Chapter 1 - Laboratory diagnosis of pulmonary mycoses*

Capítulo 1 - Diagnóstico laboratorial das micoses 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 teciduais; soromicologia; 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

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fungus in its parasitic form) and the selection of the culture medium, temperature and incubation period (isolation of the fungus for subsequent identification). All these factors are interconnected, and the absence of one or the selection of an incorrect procedure at any of these steps might jeopardize the interpretation of the final result. Therefore, it is of paramount importance that laboratory scientists remain in close communication with clinicians to ensure that all steps are correctly followed, thus making diagnosis more accurate in order to indicate the appropriate treatment. Unfortunately, clinicians, pathologists and microbiologists frequently work independently when diagnosing an infectious disease. This communication breakdown leads to the submission of material for laboratory analysis with incomplete clinical data, as well as to the delay or inability in establishing a diagnosis, which, in turn, leads to an increase in the duration of disease.

The definitive laboratory diagnosis can be made directly, by detecting the presence of the microorganism, or indirectly, by investigating the specific response of the host to the fungus (Table 1). However, it is worthy of note that laboratory findings should be interpreted considering the clinical and epidemiological history. Serological screening, histopathological examination (tissue reaction and documentation of fungal elements in the tissues) and the results of direct microscopic examination guide the correct interpretation of laboratory findings and the initial therapeutic decision. The results of cultures can corroborate or change this decision.[4] The microbiologist should inform the physician whether the test results are sufficient to guide the therapeutic actions. The ideal combination to diagnose a pulmonary fungal infection should include the following:

1) clinical evidence (fever, rales, rhonchi and radiological alteration)
2) fungal isolation and compatible fungal identification
3) identification of fungal elements invading the tissue
4) micromorphological similarity of the isolated fungus to the characteristics observed in the histopathological examination or in the direct examination
5) immunological response to the fungus identified

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.

Direct diagnosis – investigation of the agent
Mycological examination
Direct examination
Culture
Detection of antigen
Latex agglutination test
sandwich ELISA GM-D glucan
Detection of fungal DNA
Polymerase chain reaction
Histopathological examination
Grocott, Mayer's Mucicarmine, Fontana-Masson staining
Indirect diagnosis – investigation of the host response
Imaging studies
X-ray
Computed tomography
Detection of antibodies
Intradermal tests*
Immunodiffusion
ELISA
Histopathological examination
H&E

*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.[4]

The clinical sample should be appropriately packed, according to the means of transportation, the nature of the clinical sample and the

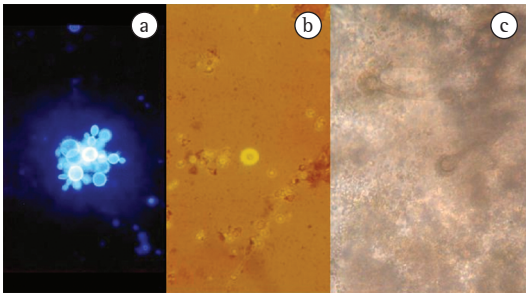

Figure 1 - a) Calcofluor white staining revealing large, multi-budding yeast-like elements characteristic of *Paracoccidioides brasiliensis*; b) Nigrosin staining revealing encapsulated yeast characteristic of *Cryptococcus* sp.; c) Clarification with potassium hydroxide revealing the conidiophores of *Aspergillus* sp.

composition of the vials, which should be properly closed.

Samples should be transported as quickly as possible. Clinical specimens should be transported according to the laboratory procedure to be performed: the material to be cultured should be transported in sterile vials packed in ice (ice box with ice packs); fixed slides are sufficient when the examination required is direct microscopy.

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.

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]

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

Material	Quantity	Reagents/stains
Bronchoalveolar lavage fluid	10-20 mL	Silver and calcofluor
Sputum	5-10 mL	N-acetylcysteine or sodium hydroxide or *sputolysin* (Stat-Pack, Caldon Biotech, Carlsbad, CA, USA), calcofluor
Cerebrospinal fluid	3-5 mL	Nigrosin, calcofluor
Pus and exudates	3-5 mL	Potassium hydroxide, silver, Kinyoun, Gram
Blood[a]	10-20 mL (adults); 1-5 mL (children)	Silver, Giemsa, calcofluor
Tissue	1-5 mm	Silver, potassium hydroxide[b]
Bone marrow	0.2 mL (smear); 1 mL (culture)	Silver, potassium hydroxide[b]
Urine	10-20 mL	Direct, calcofluor

[a]should be heparinized, [b]with the addition of Parker blue-black ink.

