigen detection.

Keywords: Mycology; Laboratories, hospital; Diagnostic techniques and procedures; Lung diseases, fungal.

Resumo

Nesta era de imunossupressão e transplantes, é imperativa a comunicação entre médicos e laboratoristas devido ao fato de que o diagnóstico de doenças fúngicas, para esses pacientes, deve ser rápido, o que é complicado e requer a cooperação e colaboração de vários profissionais com distintas especializações. Este artigo revisa as técnicas laboratoriais utilizadas para o diagnóstico de infecções fúngicas pulmonares. Os tópicos abordados incluem: fatores relacionados ao hospedeiro, como resposta imunológica e predisposições anatômicas; colheita, armazenamento, remessa e transporte das amostras; processamento laboratorial; exame microscópico direto; técnicas de coloração, cultivo e identificação fúngica; biossegurança em laboratórios; tropismo e reação tecidual; soromicrologia; e detecção de antígenos.

Descritores: Micologia; Laboratórios hospitalares; Técnicas de diagnóstico e procedimentos; Pneumopatias fúngicas.

Introduction

In recent decades, there has been a marked increase in the incidence of fungal infections due to four factors: more aggressive treatment for other conditions (use of immunosuppressants, transplantations and the use and abuse of antibiotics); the increased incidence of leukemia, lymphoma and AIDS; greater knowledge of clinical mycology; and the greater accuracy of diagnostic techniques.1–3

Pulmonary mycoses can be caused by yeast-like fungi (Cryptococcus sp.), dimorphic fungi (Histoplasma sp.) or filamentous fungi (Aspergillus sp.) and can be categorized according to the patient risk factor: changes in T lymphocytes (genera Blastomyces, Coccidioides, Cryptococcus, Histoplasma and Pneumocystis) or neutropenia (genera Aspergillus, Fusarium, Scedosporium and Trichosporon, as well as zygomycetes). The clinical and epidemiological history, as well as imaging studies, can lead to a presumptive diagnosis, which guides physicians in the collection of the clinical specimen. Such data indicate the most appropriate processing technique for the clarification of the etiology, including the selection of the technique for microscopic examination (visualization of the

* Study carried out at the Department of Internal Medicine of the Universidade Federal do Rio Grande do Sul – UFRGS, Federal University of Rio Grande do Sul – School of Medicine, Porto Alegre, Brazil, and at the Mycology Laboratory of the Santa Rita Hospital, Santa Casa Hospital Complex, Porto Alegre, Brazil. Correspondence to: L.C. Severo. Laboratório de Micologia/Hospital Santa Rita, Santa Casa - Complexo Hospitalar, Rua Prof. Annes Dias, 285, CEP 90020-090, Porto Alegre, RS, Brasil. Tel 55 51 3214-8409. Fax 55 51 3214-8435. E-mail: severo@santacasa.tche.br; severo@pesquisador.cnpq.br Financial support: L.C. Severo is a class 1B researcher for the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, National Council for Scientific and Technological Development; process no. 301624/79-8).
All steps of the laboratory diagnosis of pulmonary mycoses are discussed in this study, including the initial stage, regarding the samples, and laboratory methods, such as the principal diagnostic tests routinely performed in mycology laboratories and some new tests that have shown promising results for the early diagnosis of certain pulmonary mycoses.

**Clinical specimen**

There are three steps that are of fundamental importance to making a definitive diagnosis: the collection of specimens, the storage of specimens and the transport of specimens.

**Collection**

Specimen collection should be as aseptic as possible, avoiding contamination. All specimens should be collected in sterile and appropriately identified vials, which must be properly sealed to avoid leaking. The samples generally used for the diagnosis of pulmonary mycoses are secretions and tissue fragments of the lower respiratory tract, as well as samples obtained from sites of dissemination, blood and other body fluids.[4]

Table 1 - Tests used for the direct and indirect diagnosis of pulmonary mycoses.

