Diagnosis of Paracoccidioidomycosis

Subjects: Infectious Diseases

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Paracoccidioidomycosis (PCM) is a systemic mycosis endemic to Latin America caused by thermodimorphic fungi of the genus *Paracoccidioides.* Enhanced understanding of the phylogenetic species concept and molecular variations has led to changes in this genus' taxonomic classification. Although the impact of the new species on clinical presentation and treatment remains unclear, they can influence diagnosis when serological methods are employed. Brazil accounts for 80% of PCM cases worldwide, and its incidence is rising in the northern part of the country (Amazon region), owing to new settlements and deforestation, whereas it is decreasing in the south, owing to agriculture mechanization and urbanization. Clusters of the acute/subacute form are also emerging in areas with major human intervention and climate change. Advances in diagnostic methods (molecular and immunological techniques and biomarkers) remain scarce, and even the reference center's diagnostics are based mainly on direct microscopic examination.

Keywords: paracoccidioidomycosis ; Paracoccidioides spp. ; endemic mycosis

1. Introduction

Paracoccidioidomycosis (PCM) is a systemic fungal infection caused by thermodimorphic microorganisms belonging to the genus *Paracoccidioides*. The disease is endemic to South and Central America, but imported cases have been reported in North America, Europe, Africa, and Asia. The fungus is a soil saprophyte; classically, humans get infected through agricultural activities. Thus, socioeconomic changes in Latin America in recent decades—namely, reduction in human labor in agriculture—have directly impacted the epidemiology of PCM [1][2][3][4].

Although PCM-related mortality is low, morbidity is high once the chronic form's sequelae are present in almost 50% of patients despite treatment [5]. Often, a lack of early clinical suspicion results in delayed treatment.

A recent review of endemic mycosis in America recognized that PCM had the fewest diagnostic tools available [6]. Microbiological studies on respiratory, ganglion, or mucocutaneous samples can be easily performed with a direct microscopic examination (DME). A limitation is that expertise is required to recognize the characteristic fungal structures [6].

Commercial kits for serological diagnosis have been developed, but countries still face production, distribution, and cost problems; thus, in most centers, the antigen is produced *in-house*. Some investigations have been conducted to determine triggers that detect PCM owing to all the species of *Paracoccidioides*. Novel molecular biomarkers have been studied, but warrant further investigation to validate their use and identify the best scenarios to employ them [6][7][8][9].

Treatment-wise, *Paracoccidioides* spp. are susceptible to sulfonamides, azoles, and amphotericin B (both conventional and lipidic formulations). Although itraconazole has been proven to be more effective than cotrimoxazole to treat mild and moderate PCM, in Latin America, the last is still largely used because of its lower cost. Similarly, although the lipid formulations of amphotericin B are less toxic than the conventional one, their high price limits its use in low-income countries [10][11][12].

2. Diagnosis of Paracoccidioidomycosis

2.1. Clinical Diagnosis

The first challenge in diagnosing PCM is to think about the disease when, even in endemic countries and more so in countries outside Latin America, physicians are unfamiliar with this systemic mycosis. Consequently, late diagnosis increases morbidity and mortality rates [4][13].

PCM infection is acquired by inhaling fungal propagules found in the environment, but only 1% to 2% of infected individuals will develop clinical manifestations during their lives [3]. Those who remain asymptomatic can control fungal replication through a robust Th-1 immune response pattern, characterized by cytokine release that activates macrophages and TCD4+ and TCD8+ cells, forming compact granulomas. This stage is named PCM infection [14]. Among those individuals who progress from infection to illness, 5–25% present with the acute/subacute clinical form of PCM, in which Th-2 and Th-9 immune response patterns activate B-lymphocytes that produce high levels of antigen-specific IgA, IgE, and IgG4 [15]. The other 75–95% of cases will evolve from the latent stage to the chronic form of the disease many years later, usually after the fourth decade of life [3].