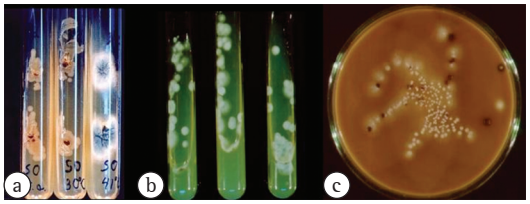

Figure 2 - a) Culture on 2% Sabouraud-glucose agar at different incubation periods, showing inhibition of bacterial growth starting at 40°C, associated with thermal tolerance of *Aspergillus fumigatus*; b) Culture on 2% Sabouraud-glucose agar with cycloheximide for the isolation of *Histoplasma capsulatum*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

Other specific culture media can be used in cases of evident clinical suspicion, such as sterilized bread for zygomycosis etiologic agents and Staib agar (seeds of *Guizotia abyssinica*) for *Cryptococcus* sp. in specimens obtained from the bronchial tree (Figure 2C).[6] The incubation period and the temperature also depend on the etiologic agent to be isolated. In general, yeast-like and filamentous fungi present rapid growth, being isolated in up to one week, whereas dimorphic fungi need a longer incubation period (two to three weeks for *H. capsulatum* and *P. brasiliensis*). With regard to temperature, the material should ideally be incubated at 25°C and 35°C, allowing the isolation of all microorganisms and the prompt identification of thermally dimorphic fungi.[7] Table 3 shows the culture media most frequently used in clinical mycology.

The identification of most genera of filamentous fungi that cause pulmonary mycoses is based on the morphological characteristics found in the culture media routinely used. However, to identify certain species, special media are needed, such as the Czapek-Dox medium for the *Aspergillus* species[8] and the canavanine-glycine-bromothymol blue to distinguish between *C. neoformans* and *C. gattii*.[9] Another useful technique to identify filamentous fungi is slide culture on potato carrot agar medium, which stimulates conidiogenesis and facilitates the visualization of complete sporulation structures. To identify the yeast, biochemical tests, including determination of carbohydrate assimilation/fermentation, determination of urease activity, germ tube test and an automated assay, such as the ID 32C and ATB Expression 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).[7] 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.[10,11] 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.[12,13]

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-

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

Mycosis	Material	Culture media								Growth period
		S	SCl	My	BHI	AN	U	CGB	Bread	
Histoplasmosis	Sterile	x			x					7-14 days
	Non sterile		x	x						
Paracoccidioidomycosis	Sterile	x			x					21-30 days
	Non sterile		x	x						
Cryptococcosis	Sterile	x			x	x	x	x		1-10 days
	Non sterile	x	x		x	x	x	x		
Aspergillosis	Sterile	x			x					3-5 days
	Non sterile		x							
Zygomycosis	Sterile	x			x				x	1-3 days
	Non sterile		x							
Hyalohyphomycosis: *Scedosporium/Fusarium*	Sterile	x			x					2-6 days
	Non sterile		x	x						

S: Sabouraud (25°C); SCl: 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).

mated systems, continuously controlled, such as BACTEC (Becton Dickinson, Sparks, MD, USA) and BacT/ALERT (bioMérieux). For the isolation of yeast (genera *Candida*, *Trichosporon* and *Cryptococcus*), these methods are equivalent; however, the lysis-centrifugation blood culture system is considered the gold standard for the isolation of thermally dimorphic fungi, especially *H. capsulatum*, in blood samples.[4,14-18]