<table>
<thead>
<tr>
<th>Direct diagnosis – investigation of the agent</th>
<th>Indirect diagnosis – investigation of the host response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycological examination</td>
<td>Imaging studies</td>
</tr>
<tr>
<td>Direct examination</td>
<td>X-ray</td>
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<tr>
<td>Culture</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>Detection of antigen</td>
<td>Detection of antibodies</td>
</tr>
<tr>
<td>Latex agglutination test</td>
<td>Intradermal tests*</td>
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<tr>
<td>sandwich ELISA GM-D glucan</td>
<td>Immunodiffusion</td>
</tr>
<tr>
<td>Detection of fungal DNA</td>
<td>ELISA</td>
</tr>
<tr>
<td>Polymerase chain reaction</td>
<td>Histopathological examination</td>
</tr>
<tr>
<td>Histopathological examination</td>
<td>Grocott, Mayer’s Mucicarmine, Fontana-Masson staining</td>
</tr>
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<td></td>
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</tbody>
</table>
*No diagnostic value.*
Respiratory secretions

Sputum samples are quite useful for the diagnosis of systemic mycoses, especially paracoccidioidomycosis. The material should be collected in the morning, before the first meal and after oral hygiene with water, by asking the patient to take a deep breath and cough. Expectoration is facilitated by previous hydration, nebulization with warm hypertonic saline and postural drainage with fist-percussion.

Bronchial lavage and bronchoalveolar lavage (BAL) are useful for the diagnosis of a wide range of pulmonary infections, especially in cases of opportunistic infections in immunocompromised patients (pneumocystosis).

Tissue fragment

The combination of mycological and histopathological examinations is highly advantageous, since they are complementary examinations. After the tissue fragment is collected, it should be separated into aliquots in the surgical ward. One of these aliquots should be placed in a vial containing sterile saline solution or distilled water (for mycological examination) and another aliquot should be placed in a vial containing formalin (for histopathological examination).

Fine needle aspiration biopsy

The aspirate from lymph nodes, lung and other organs allows the evaluation of the cellularity, microscopic diagnosis and culture. The collected material, when scarce, should be immediately placed on a microscope slide and fixed for subsequent staining. When it is possible to collect tissue fragments or a larger aliquot, the material should be left in the syringe or placed in sterile vials containing saline solution or distilled water to avoid dehydration.

Blood

Blood is used for cultures or serological examinations. It should be aseptically collected, through peripheral venous puncture, in blood culture tubes or tubes not containing EDTA (vacutainer system) for serum separation.

Storage

All clinical specimens should be sent to the laboratory immediately after collection to avoid the proliferation of other microorganisms that might interfere with fungal isolation, which can hinder or impede the development or detection of the real agent causing pulmonary infection. When immediate transport of the material is not possible, the samples should be refrigerated and maintained at 4°C. Cerebrospinal fluid samples should be maintained at room temperature, since refrigeration might delay the growth of Cryptococcus sp.

Any material that has been in contact with mucosae can be contaminated with the local microbiota, as occurs with sputum, and should be processed within 2 h. If this is not possible, the material should be refrigerated for a maximum of 24 h, bearing in mind that this will delay fungal growth.

Transportation

The material should be identified with basic information, as follows: name of the patient, city of origin, current/previous address, trips taken (to include imported mycoses in the diagnosis), occupational/leisure history (such as animal husbandry, including animals such as chicken and pigeons—histoplasmosis and cryptococcosis, respectively), type of material, date of collection, diagnostic hypothesis, predisposing conditions or associated conditions, therapeutic history, laboratory tests required, physician in charge, service of origin and telephone number or e-mail address.

The clinical sample should be appropriately packed, according to the means of transportation, the nature of the clinical sample and the...
Tissue fragments, sputum and BAL fluid can be analyzed by adding a solution containing 20% potassium hydroxide, with or without Parker blue-black ink, calcofluor white or nigrosin (Figure 1). Pus, blood, exudate, BAL fluid and organ aspirate smears can be fixed and stained with Giemsa or Grocott staining (Table 2).

**Culture**

The culture consists in providing the fungus present in the clinical sample with the necessary nutrients in vitro, so that it can multiply, form reproductive structures and be identified. This examination should be done even when the fungus can be microscopically identified for having a peculiar morphology. When the fungus cannot be microscopically identified, it must be isolated in the culture medium and identified in order to establish a diagnosis of the mycosis.