Chronic PCM manifests gradually and may occur years after exposure to *Paracoccidioides* when the patient is already living in urban areas or outside endemic regions [3]. This form mainly affects the lungs; mucous membranes; skin; and, eventually, the adrenal and central nervous systems [2][16][17]. The main manifestation of chronic PCM in approximately 90% of patients is pulmonary, with symptoms of cough, dyspnea, and sputum expectoration [3][16]. A recent study by Dutra et al. [18] in a southeastern Brazilian hospital found that 59.6% of the patients with PCM had granulomatous ulcerated oral lesions. The oral lesions may be the first visible physical manifestation of the disease noticed by the patient, and may lead to a prompt diagnosis. However, even with adequate treatment, patients may develop the residual form of PCM, owing to fibrosis of the affected organs [3][5].

The acute/subacute form of the disease shows rapid and disseminated progression in the form of skin lesions; lymphadenopathy; and eventual suppuration, fever, and anorexia [3][16][19]. This form characteristically develops a few weeks or months after fungal exposure [2].

In immunocompromised patients, a mixed clinical form of PCM, with characteristics of both chronic and acute forms of the disease, has been observed. Pulmonary involvement can coincide with generalized adeno- and hepatosplenomegaly. In patients with the mixed form, multiple, exuberant skin involvement; lytic bone lesions; and central nervous system involvement can be present, indicative of severe disease. Patients with HIV co-infected with PCM make up the bulk of immunosuppressed patients with PCM; however, cases of PCM in transplant patients and those receiving immunobiological therapy have also been reported [19][20][21][22].

PCM is often confused with tuberculosis in Latin America, owing to its high prevalence and the similar clinical presentations of both diseases [23]. Besides tuberculosis, the most relevant differential diagnoses of chronic pulmonary PCM are other fungal infections, such as coccidioidomycosis and histoplasmosis. Sarcoidosis, pneumoconiosis, and interstitial pneumonitis should also be considered. Moreover, it is essential to rule out concomitant diseases. Tuberculosis and PCM can occur simultaneously or sequentially in 5.5–19% of cases [16][17][24][25]. Additionally, PCM and solid cancers of the respiratory and gastrointestinal tracts share similar risk factors (male sex, smoking, and alcohol intake). Solid neoplasias and *Paracoccidioides* infection have been shown to co-exist in 0.16–11% of patients [26].

In patients with mucocutaneous PCM, the differential diagnosis should include leishmaniasis, tuberculosis, chromoblastomycosis, leprosy, syphilis, and neoplasia. In individuals with acute PCM, clinicians should be concerned about hematologic neoplasms, histoplasmosis, tuberculosis, and visceral leishmaniasis [3]. It is important to remember that many infectious diseases share the same endemic areas as PCM.

Finally, patients with chronic pulmonary PCM are at higher risk of more severe illness with COVID-19 coinfection. Despite that, during the pandemic, only one case of SARS-CoV-2 and *Paracoccidioides* spp. co-infection was described in an individual with the acute form of PCM. The scarcity of documented cases probably reflects underdiagnosis or underreporting in Latin American countries that had their health systems overwhelmed by COVID-19 [27][28][29].

2.2. Laboratory Diagnosis

Laboratory diagnosis via microscopy remains the gold standard method for diagnosing PCM. This may show the presence of the etiologic agent in biological fluids and tissue sections or the isolation of the fungus from clinical specimens, owing to the characteristic appearance of typical *Paracoccidioides* spp. yeast forms [30]. Other tools, such as cultures, immunodiffusion assays, and polymerase chain reaction (PCR) tests [7][8][9][31][32], are also used. Different types of clinical samples may be collected for testing. Mucocutaneous scrapings, sputum, bronchoalveolar lavage (BAL), cerebrospinal fluid (CSF), lymph node aspirate, biopsy, and tissue samples are those most frequently collected [33][34]. Prior processing of some samples is needed to increase their sensitivity to detection methods, including centrifugation (for sputum, BAL, CSF, and lymph node aspirate) and maceration of fragmented, biopsied tissues [7]. The sputum sample should be prepared with potassium hydroxide, sodium hydroxide, and N-acetyl-L-cysteine before being added to a suitable culture medium at 25 °C [30].

