# Diagnostic Aspects of Paracoccidioidomycosis

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**Abstract** The gold standard for the diagnosis of paracoccidioidomycosis is direct examination and culture; however, serologic, histopathologic, and molecular approaches have been recently adopted for the diagnosis of this mycosis. Few molecular methods have been applied to the diagnosis of paracoccidioidomycosis to detect *Paracoccidioides* spp. DNA from clinical specimens, and to identify the same fungi in culture. In this review, we focus on the current diagnosis of the paracoccidioidomycosis, and discuss the current molecular tools applied to the diagnosis and identification of the *Paracoccidioides* complex species.

**Keywords** Paracoccidioidomycosis · *Paracoccidioides* spp · Diagnose · Mycology, serology, molecular techniques · Tropical mycosis · Tropical medicine · Review

## Introduction

The endemic mycosis paracoccidioidomycosis (PCM) has the genus *Paracoccidioides* as its etiologic agent, and occurs as an active disease in up to 2 % of infected individuals [1]. Phylogenetic and population studies of *Paracoccidioides* species indicate that the genus comprises at least four phylogenetic lineages: S1 (species 1 from Brazil, Venezuela, Peru, Paraguay, and Argentina), PS2 (phylogenetic species from Brazil

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and Venezuela), PS3 (phylogenetic species from Colombia), all three of which are included in the *Paracoccidioides brasiliensis* species [2, 3], and *Pb*01, now proposed to be *P. lutzii* [4••]. The disease is one of the most prevalent systemic mycoses in Latin America [5], and some cases have been reported outside of the American continent, both in individuals from endemic areas and in returning travelers from those regions [6]. Despite the large number of cases frequently diagnosed, the exact epidemiology of PCM is not known since there is no compulsory notification of identified cases in many countries. Due to the lack of available data, PCM was recently added to the list of neglected diseases [7].

PCM is a typical occupational infection of farmers and outdoors workers, preferentially affecting men, mostly in the third and fourth decades of life. As a rule, *P. brasiliensis* infection is acquired by the inhalation of airborne fungal particles after soil is turned over; the conidia can easily reach the alveoli when inhaled, and a lung–lymph node primary complex is developed. These early lesions may remain silent for many years and, in a manner similar to tuberculosis and other systemic mycoses, they may at any time progress in the lungs or disseminate by the lymphohematogenic route.

Pathogenic fungi of the genus *Paracoccidioides* are dimorphic in the environment or when cultured in the laboratory at 25–30 °C. In the filamentous form, the fungus grows slowly, with a wide variety of colonial morphologies presenting as forms ranging from a glabrous leathery, brownish, flat colony with a few tufts of aerial mycelium to a wrinkled, folded, floccose form, to velvety, white, pink, and beige forms. Microscopically, a variety of conidia is seen, none of which is characteristic of the species. In contrast, the parasitic phase and the yeast phase of *Paracoccidioides* that develops when the fungus is cultured in appropriate culture media at 35–37 °C, present 2–30 μm or more yeast cells, which may be oval or irregular shapes with one or more buds [8]. Macroscopically, colonies of *Paracoccidioides* have a yellowish-beige color and a creamy texture.

Mycoses can be challenging to diagnose, and accurate interpretation of laboratory data is important to ensure appropriate treatment. Although the clinical manifestations of PCM are well-described, the diagnosis of this mycosis cannot be based on clinical information alone because the symptoms of PCM overlap with those of other diseases.

PCM is classically diagnosed by correlation of clinical, epidemiologic, and laboratory data. To confirm the diagnosis, laboratory examinations must be performed. Typical laboratory analyses involved in PCM diagnosis include microscopic examination and cultures of clinical specimens, such as skin biopsies or pus, sputum, urine, blood, synovial and cerebrospinal fluids, can be analyzed depending on the affected organs or systems. Currently, there are additional diagnostic tools available for diagnosis of PCM to supplement culture and microscopic examination. These laboratory tests have a rapid turnaround time and reasonable specificity and sensitivity. For instance, serologic techniques involving antibody and antigen detection have been developed using different methodologies. Molecular methods to detect *Paracoccidioides* DNA in clinical specimens, including tissue fragments, are also being studied in several laboratories to facilitate rapid diagnosis of the infection. The accuracy of routine diagnostic tests applied for PCM was recently reviewed in a university hospital in Brazil, demonstrating that the association of conventional tests with serologic tests is sufficient to establish laboratory diagnosis of PCM [9].