The culture medium most commonly used in mycology laboratories is 2% Sabouraud glucose agar (Figure 2a) with cycloheximide (Figure 2b), chloramphenicol or a combination of the two. The selection of the culture medium depends on the clinical data, the results of the microscopic examination or both. Sabouraud glucose agar is used to isolate fast-growing fungi, whereas Sabouraud glucose agar with cycloheximide or chloramphenicol, which are antifungal and antibacterial, is used in order to isolate fungi that need a longer incubation period, such as dimorphic fungi (e.g., *H. capsulatum*). In these cases, the antimicrobial agents added to the medium inhibit the growth of the “contaminating” fungi and of bacteria, both of which impede the growth and isolation of the true etiologic agent. (5)

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**Material inappropriate for examination**

Sputum collected more than 24 h prior, unidentified vials, vials containing information different from that observed in the clinical form, material collected using swabs and material sent in inappropriate vials (with suspected contamination) are inappropriate for laboratory examination. The microbiologist should contact the clinician and request the collection of another sample. (4)

**Laboratory diagnosis**

**Direct examination**

Direct examination consists in microscopically evaluating the clinical sample, placed between the slide and the coverslip, using reagents or stains to visualize fungal structures. Direct examination is conclusive for the diagnosis of certain pulmonary mycoses, such as pneumocystosis, paracoccidioidomycosis (Figure 1a), cryptococcosis (Figure 1b) and fungus ball caused by *Aspergillus* (Figure 1c). However, in most cases, direct examination does not suffice to identify the etiologic agent.

**Table 2** - Reagents and stains for the direct mycological examination of different clinical specimens.

<table>
<thead>
<tr>
<th>Material</th>
<th>Quantity</th>
<th>Reagents/stains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchoalveolar lavage fluid</td>
<td>10-20 mL</td>
<td>Silver and calcofluor</td>
</tr>
<tr>
<td>Sputum</td>
<td>5-10 mL</td>
<td>N-acetylcysteine or sodium hydroxide or <em>spatulysin</em> (Stat-Pack, Caldon Biotech, Carlsbad, CA, USA), calcofluor</td>
</tr>
<tr>
<td>Cerebrospinal fluid</td>
<td>3-5 mL</td>
<td>Nigrosin, calcofluor</td>
</tr>
<tr>
<td>Pus and exudates</td>
<td>3-5 mL</td>
<td>Potassium hydroxide, silver, Kinyoun, Gram</td>
</tr>
<tr>
<td>Blood&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10-20 mL (adults); 1-5 mL (children)</td>
<td>Silver, Giemsa, calcofluor</td>
</tr>
<tr>
<td>Tissue</td>
<td>1-5 mm</td>
<td>Silver, potassium hydroxide&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>0.2 mL (smear); 1 mL (culture)</td>
<td>Silver, potassium hydroxide&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Urine</td>
<td>10-20 mL</td>
<td>Direct, calcofluor</td>
</tr>
</tbody>
</table>

<sup>a</sup>should be heparinized, <sup>b</sup>with the addition of Parker blue-black ink.
System (bioMérieux, Marcy l’Étoile, France), are required.

Although there is still the incorrect idea that it takes a long time to obtain the results of a mycological examination, most fungi that cause diseases in humans can be isolated within 7 days (yeast-like and filamentous fungi), except for dimorphic fungi, which might need a longer incubation period (two to three weeks) for the development of characteristic structures (Table 3). Therefore, not sending the material for culture growth can result in diagnostic failure, treatment failure and patient suffering.

It is of note that various fungi that cause pulmonary mycoses are opportunistic microorganisms, i.e., microorganisms which were previously considered contaminating and of no clinical importance. Such fungi have been recognized as pathogens and are extremely relevant etiologic agents in immunocompromised individuals. Fungi of the genus Aspergillus fit this description; they are anemophilous and ubiquitous fungi that are associated with high mortality rates in certain groups of patients, such as hematological patients and bone marrow transplant recipients. Therefore, fungal cultures should be carefully interpreted when fungi such as Aspergillus sp., Fusarium sp. and zygomycetes are isolated. In addition, the hypothesis of sample contamination and the hypothesis that these fungi represent the true pathogen should both be considered. In these cases, definitive diagnosis requires the association of other tests, such as histopathological examination or serological examination.

We must bear in mind that the dimorphic fungi Histoplasma sp. and Coccidioides sp. (endemic in the Brazilian northeast) are highly virulent in the filamentous phase; therefore, manipulation of fungal colonies should be carried out using a safety hood and, in cases of Coccidioides sp., it is preferable to work with formalin cultures.