2.2.1. Mycological Diagnosis

This method includes visualizing fungal elements through DME, followed by isolation of the agent in culture media [35]. DME of the sputum, BAL, CSF, lymph node aspirate, and mucocutaneous scraping are prepared with the addition of 10–20% KOH or calcofluor, making it possible to visualize *Paracoccidioides* spp. in their parasitic form with multiple budding cells (blastoconidia) surrounding it, connected by short cellular bridges [36][37][38][39][40]. However, it is important to mention that for biopsy and tissue samples, the slides are mounted using 40% KOH [36]. *Paracoccidioides* cells have a thick mucopolysaccharide wall with a double-contour appearance that is birefringent under light microscopy [41][42]. *Paracoccidioides* structures resembling a “ship’s wheel” or “Mickey Mouse” are deemed pathognomonic findings [9][37][40].

The size and multiple budding distinguish *Paracoccidioides* spp. from other fungi. Nevertheless, *Paracoccidioides* isolates can be mistaken for *Histoplasma capsulatum* and *Cryptococcus* spp. when it produces small, non-budding cells, and when *Cryptococcus* does not produce its capsule efficiently [42][43].

Moreto et al. [7] evaluated the diagnostic methods for PCM at a university hospital between 1976 and 2004. They observed that in the DME of 51 different tissue specimens and 112 sputum samples, the sensitivity was 75% and 63%, respectively. For 483 sputum cell blocks, the values found were 55%. Since the PCM chronic form is the most frequent form encountered, sputum is the biological material most commonly evaluated under DME, and the sensitivity will depend on the processing method of that material [13][17][44]. Although DME is a simple, fast, and low-cost technique implemented in small laboratories, its sensitivity is low [13][17][44][45][46]. Thus, DME cannot provide a conclusive diagnosis in cases of negative results. Owing to the heterogeneity of the sample fractions, DME can mistakenly lead to the assumption that the fungus does not exist in the entire sample [32].

2.2.2. Cultures

Paracoccidioides take an average of 3–6 weeks to grow on fungal culture media [47][48][49]. Nonetheless, the growth of this fungus varies in the different studies, with a sensitivity of 80 to 97% of cases [49][50][51][52]. The results should be evaluated about 4 weeks after the cultivation of the sample at 25 °C. To reduce the running cultivation time, samples must be cultured simultaneously at 25 °C and 37 °C. The culture media most frequently used are Sabouraud, Mycosel, and Fava-Netto agar. Other media, such as mycobiotic agar, brain heart infusion agar (BHI), Sabouraud dextrose plus BHI broth (SABHI), agar-yeast extract-phosphate, agar-yeast extract, agar-yeast extract-penicillin plus streptomycin and cycloheximide, and Kelley medium with hemoglobin, are also employed [32][36][37][42][53]. According to Hahn et al. [36], the successful recovery of *Paracoccidioides* from clinical specimens depends on several factors, including the culture media and the number of tubes or plates seeded, besides the decontamination with antibiotics of sputum and bronchial lavage fluid. Despite its slowness, culture is always recommended because it allows species identification using molecular biology [9][54][55][56].

2.2.3. Histopathological Diagnosis

Histopathological examination is a valuable tool (≥95% sensitivity) for PCM diagnosis. It can also determine disease severity [7][17][57]. The sections can be stained with hematoxylin/eosin (H & E), Grocott’s methenamine silver, and periodic acid–Schiff (PAS). These last two are specific stains that increase sensitivity [18]. When stained with H&E, an inflammatory response can be observed in the parasite–host interaction. Organizing granulomas or a combination of suppurative and granulomatous infiltrates can also be seen [7][32][58]. If the samples are sufficient, DME and tissue culture can be performed [44]. It is worth mentioning that biological samples for histological examination are fixed in formaldehyde solution. For culture, the biopsy and tissue samples must be placed in a sterile container under a sterile physiological saline solution.