The following sections focus on these applications for diagnosis of PCM. Some of the well-established clinical and laboratorial diagnoses are discussed and current conventional diagnostic tools are reviewed. Additionally, we outline the development of novel molecular methods and discuss their relative merits.

## Clinical Diagnosis

PCM has clinical manifestations that require differentiation from tuberculosis, Hodgkin disease, several systemic and subcutaneous mycoses, and squamous cell carcinoma [10]. The primary pulmonary infection is usually subclinical, and individuals may never develop clinical signs. A small percentage of patients present with clinical symptoms, most of whom have lung involvement [11].

The most common form of this infection in adults is the chronic multifocal form, in which there is dissemination to the lungs, lymph nodes, skin, and mucosae. Cough, dyspnea, weight loss, and cutaneous and mucosal lesions are the major complaints reported by patients with PCM. Chest X-rays reveal diffuse reticulonodular infiltrates, which are more evident in the upper lobes. The acute form usually presents as lymph node enlargement and cutaneous lesions, with digestive and osteoarticular symptoms. Other manifestations

include anemia, fever, and weight loss, with the overall health of patients deteriorating rapidly. Pulmonary involvement in the acute form is rare. The initial investigation should include the following non-specific tests: chest X-ray, blood cell counts, liver function tests, urea, creatinine, sodium, and potassium [12].

## Conventional Diagnosis

Depending on which clinical specimen is sent to the mycological laboratory for diagnosis, some procedures take place before microscopic and culture analysis. Skin biopsies should be sent for culture under sterile physiological saline solution. Water and formaldehyde are not suitable as transport liquids, since they interfere with the microbiological tests. However, formaldehyde must be used when sending a tissue sample for histopathological examination. In the laboratory, skin biopsies have to be triturated using surgical scissors. Grinding them with mortar and pestle must be avoided since this procedure can destroy some fungal structures that can be present on the material. On the other hand, pus from ulcerated lesions or skin scrapings do not need any previous treatment, and can be analyzed after sample collection. Liquid samples such as sputum, bronchoalveolar lavage, and cerebrospinal fluid must be centrifuged and the supernatant discarded before mycological examination [13].

Direct microscopic examination of the specimens is typically made with 10 % potassium hydroxide (NaOH) or 4 % sodium hydroxide (KOH) in order to detect parasitic multiple budding yeast cells using a light microscope under a 400–1,000× magnification. These cells are large (3–30 μm in diameter), round to oval, and multiple buds are attached to the parent cell by narrow connections (see Fig. 1a) [14]. Differentiation between *P. brasiliensis* and *Blastomyces dermatitidis* yeast cells is not usually a problem because the endemic areas of the diseases caused by these fungi do not overlap [15]. However, for the diagnosis of imported mycoses, the differentiation is easy because *B. dermatitidis* yeasts present a single bud with a broad connection to the parent cell [14].

Histopathological examination is of great value for the diagnosis of PCM. Although *P. brasiliensis* yeast cells might be seen routinely using hematoxylin and eosin (H&E) stain, other stains such as Gomori methenamine silver (GMS), periodic acid-Schiff (PAS), and immunohistochemical staining can be used to enhance detection of fungal elements. Tissues present granuloma formation with multinucleated giant cells, a polymorphonuclear infiltrate, and the pathognomonic "ship's wheel" budding yeast cells [16].

Definitive diagnosis of PCM is based on the isolation and identification of its etiological agent in culture. Isolation of *P. brasiliensis* can be obtained after spreading the clinical