**Blood culture systems**

In the last two decades, there has been major progress in systems for fungal isolation using peripheral blood. The first was the biphasic medium. Currently, the following systems are available: lysis-centrifugation blood culture system (Wampole Isolator; Wampole Laboratories, Cranbury, NJ, USA) and auto-

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Figure 2 - a) Culture on 2% Sabouraud-glucose agar at different incubation periods, showing inhibition of bacterial growth starting at 40°C, associated with thermal tolerance of Aspergillus fumigatus; b) Culture on 2% Sabouraud-glucose agar with cycloheximide for the isolation of Histoplasma capsulatum from nonsterile samples; c) Culture on Staib agar showing melanin production by Cryptococcus sp., which makes it different from the other yeasts (white).
Histopathological examination

Fragments obtained through biopsy or surgical excision should be fixed in formalin, embedded in paraffin and, after sectioned, stained for histopathological evaluation. Histopathological evaluation can be classified as a direct test for the diagnosis of pulmonary mycoses, useful in the identification of the fungal agent using Grocott staining, or as an indirect test for the diagnosis of pulmonary mycoses, useful in the investigation of the host response, through tissue response evaluation, using H&E staining. Tissue response is nonspecific and does not suffice for the etiologic diagnosis of pulmonary mycoses. However, histopathology with the detection of the fungal agent in the tissues is central to diagnose opportunistic infections, since it confirms that the isolated fungus is not a contaminant agent. The combination of these two tests is frequently considered the gold standard for the diagnosis of opportunistic mycoses such as aspergillosis, zygomycoses, hyalohyphomycoses and phaeohyphomycoses. (2,6,21,22)

Histopathological examination greatly contributes to the mycological diagnosis. However, we should bear in mind that this technique, based on micromorphology and tissue reaction, has limitations. In addition, more than one microorganism might be involved in the infectious process and, in such cases, only the combination of mycological culture

Table 3 - Culture media recommended for fungal isolation according to the clinical specimen and fungal growth period.

<table>
<thead>
<tr>
<th>Mycosis</th>
<th>Material</th>
<th>Culture media</th>
<th>Growth period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>SCI</td>
</tr>
<tr>
<td>Histoplasmosis</td>
<td>Sterile</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>Non sterile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paracoccidioidomycosis</td>
<td>Sterile</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>Non sterile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptococcosis</td>
<td>Sterile</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Non sterile</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Aspergillosis</td>
<td>Sterile</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Non sterile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zygomycosis</td>
<td>Sterile</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Non sterile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyalohyphomycosis:</td>
<td>Sterile</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Scedosporium/Fusarium</td>
<td>Non sterile</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

S: Sabouraud (25°C); SCI: Sabouraud with chloramphenicol (25°C); My: Sabouraud with chloramphenicol and cycloheximide (25°C); BHI: brain-heart infusion (35°C); AN: Staib agar (25°C); U: urea (25°C); CGB: canavanine-glycine-bromothymol blue (25°C).
and histopathological examination can clarify the diagnosis. The histological examination and mycological investigation should never be neglected, especially in cases of opportunistic infections.

The communication between the pathologist and the clinician, the surgeon, the radiologist and the microbiologist is highly beneficial for the diagnosis of pulmonary mycoses. In addition to showing the fungal elements in the tissues and evaluating the structural consequences of the disease, the pathologist can help elucidate the pathogenesis and predict functional alterations and prognosis. In tissue sections, tissue reaction can be classified as granulomatous, necrotic or purulent; the etiologic agent can be initially classified, according to the fungal elements observed, as filamentous or round fungal elements (yeasts, sporangia, spherule, conidia and spores).\(^{(21-24)}\)

In addition to the histopathological examination of tissue fragments obtained in vivo, autopsy studies (histopathological and mycological examinations) are also important, since they can confirm a previous diagnosis of mycosis or reveal an infection that was not suspected prior to death. In this case, the relevance of autopsy is to elucidate the presence of a mycosis, so that this condition is included in the differential diagnosis in vivo and attempts are made to treat it properly, avoiding the occurrence of similar cases.