In the lysis-centrifugation blood culture system, the blood obtained by means of sterile venous puncture should be inoculated into a specific vial, containing sodium polyanethol sulfonate, polypropylene glycol and saponin, and then homogenized and centrifuged (5,100 rpm). Subsequently, the sediment obtained should be seeded onto two culture media (Sabouraud agar at 25°C and brain-heart infusion agar at 35°C) and cultured for up to three weeks.[19] Sodium polyanethol sulfonate works as an anticoagulant agent and polypropylene glycol as an antifoam substance. Saponin is the key component in this system, since it promotes the lysis of white and red blood cells and inactivates the complement and other plasma antimicrobials, allowing the intracellular release of the fungi, which increases growth rate and abbreviates culture incubation period (2-10 days), for both yeast-like fungi and filamentous fungi. This technique has greater sensitivity than the other blood culture techniques.[20]

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]

The 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

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 4 - Some characteristics of tissue reaction in fungal infections.

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

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

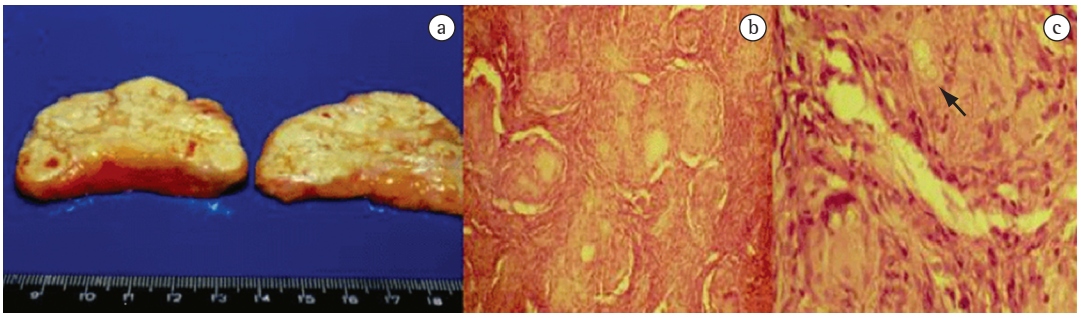

Figure 3 - a) Adrenal gland with severe caseous necrosis (Addison's disease) caused by paracoccidioidomycosis; b) Sarcoid granuloma in paracoccidioidomycosis; c) Spherical, multi-budding, yeast-like elements of *Paracoccidioides brasiliensis* evidenced by H&E staining (arrow).

diagnosis can be established for certain mycoses. As can be seen in Figure 3C, *P. brasiliensis* 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.[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. brasiliensis*. 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 the 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.[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.

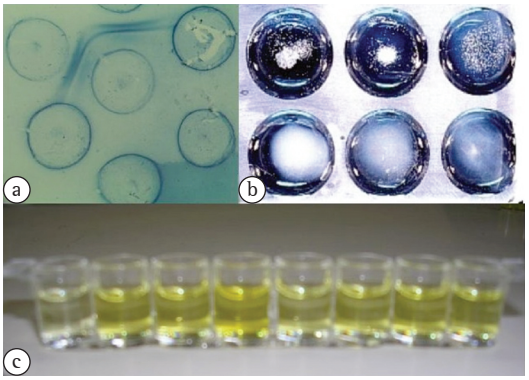

Figure 4 - a) Lines of identity observed in positive immunodiffusion test for paracoccidioidomycosis; b) Latex agglutination test for cryptococcosis, revealing agglutination in the upper holes, corresponding to the positive serum samples and an opaque aspect in the lower holes, corresponding to the negative serum samples; c) Sandwich ELISA for the detection of galactomannan antigen in patients with suspected invasive aspergillosis (from left to right: negative control sample, cutoff control sample, positive control sample and serum samples being investigated).

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 serums 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 hyphae 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.[37,39,41-44] Recently, cross-reaction with *Histoplasma* sp. has also been demonstrated, resulting in positive galactomannan results in patients with histoplasmosis.[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%.[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.[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.[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.[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%.[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.[32,34,43]

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. *Fundação Universidade Federal do Rio Grande* – FURG, Federal University of Rio Grande Foundation – Rio Grande, Brazil.

Flávio de Mattos Oliveira
Researcher. Santa Rita Hospital Mycology Laboratory, Santa Casa Hospital Complex, Porto Alegre, Brazil.

Luiz 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.