2.2.4. Immunological Diagnosis: Antibody Detection

In 1916, Arthur Moses, an assistant physician at the Oswaldo Cruz Foundation, isolated an antigen from *P. brasiliensis*-infected cells used in a complement fixation (CF) test to diagnose PCM [59]. Since then, numerous antigens and antibody-based serological assays have been developed as alternative methods for detecting fungal structures in biological fluids/tissues and disease monitoring [60][61]. Double immunodiffusion (DID), counterimmunoelectrophoresis (CIE), immunofluorescence, radioimmunoassay, enzyme-based immunoassays (ELISA), immunoblotting, dot immunoassay, western blotting, and latex particle agglutination (LA) are some important techniques in use [9][60][61][62][63][64][65][66][67][68][69]. Though many validated methods for detecting anti-*Paracoccidioides* serum antibodies exist, most are conducted only in research centers. There also remains significant limitations, owing to cross-reactivity with other infectious fungi, such as *Histoplasma* and *Aspergillus* [35][60][70].

DID is the most widely used method for detecting anti-*Paracoccidioides* serum antibodies in endemic countries [60][71]. Its advantages include the ease of performing the quantitative techniques, low cost, and high specificity (85–100%) [17][23][48][49]. However, its sensitivity can range from 80 to 95% depending on the antigen applied [72][73]. CIE has similar specificity to DID (>95%) with slightly higher sensitivity (77–100%). Both techniques are recommended as serological screening tests for patients with suspected PCM because of their faster turnaround time than microbiological methods [35][60][72][74][75].

By applying the immunoblotting technique, de Camargo et al. [76] assessed several exoantigens produced by *P. brasiliensis* isolates against serum from PCM-positive patients. IgG anti-*P. brasiliensis* was discovered in some cell surface components, but the most promising were glycoproteins gp70 (70 KDa) and gp43 (43 KDa). The latter is commonly recognized by IgG antibodies, and is reactive in 100% of patients with PCM caused by *P. brasiliensis sensu stricto*.

Several components with antigenic ability to distinguish circulating antibodies in patients with suspected PCM have been tested. Glycoprotein gp43 is the most commonly used component that can be presented as a cell-free antigen (CFA), exo-antigen (ExoAg), and recombinant or purified antigen [9][71][76][77]. Throughout the exponential growth phase of *P. brasiliensis*, its cells secrete this antigen, found in almost all isolates [32]. However, there is decreased expression of this antigen in patients infected with *P. lutzii*, and false-negative results may result [9][71][76][77]. These differences in antigenic composition are probably related to phylogenetic peculiarities [78]. In addition, gp43 may trigger cross-reactivity in patients with histoplasmosis or lobomycosis because its epitope is a galactose-containing carbohydrate, common among pathogenic fungi [32][79].

By evaluating different antigenic preparations from *P. lutzii* using the immunodiffusion technique, Gegembauer et al. [73] demonstrated that tests employing *P. brasiliensis* antigens might yield false-negative results when *P. lutzii* is the causative agent [70][80]. Maifrede et al. [70] showed that 7 of 21 sera samples negative for *P. brasiliensis* antigen were positive for *P. lutzii* when the Pb339 exoantigen and PIEPM208 CFA were applied. It can infer that the frequency of *P. lutzii* may be higher than reported in endemic areas because gp43 is the most commonly used antigen in routine laboratory examinations [9][34][70].

In 2021, an American company began commercializing a DID-based test to detect *Paracoccidioides* serum antibodies (ID Antigen®). In the same year, Cocio and Martinez [71] used CIE and DID to evaluate the sensitivity and specificity of the antigen in ID Antigen®. They found that of the 24 PCM-positive serum samples of patients with active PCM, 100% were reactive in CIE methodology using ID Antigen®, including 11 cases of infection by *P. brasiliensis sensu stricto*, one by *P. americana* and one by *P. lutzii*. The test's specificity was 100%, with negative results for histoplasmosis, aspergillosis, and other diseases, and an overall 75% sensitivity with PCM sera. Therefore, the antigen available in the commercial test could diagnose PCM caused by three different species.

Considering that five *Paracoccidioides* species have been recognized as PCM agents in endemic areas, new antigen preparations must be, and are, being investigated to expand the use of PCM serology with increased sensitivity and specificity.