**Staining techniques**

The H&E staining technique is used to observe tissue reaction. In the diagnosis of pulmonary mycoses, this technique is not ideal, because fungal structures are not usually visualized or properly stained. The advantage of this staining technique is that it allows us to distinguish between hyaline and dematiaceous fungal structures, differentiating hyalohyphomycoses from phaeohyphomycoses and aiding in the identification of the etiologic agent. Among the special staining techniques used for the visualization of fungal elements in tissues and smears, silver staining (Grocott) is the most commonly used, the most sensitive and the most specific for mycological diagnosis because it provides

<table>
<thead>
<tr>
<th>Mycosis</th>
<th>Tissue tropism</th>
<th>Reaction</th>
<th>Parasitic form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillosis</td>
<td>Lungs, paranasal sinuses, blood vessels</td>
<td>Suppurative</td>
<td>Septate, hyaline hyphae with 45° ramifications</td>
</tr>
<tr>
<td>Paracoccidioidomycosis</td>
<td>Lungs, adrenal glands, mucocutaneous junction, striated muscle, lymphoid tissue and cooler regions of the body</td>
<td>Mixed (pyogenic and granulomatous); pseudoeoepitheliomatous hyperplasia; sarcoid granuloma</td>
<td>Spherical, multi-budding, yeast-like element (15-30 µm)</td>
</tr>
<tr>
<td>Histoplasmosis</td>
<td>Lungs, spleen, lymph nodes, liver, bone marrow and skin</td>
<td>Histiocytic; granuloma with caseous necrosis; sarcoid granuloma; granuloma with calcification</td>
<td>Small, yeast-like, single-budding element (2-5 µm)</td>
</tr>
<tr>
<td>Zygomycosis</td>
<td>Lungs, paranasal sinuses, blood vessels, subcutaneous tissue, gastrointestinal tract</td>
<td>Thrombotic angiitis, sarcoid granuloma</td>
<td>Seldom septate hyaline hyphae, 5-20 µm in diameter, with 90° ramifications</td>
</tr>
<tr>
<td>Cryptococcosis</td>
<td>Central nervous system, lungs</td>
<td>No reaction; sarcoid granuloma</td>
<td>Commonly encapsulated yeast (4-6 µm)</td>
</tr>
<tr>
<td>Pneumocystosis</td>
<td>Lungs, alveolar space</td>
<td>Granuloma without caseous necrosis presenting scattered giant cells—granuloma with central calcification: foamy alveolar exudate; interstitial thickening caused by edema; and mononuclear infiltrate</td>
<td>Fungal elements present as ascii containing ascospores</td>
</tr>
</tbody>
</table>

Table 4 - Some characteristics of tissue reaction in fungal infections.
better contrast between the fungal elements and the surrounding tissue. In cases in which only the material is sent to the pathologist, special staining techniques are used to elucidate the etiologic agent. For pulmonary mycoses caused by *Cryptococcus* sp., there are staining techniques that make the identification of this agent easier: Mayer’s mucicarmine staining, which stains the mucopolysaccharide capsule bright red; and the Fontana-Masson staining, which stains the melanin on the fungal wall.[13]

**Interpretation of the histopathological findings**

The success of the histopathological diagnosis of the mycoses depends not only on the knowledge of the pathologist but also on the quality of the staining technique, as well as on the presentation and number of fungal elements in the clinical sample.

One characteristic of infectious diseases is the tissue tropism presented by the different etiologic agents (Table 4). This characteristic is especially observed in cases of cryptococcosis, histoplasmosis and paracoccidioidomycosis, in which the etiologic agents affect, respectively, the central nervous system, the monocytic/macrophagic system (liver, spleen, ganglia and bone marrow) and the adrenal gland (Figure 3a). Angiotropism is observed in cases of aspergillosis, hyalohyphomycosis and zygomycosis, causing thrombotic angiitis.[22,25,26]

Although tissue reaction is nonspecific for the diagnosis of fungal infections and varies according to the individual, the location and the duration of infection, certain reactions are more commonly observed in specific types of mycoses (Table 4). An acute or chronic pyogenic reaction with neutrophilic infiltrate (abscess) is commonly seen in cases of invasive aspergillosis, but can also be observed, as a rule, in cases of actinomycosis, nocardiosis and rhodococcosis; therefore, these bacterial infections should be included in the differential diagnosis. A mixed, pyogenic and granulomatous reaction with pseudoepitheliomatous hyperplasia is commonly seen in cases of paracoccidioidomycosis. Histiocytic granuloma with Langhans giant cells and central necrosis (tuberculous granuloma with caseous necrosis) is characteristic of histoplasmosis, especially in its acute pulmonary form. Sarcoid granuloma can be seen in cases of paracoccidioidomycosis (Figure 3b), cryptococcosis and histoplasmosis, which is extremely relevant; therefore, these diseases should be ruled out before confirming the diagnosis of sarcoidosis, a diagnosis based on the exclusion of other possible diagnoses. Fibrotic granuloma with caseous necrosis, frequently diagnosed by means of imaging techniques, such as a nodule(s) in the pulmonary cortex, can be observed in cases of paracoccidioidomycosis, cryptococcosis and histoplasmosis. In cases of histoplasmosis, the fibrotic granuloma with caseous necrosis is usually calcified.[21-23]