**Fig. 1** *Paracoccidioides brasiliensis* yeast cell microscopic morphology: (**a**) sodium hydroxide 10 % preparation; (**b**) typical "pilot-wheel" shape stained with lactophenol cotton blue
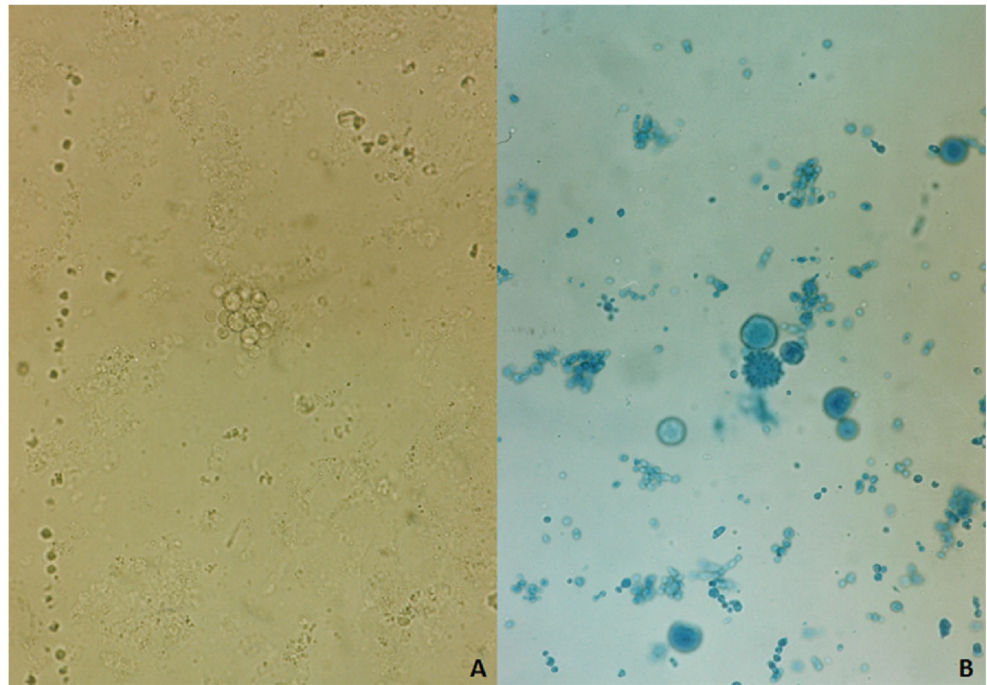


specimens on routinely used Sabouraud Dextrose Agar supplemented with 400 mg/L of chloramphenicol to avoid bacterial contamination and on cycloheximide-containing media such as Mycosel or Mycobiotic agar. Cycloheximide inhibits growth of several anemophilous fungi that can contaminate cultures from clinical specimens obtained from non-sterile sites; however, this drug does not impair the growth of *P. brasiliensis* [17]. Although *P. brasiliensis* can grow at around body temperature, this is not the optimal temperature for fungal growth. Therefore, to enhance the odds of fungal isolation, cultures must be incubated at 25–30 °C. After 2–3 weeks of incubation, filamentous colonies start to grow. However, some strains require extended incubation for growth. These colonies present a white mycelium that often turns brown after some time and microscopically septate branched hyphae can be observed. Infrequently, a few microconidia are observed along the hyphae [14]. For final identification, conversion to the yeast phase must be performed by subculturing the fungus at 37 °C in brain heart infusion or Fava-Netto's Agar (proteose peptone 3 g/L, peptone 10 g/L, beef extract 5 g/L, NaCl 5 g/L, yeast extract 5 g/L, glucose 4 %, pH 7.2). In these media, yeast cells, similar in size and shape to those observed in parasitism, must be observed (see Fig. 1b). If there is no growth of *P. brasiliensis*-resembling fungal colonies after a 6-week incubation period, cultures can be considered negative.

Despite being the gold standard, direct examination requires skilled professionals to read the test, the culture takes a long time, and handling this fungus raises biohazard concerns [18]. In addition, these methods are not enough to differentiate the new cryptic species on the *P. brasiliensis*

complex, since the only morphological difference between them is conidial morphology: *P. lutzii* isolates present elongated conidia, while *P. brasiliensis* isolates have short round conidia [19]. Cultures from clinical specimens on routine mycological media usually lack conidial formation.

Recently, a new microdilution method for antifungal susceptibility of *P. brasiliensis* isolates was described. This proposed protocol employs an inoculum concentration of $1 \times 10^5$ cells/mL in Müeller-Hinton medium and an incubation temperature of 35–37 °C for 15 days. This method was able to determine reliable minimal inhibitory concentrations of amphotericin B, itraconazole, ketoconazole, fluconazole, and terbinafine for 22 *P. brasiliensis* isolates [20•].

## Serology

Serologic techniques are usually simpler than culture and very useful in the diagnosis and follow-up of patients with PCM. The following serologic tests have been used for diagnosing PCM: agar gel immunodiffusion (ID), tube precipitin, complement fixation (CF), counter-immunoelectrophoresis (CIE), immunofluorescence, radioimmunoassay, passive hemagglutination inhibition, immunoenzyme assays, such as ELISA, MELISA, dot blot [18], and inhibition-ELISA [21], and, more recently, the latex agglutination test [22]. These techniques have had considerable advances in the past decades because of the development of innovative detection schemes, identification of relevant *P. brasiliensis* antigens, and increasing use of monoclonal antibody technology for application in immunoassays [23••]. The literature concerning the serodiagnosis of

PCM is extensive and diverse, extending back to the first description of a complement reaction by Moses in 1916 [24]. For detailed summaries of the work in this field up to 2011, the reviews by Teles and Martins [18] and Camargo [25] are recommended. However, in the last 3 years some important contributions to this field have been made, and these are discussed in this section.