2.2.5. Antigen Detection

Antibody detection is not the best choice for all patients with PCM. To illustrate, in many studies, immunocompromised patients and those with severe forms of acute/subacute disease showed decreased antibodies, with half showing none [42][81][82]. Therefore, identifying antigens instead in these cases would be more suitable.

In 1997, using the inhibition ELISA technique (inh-ELISA), Colombian researchers discovered a monoclonal antibody to detect the 87 kDa antigen in patients with PCM. In patients with the disease, acute, multifocal, and unifocal forms were detected in 100%, 83.3%, and 60% of patients, respectively [83]. Since then, several antigenic molecules and tools have been characterized and evaluated [31][36][66][67][68][69]. Notably, anti-gp43 and anti-gp70 monoclonal antibodies are still the most frequently investigated glycoproteins [44]. However, cross-reactions have been obtained with heterologous sera, such as sera from patients with aspergillosis, cryptococcosis, and histoplasmosis [56].

Xavier et al. [67] analyzed the Platelia™ *Aspergillus* enzyme immunoassay (EIA) (Bio-Rad, Marnes-la-Coquette, France) as a diagnostic tool for 30 PCM patients and found a positivity rate of 50%. This method is widely used to detect galactomannan in patients suspected of having invasive aspergillosis [84].

Recently, Melo et al. [69] investigated the performance of (1,3)-β-D-glucan assays (BDG), a test used to diagnose invasive fungal infections in patients with PCM. Fifty-two serum samples from 29 patients with acute and chronic PCM were evaluated. Despite its excellent diagnostic sensitivity (96.5%), it did not contribute to disease monitoring.

Some commercial methods have been validated and are currently available for detecting specific fungal antigens in patients with cryptococcosis, histoplasmosis, coccidioidomycosis, blastomycosis, aspergillosis, and candidiasis. However, progress has not been made on making commercial tests for detecting *Paracoccidioides* antigens available. It is essential to highlight that if the antigen tests were commercially available, they could make the serological diagnosis of PCM more accessible to patients who live far from referral centers.

2.2.6. Molecular Detection

In the last century, molecular tools have provided crucial information for the taxonomic classification and epidemiological, diagnostic, and therapeutic management of pathogenic fungi. Several molecular methods, including PCR-derived techniques, have opened doors for the early diagnosis of fungal diseases and the identification of etiologic agents [9]. PCR, loop-mediated isothermal amplification (LAMP), quantitative real-time PCR (qPCR), nested and semi-nested PCR, and duplex PCR-assay have been found to detect *Paracoccidioides* genetic material directly from clinical samples [7][9][34][85][86][87][88][89]. Most assays are based on primary markers, such as the *GP43* gene and the internal transcribed spacer (ITS) region of ribosomal DNA [34].

In 2021, Pinheiro et al. [34] developed a duplex PCR single-assay capable of detecting and differentiating members of the *P. brasiliensis* complex and *P. lutzii* from paraffin-embedded tissue blocks [18]. This methodology became vital in clinical laboratory practice, particularly in diagnosing atypical cases, such as those with seronegative yet positive DME results, and in examining patients with co-infections.

Despite having similar sensitivity and specificity as DME and histopathological techniques, molecular methods might have a better yield with materials with a low burden of infection (serum, BAL, CSF), and may be more sensitive than DID. However, the molecular approach is performed based on *in-house* tests, for which currently external quality assessments are lacking [7][34][89]. Moreover, using molecular techniques for diagnosing disease-causing fungi directly from the clinical sample is challenging because of the complexity of DNA extraction. In addition, databases with genome sequences for these microorganisms are under construction. The very nomenclature of these agents requires continuous updating in the laboratory. In other words, the molecular tools, to be successfully used, have to adapt to the objectives of the study. Knowledge is constantly evolving in the study of fungi, and these techniques are not yet validated for use in the routine diagnosis of PCM. Furthermore, it is worth emphasizing that molecular tests are expensive, and most of the population affected by PCM belongs to developing countries.

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