Phagocytosed elements within macrophages can be observed in cases caused by *H. capsulatum* or *Cryptococcus* sp. (when the etiologic agent is small and capsule-deficient). Within giant cells, *P. brasiliensis*, *Cryptococcus* sp. and *H. capsulatum* have been observed.[21,23]

According to the size, micromorphological aspect (budding, hyphae and pseudohyphae) and staining aspect of the fungal elements in the histopathological examination, presumptive

**Figure 3** - a) Adrenal gland with severe caseous necrosis (Addison’s disease) caused by paracoccidioidomycosis; b) Sarcoid granuloma in paracoccidioidomycosis; c) Spherical, multi-budding, yeast-like elements of *Paracoccidioides brasiliensis* evidenced by H&E staining (arrow).
diagnosis can be established for certain mycoses. As can be seen in Figure 3C, P. brasilensis is observed as spherical, yeast-like, multi-budding fungal elements (buds, 15-30 µm); H. capsulatum is characterized as small, yeast-like, single-budding fungal elements (buds, 2-5 µm); Cryptococcus sp. is commonly observed as an encapsulated and single-budding yeast (buds, 4-6 µm). The presence of regularly septate hyaline hyphae with ramifications can suggest an infection caused by Aspergillus sp., an infection caused by Scedosporium sp. or any other hyalohyphomycosis. In aspergillosis, in cases of colonization of aired cavity (fungus ball) in which visualization of the conidiophores of Aspergillus sp. is possible, the diagnosis is definitive; however, only the genus can be identified.\textsuperscript{[21,27]}

Phaeohyphomycoses, zygomycoses and fungus balls are collective terms that characterize groups of mycoses caused by different fungal genera. In such cases, only histopathology does not suffice to establish an etiologic diagnosis; the isolation and the identification of the fungus through mycological examination are also required.

In most cases, the histopathological examination serves as a reference and an aid to determine the etiology of the disease, since it reveals characteristic structures of a specific agent. However, although the detection of small yeast-like fungal elements in the tissues is suggestive of infection caused by H. capsulatum, it might indicate the presence of capsule-deficient Cryptococcus sp., Candida glabrata, Sporothrix schenckii or small forms of P. brasilensis. In such cases, if the agent is not isolated in the culture, the diagnosis can only be confirmed by direct immunofluorescence, a technique restricted to referral centers and only available for the detection of certain fungi.

Other tests might confirm a diagnostic hypothesis, such as the immunodiffusion test for the detection of antibodies, the latex agglutination test for the detection of the capsular antigen of Cryptococcus sp. and, more recently, the sandwich ELISA for the detection of galactomannan antigen of Aspergillus sp., the detection of β-glucan and the use of molecular techniques for the detection of fungal DNA in the clinical sample.\textsuperscript{[28-34]} These tests can present different efficacy rates (sensitivity, specificity, positive predictive value and negative predictive value) according to the clinical sample and the individuals tested. The probability of distinguishing between patients with or without mycosis is the function of the sensitivity and specificity of a test. However, the most important feature to clinicians is the predictive value, i.e., the probability that a patient who tested positive actually has the disease and the probability that a patient who tested negative does not have the disease. The predictive value depends on the sensitivity and specificity but also on the prevalence of the disease in the population studied.

**Immunodiffusion**

The immunodiffusion test is based on the formation of immunocomplexes that, due to their high molecular weight, precipitate and form a precipitation line that can be macroscopically observed. It is a relatively fast and easy test. The results can be obtained within one week; however, they are only qualitative results.

The immunodiffusion test can be performed in slides or Petri dishes containing agar gel at 1%, in phosphate buffer or Tris buffer. Wells are created in the gel, using special perforators, to place the antigen in the center and the serum around the center. The serum and the antigen
diffuse in the agar and, when they meet, they react and precipitate, forming an opaque line that can be visualized through indirect illumination against a dark back. A positive control serum sample (standard serum) should be used in this reaction to facilitate the reading and final interpretation of the test. A positive sample will form a line of precipitation linked to the line formed by the standard serum, also known as “line of identity”, which means that the two sera have antibodies against the same antigen. In addition to serum, other clinical samples, such as cerebrospinal fluid and urine, can be used (Figure 4a).