A range of antigenic preparations, derived both from whole yeast cells and culture filtrate, in their crude and/or purified state, have been applied in the serologic tests, which resulted in high cross-reactivity, one of the most persistent problems found in the serodiagnosis of PCM. Moreover, these antigenic preparations present antigenic variability, making it very difficult to standardize diagnostic techniques in different laboratories [26•]. For these reasons, several groups have been working with pooled crude exoantigens of *P. brasiliensis* in conventional serologic techniques such as ID, CIE, ELISA [27, 28], and western blot [29]. Recently, corroborating these findings, Machado et al. [30••] analyzed the impact of cryptic species of *P. brasiliensis* on the PCM immunodiagnosis. The authors emphasize that the use of a single antigen preparation in the conventional serologic tests is not recommended, since a small concentration of glycoprotein gp43, the main *P. brasiliensis* antigen, was found in culture filtrates of *P. lutzii* strains, and the production of this antigen varied between isolates belonging to the same species.

Production of several antigenic preparations has improved sensitivity and specificity of the serologic tests focused on antibody detection. Some purified antigens from *P. brasiliensis*, such as gp43, have been widely applied in the immunodiagnosis of PCM. Patients affected by severe PCM forms show high levels of anti-gp43 antibodies, which decrease over the course of disease treatment in most cases [31, 32]. In fact, 90 % of PCM patients present positive results for gp43-based ID assays and around 100 % for immunoblotting assays [32]. The protein p87 [33] has also been considered as a suitable alternative antigen for serologic diagnosis of this mycosis.

Fusion molecules from the genus *Paracoccidioides* obtained by recombinant technology have been also used as alternative antigens for the immunodiagnosis of PCM. The gene encoding gp43 was the first antigen cloned and expressed as a recombinant fusion protein [34], and it showed high reactivity when evaluated with sera from patients with PCM. McEwen et al. [35] cloned the protein p27 (rPb27), which has been applied to detect immune responses by ELISA [36, 37]. Recently, the utility of rPb27 and the secreted and surface Mexp antigen was evaluated in an in-house ELISA for detection of the IgG profile and its subclass levels in 92 serum samples from 54 chronic PCM patients at different points during or after treatment. There was a tendency towards decreasing antibody levels during treatment, but these levels do not clear in most patients after treatment is stopped [38].

Besides gp43 and p27, other recombinant fusion proteins such as heatshock protein (hsp) 60 [39] and hsp70 [40] have been described as an interesting tool for PCM diagnosis. More recently, combined use of *P. brasiliensis* recombinant rPb27 and rPb40 antigens in an indirect ELISA provided an excellent immunodiagnosis assay for PCM, showing 96 and 100 % sensitivity and specificity, respectively [26•]. These data indicate that the use of a multiantigenic preparation is an efficient strategy to improve the sensitivity and specificity of an immunoassay, corroborating data suggested in the early 2000s [41]. In addition, a synthetic peptide named P2 that mimicked the epitope of the 75 kDa protein of *P. brasiliensis* was recognized in untreated PCM patients' sera from acute and chronic forms of PCM in an ELISA, showing 100 % sensitivity and 94.59 % specificity in relation to human sera control [23••].

Although antigen detection has some important advantages over antibody detection in the diagnosis of PCM, particularly in immunocompromised individuals [18, 25, 42], there remains considerable scope for improvement in the area of antigen detection since the last important contributions to this field were made in 2011.

## Molecular Methods for Paracoccidioidomycosis Diagnosis

Nucleic acid-based assays have a good potential to complement and enhance the sensitivity and rapidity of conventional methods used in diagnostic mycology [43]. The majority of molecular tests are polymerase chain reaction (PCR) methods based on the amplification of the fungal gene sequences, and the ribosomal DNA gene was the main target applied in the early scientific work described in the literature [44, 45].