The immunodiffusion test for the diagnosis of histoplasmosis produces two lines of precipitation known as H and M bands. The H band establishes the diagnosis of the disease and is detected in 10-25% of the positive samples, as opposed to the M band, which does not allow the distinction between a previous and a current infection and can be found in 75-85% of the positive samples.[6]

This test is routinely used to identify antibodies in pulmonary mycoses caused by *H. capsulatum*, *P. brasiliensis* and *Aspergillus* sp. (allergic aspergillosis and fungus ball).[2] False-negative results are frequently observed in immunocompromised patients.

**Latex agglutination test**

The latex agglutination test for the detection of capsular antigens of *Cryptococcus* sp is highly sensitive (95%) and specific (98%).[2,12,35] It is an extremely fast and easy test that yields a qualitative initial result, through a reaction of passive agglutination. However, by means of serial dilutions of the clinical sample, the final result can also be quantified. Various specimens, such as serum, cerebrospinal fluid, urine and BAL fluid, can be used for this test.

The latex agglutination test consists in homogenizing the clinical sample with an inert particle (latex) sensitized with an antibody recognizing the capsular antigen of *Cryptococcus* sp. The result is visually interpreted; negative samples have an opaque aspect, whereas positive samples form clots, which correspond to the agglutination of various antigens interconnected through Fab fragments (Figure 4B). False-negative results can occur in cases of infections caused by capsule-deficient *Cryptococcus* sp. or in cases in which there is an excess of antigen in the sample, which impedes the formation of bridges and agglutination, a phenomenon known as the “prozone effect”. When this phenomenon is suspected, the serum samples should be diluted and the test repeated.

**Detection of galactomannan through sandwich ELISA**

Definitive diagnosis of invasive aspergillosis is limited because of various factors.[33] Cultures of respiratory secretions have extremely low diagnostic sensitivity: *Aspergillus* sp. is recovered in sputum cultures of only 8-34% of the patients with invasive aspergillosis and in BAL cultures of only 45-62% of the patients with invasive aspergillosis. Diagnostic confirmation usually requires a histopathological evaluation. However, severe cases of neutropenia or thrombocytopenia are usually a contraindication to invasive surgical procedures. Transbronchial biopsy is associated with a high frequency of false-negative results. Cultures of blood, cerebrospinal fluid and bone marrow are rarely positive to *Aspergillus* sp. HRCT scans are quite useful for the early diagnosis of invasive pulmonary aspergillosis in neutropenic individuals, particularly when the “halo sign” (halo of necrosis surrounding a pulmonary nodule) is observed.[10,11,36]

Specific tests have been developed to aid the conventional methods commonly used in mycology. One of the tests recently approved by the Brazilian National Health Products Oversight Agency is the sandwich ELISA technique, developed to detect galactomannan, a hydrosoluble polysaccharide present in the cell wall of fungi of the genus *Aspergillus* that is released in the blood during hyphal growth in the host tissues (Figure 4C).[37,38] This technique can detect low concentrations of galactomannan (0.5 ng/mL) in clinical samples and is commercially available (Platelia *Aspergillus* EIA; Bio-Rad, Hercules, CA, USA). Sandwich ELISA yields fast results (approximately 4 h); however, its use as a diagnostic tool is approved only for serum samples of neutropenic patients (hematologic patients or bone marrow transplant recipients). Its efficacy for other clinical specimens and for different invasive aspergillosis risk groups is currently under study.[39,40]

When serially monitored, the detection of galactomannan allows the diagnosis of invasive
Aspergillosis to be established 6-14 days earlier in neutropenic individuals. Because of the probable intermittent release of antigen, the test should be performed twice a week. The diagnosis is confirmed if positive results are obtained in two consecutive samples. The rate of false-negative results ranges from 8% to 10% and is related to the encapsulation of the infection, formation of immunocomplexes by anti-Aspergillus antibodies (in less immunocompromised patients), or exposure to antifungal agents (as prophylaxis). The frequency of false-positive results usually ranges from 8% to 14% and is mainly caused by the use of cytotoxic chemotherapeutic agents (causing damage to the intestinal mucosa), host versus graft disease, autoreactive antibodies, infection caused by other fungi such as those of the genera Penicillium and Paecilomyces, transfusions, antibiotics of fungal origin (piperacillin-tazobactam, amoxicillin with clavulanic acid) and laboratory contamination.\textsuperscript{[17,39,41-44]} Recently, cross-reaction with Histoplasma sp. has also been demonstrated, resulting in positive galactomannan results in patients with histoplasmosis.\textsuperscript{[45]}