The reliability of the internal transcribed spacer (ITS) region for molecular detection of *P. brasiliensis* was also demonstrated in a real-time PCR [46, 47], being a sensitive methodology for rapid diagnosis of PCM. Recently, this methodology was adapted for a classic PCR format, using *P. lutzii* instead of *P. brasiliensis*. Despite its high sensitivity (1.1 pg DNA) and specificity in detecting *P. lutzii* DNA from fungal cultures, this format was not suitable for DNA detection in serum samples, and its utility in other types of clinical samples such as biopsies needs to be further evaluated [48]. Also, a semi-nested PCR with primers ITS1 and ITS4 in the first reaction and MJ03 and ITS1 in the second reaction [49] presented high sensitivity and specificity for detection of *P. brasiliensis* in tissue. The ITS1–5.8S–ITS2 region of ribosomal RNA was also applied in a nested PCR in a collection of aerosol samples as an optional methodology for environmental detection of *Paracoccidioides* spp. The molecular detection proved the best method for discovering these fungi in the environment when compared with culturing strategies [50].

After molecular characterization of the antigen gp43, the majority of the PCR assays for the diagnosis of PCM were based on oligonucleotide primers derived from the sequence of the gene encoding the gp43 antigen. Several formats of PCR were developed prior to 2011 for detection of *P. brasiliensis* in biological specimens, such as conventional PCR [51], nested PCR assays [52–55], and semi-nested PCR [49], presenting good sensitivity and specificity.

The gp43 sequence was also the target of a 5′ nuclease assay using fluorescent probes that was found to be straightforward, sensitive, and reproducible for *P. brasiliensis* detection [56]. This sequence was further shown to be useful in the characterization of atypical *P. brasiliensis* strains [57].

In the last decade the potential diagnostic application of a heminested PCR targeting a new molecular *P. brasiliensis* marker named ceja-1 was also evaluated. This methodology was able selectively to detect only DNA from virulent *P. brasiliensis* strains. Non-virulent *P. brasiliensis* strains and other organisms, including several pathogenic systemic fungi, mycobacteria, protozoa, nematodes, mouse and human DNA, were unable to generate amplicons with this protocol. Moreover, the feasibility of this method in specimens from PCM patients was demonstrated, although a large number of clinical samples should be further evaluated for the establishment of sensitivity of this test [58].

## Molecular Typing

*P. brasiliensis* has shown extensive genetic variability as determined by molecular tools such as restriction fragment length polymorphism (RFLP) analysis [59], electrophoretic karyotyping [60], random amplified polymorphic DNA (RAPD) [61, 62], and microsatellites [63].

The pathogenic fungus *P. brasiliensis* from geographically separated regions of South America was probed by RFLP using *Hinf*I and *Hinc*II, showing clear RFLP patterns. Computational analysis of the RFLP patterns for the 32 isolates suggested that at least five groups of strains existed that were geographically distinct and corresponded closely with present country borders [59].

*P. brasiliensis* typed by RAPD presented marked differences in its virulence patterns [61]. The ability of *P. brasiliensis* isolates to invade tissues was studied in an experimental model using susceptible B10.A mice. The analysis was performed according to the severity of the lesions, including the number and size of granuloma, and the number and dissemination of fungi to different organs. Another relevant example of RAPD performance was the results of the analysis of the complex RAPD profiles carried out using humans isolates with three arbitrary primers. The methodology demonstrated limited intraspecific genomic variations in *P. brasiliensis* isolates, indicating that such methodology

could be useful for the analysis of the *P. brasiliensis* genome for characterization or differentiation within this genus [62].

The electrophoretic pattern of contour-clamped homogeneous electric field gel electrophoresis (CHEF) applied to 12 environmental isolates of *P. brasiliensis* has shown five bands, with molecular sizes ranging from 3.2 to 10 Mb, and a similar model previously tested and used the clinical isolates as controls. However, one of the bands in the environmental isolates had a lesser weight (7.2 Mb) than the one corresponding to the clinical isolate (8.8 Mb), resulting in a smaller genome of approximately 29.7 Mb [60], and indicating the presence of chromosome polymorphism in this fungus.

Despite the number of available molecular methods developed for strain typing and characterization, they have not been useful for assigning isolates to the described species, thus emphasizing the need for molecular markers capable of distinguishing genetically isolated groups. A PCR- and sequencing-based microsatellite marker system that is easy to perform, stable, adaptable to large numbers of isolates, and discriminatory enough to be used as a typing system in identifying the three proposed phylogenetic species of *P. brasiliensis* was evaluated. This technique was shown to be an unambiguous tool for strain discrimination between two (S1 and PS2) of the cryptic species [2]. Subsequently, genotyping was performed on ten paraffin *Paracoccidioides*-embedded tissue samples by a nested PCR based on the polymorphism of the gene encoding the gp43 and provided good correlation of histopathological findings with genotypes [63].

## Molecular Phylogenetics and the *Paracoccidioides brasiliensis* Species Complex

*P. brasiliensis* contains several different cryptic species, as stated in phylogenetic studies using Multi-Locus Sequence Type (MLST) [2, 3], microsatellite analysis [64], and PRP8 intein sequencing [65].

*P. brasiliensis* isolates from the Brazilian Central Region showed a significant genetic divergence when compared with other S1, PS2, and PS3 isolates in MLST [3]. This phylogenetic analysis was investigated for coding and non-coding regions from various genes (*CHS4, Actin, ODC, URA3, CHS2, HSP70, FKS1, Hydrophobin, Kex1, Catalase A, Catalase P, Formamidase, Glyoxalase*) and the ITS sequences of seven new and 14 known isolates of *P. brasiliensis* showing a significant genetic distance between Pb01 and the remaining genetic groups [3], suggesting the existence of cryptic species of *P. brasiliensis* [2, 3]. Subsequently, the genealogical concordance method of phylogenetic species recognition (GCPSR) identified a clade of 17 genotypically similar isolates, including Pb01, which are distinct from the S1/PS2/P3 clade. The authors, based on the results obtained with

GCPSR, considered the "Pb01-like" group to be a new phylogenetic species, and proposed a new species called *Paracoccidioides lutzii* [4••].

Subsequently, an additional molecular marker, the PRP8 intein, was used to recognize *P. brasiliensis* isolates of the different genetic groups [65]. PRP8 inteins of 22 *P. brasiliensis* isolates belonging to all four cryptic species were sequenced. Phylogenetic analysis clearly separated the isolates from the four species and revealed a significant difference between Pb01-like and the remaining species [65]. More recently, the same group concluded that single nucleotide polymorphism analysis of *gp43*, *ARF*, and *PRP8* intein genes is useful to distinguish among the four cryptic species of the genus *Paracoccidioides* [66••]. Subsequently, the same authors also demonstrated that nested PCR amplicons from the ITS region from most environmental aerosol samples presented 100 % similarity to *P. lutzii*, showing that this species may occur in southeastern Brazil. Therefore, the development of molecular techniques should not only detect *Paracoccidioides* spp. in soil, but also distinguish the isolate in relation to species [50].

## Conclusions

PCM results from infection by *Paracoccidioides* spp., and continues to cause significant morbidity and mortality, especially in some specific regions of the world. The diagnosis of PCM is classically achieved by a correlation of clinical, epidemiological, and laboratory data. Considerable advances have been made in non-culture-based diagnosis of this systemic mycosis with the development of a variety of methods for the detection of antibodies, antigens, and nucleic acids. The methods described for the diagnosis of PCM each have their strengths and weaknesses and require critical analysis by microbiologists and clinicians. However, not all of the tests described are universally available, which complicates the capacity to diagnose and treat individuals with PCM. In addition, the immunological status of the patient and manifestation of the disease influence the efficacy of the diagnostic test. Continuing efforts to improve or develop diagnostic tests will facilitate our diagnostic capacity. However, such assays will require validation in populations from diverse regions of the world prior to their general application in routine diagnosis. Results obtained from a panel of serologic diagnostic tests play an important role in the diagnosis of PCM. Nevertheless, nucleotide probes specific for the *Paracoccidioides* species complex, and DNA amplification procedures such as PCR, allow more rapid and precise diagnosis, which would lead to earlier treatment. However, the gold standard for diagnosis continues to be the culture, and the correlation between molecular data and phenotypic characteristics are crucial in identifying the etiological agents of PCM.

Molecular methodologies have also demonstrated several important biological questions related to speciation, mode of reproduction, and genetic population. The basic information obtained from these approaches has implications for the understanding of *Paracoccidioides* spp. in relation to their behavior and the development of pathogenic features, such as resistance to antimicrobials and virulence factors used for colonization of mammalian hosts. The knowledge obtained from these studies could also be used for the development of innovative diagnostic methods, as well as for novel therapeutic approaches and production of vaccines.

**Compliance with Ethics Guidelines**

**Conflict of Interest** Rosely Maria Zancope-Oliveira, Claudia Vera Pizzini, Mauro de Medeiros Muniz, Antonio Carlos Francesconi do Valle, and Rodrigo de Almeida-Paes declare that they have no conflict of interest

**Human and Animal Rights and Informed Consent** This article does not contain any studies with human or animal subjects performed by any of the authors.

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• Of importance
•• Of major importance

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