Although sandwich ELISA is a highly specific test, the sensitivity of this test has much greater variation, with rates ranging from 17% to 100%.\textsuperscript{[46]} The principal reason for such variability is related to the test cut-off value, which determines the positivity of the samples. With a cut-off value between 1.0 and 1.5, sensitivity to galactomannan in BAL samples varied from 85% to 100% in most studies.\textsuperscript{[47-49]} Another key factor for the efficacy of this technique is the prevalence of the disease in a specific population, which influences the positive predictive value observed. Consequently, the test to detect Aspergillus sp. antigen should be reserved to high-risk patients, in situations that are cost-effective.\textsuperscript{[46]}

**Polymerase chain reaction in mycological diagnosis**

The application of molecular techniques, such as polymerase chain reaction (PCR), is scarce in the diagnosis of pulmonary mycoses. Performed only in research, these techniques present various drawbacks. Protocols are not available, methods are not standardized, there is no consensus regarding the techniques for extraction and amplification of the nucleic materials, and commercial kits are not available. Therefore, it is difficult to compare intralaboratory and interlaboratory results.\textsuperscript{[33,35]}

The PCR technique is characterized by the enzymatic amplification of short DNA regions in vitro. The principal target is the 18S ribosomal gene. Primers (oligonucleotides or short filaments of nucleic acids) are used to specifically recognize and hybridize DNA-target sequences. After recognized, the DNA-target molecules are copied by a thermostable DNA polymerase enzyme (Taq polymerase), in the presence of optimal concentrations of magnesium and deoxynucleotides (bases A, C, T and G). Through repeated heating and cooling cycles, performed in a thermocycler (PCR machine), the number of DNA molecules in the reaction is exponentially multiplied. This multiplication occurs by means of the separation of DNA strands (denaturation) with subsequent bonding of the primers to the DNA molecule (annealing) and the action of the DNA polymerase enzyme (extension), which extends the forming molecule, using the free deoxynucleotides in the reaction. At the end of each cycle, every new DNA molecule synthesized acts as a new target for the next cycle. Theoretically, this method allows the generation of billions of copies of the target DNA from a single copy of DNA, at an extremely rapid pace. The final product of the reaction is analyzed by agarose gel electrophoresis, the size of the DNA fragment obtained being determined by comparison with markers of known size. This final product can also be purified and used for other applications in molecular biology, such as genomic sequencing (determination of the molecular composition of the product, or its nucleotide sequence).

The critical points of this technique are related to the DNA extraction method and to the selection of the reaction primers, which should amplify, specifically, fungal DNA instead of human DNA. Promising results have been obtained in cases of invasive aspergillosis, using samples of serum and BAL, with sensitivity ranging from 79% to 100% and specificity ranging from 81% to 100%.\textsuperscript{[10,41,42,50-52]} However, because of the current difficulties in standardizing PCR tests, this technique has not yet been included as a diagnostic test for pulmonary mycoses.\textsuperscript{[32,34,43]}

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Mycological report

After all of the tests are performed, a report is issued. This report should present clear terminology, notify the existence of improper material, report findings, include an interpretation of the findings and inform about rare microorganisms. This type of report can only be made when appropriate clinical information is available.

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About the authors

Melissa Orzechowski Xavier
Substitute Professor of Mycology. Fundаога Universidade Federal do Rio Grande – FURG, Federal University of Rio Grande Foundation – Rio Grande, Brazil.

Flавіо де Mattos Oliveira
Researcher. Santa Rita Hospital Mycology Laboratory, Santa Casa Hospital Complex, Porto Alegre, Brazil.

Luіz Carlos Severo
Associate Professor (Level 2). Department of Internal Medicine of the Universidade Federal do Rio Grande do Sul – UFRGS, Federal University of Rio Grande do Sul – School of Medicine, Porto Alegre, Brazil.