

# Serology of paracoccidioidomycosis

Zoilo Pires de Camargo

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**Abstract** This review provides the background for understanding the role of a battery of diagnostic methods in paracoccidioidomycosis (PCM). This systemic mycosis is a disease endemic in many regions of Latin America, with sporadic cases also occurring throughout the world (mycosis of importation). Although excellent laboratory methods for diagnosis are available, there are deficiencies that must be met by continued research. Understanding the uses and limitations of a battery of laboratory methods is essential to diagnose PCM. Clinicians and laboratory directors must be familiar with the uses and limitations of a battery of serologic and mycological tests to accurately diagnose of PCM. Antibody and antigen detections are valuable adjuncts to histopathology and culture. More recently, the gp43 and gp70 antigen detection assay have improved the methodology of diagnosis of this mycosis, which improves reproducibility and facilitates monitoring antigen clearance during antifungal treatment. Furthermore, detection of antigen in cerebrospinal fluid and in bronchoalveolar lavage fluid increases the sensitivity for diagnosis of PCM in central nervous system and in pulmonary infections, respectively.

**Keywords** Antigens · Mycosis · Paracoccidioidomycosis · *Paracoccidioides brasiliensis* · Serology

## Introduction

The fungus *Paracoccidioides brasiliensis* is ensconced in the nature in Latin America, but its exact niche is not known. The expanding human and non-human populations of endemic areas provide a continuing supply of individuals who are susceptible to infection with *P. brasiliensis*. Paracoccidioidomycosis (PCM) is caused by the inhalation of conidia found in nature and under adverse nutritional conditions, the fungus sporulates forming conidia which are less than 5 µm in diameter and can easily reach the alveoli when inhaled, and a lung-lymph node primary complex develops. 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 lymphohematogenic route.

Serologic tests have served for several decades as aid in the diagnosis and management of paracoccidioidomycosis. Among the serologic tests available for mycosis, those for paracoccidioidomycosis have been the most reliable and have been useful in human medicine.

The following serologic tests have been used for diagnosing PCM: tube precipitin, complement

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Z. P. de Camargo (✉)  
Department of Microbiology, Immunology and  
Parasitology, Cellular Biology Division, Federal  
University of São Paulo (UNIFESP), Rua Botucatu 862/8º  
andar, São Paulo, SP 04023-062, Brazil  
e-mail: zoilo@ecb.epm.br

fixation (CF), double immunodiffusion, counter-immunoelectrophoresis (CIE), immunofluorescence, radioimmunoassay, passive hemagglutination inhibition, immunoenzyme assays, such as ELISA, MELISA, Dot Blot, Western Blot, and more recently inhibition-ELISA.

### Antigen preparation

In 1916, Moses [1] was the first to use *P. brasiliensis* antigens for serologic testing, using a saline extract from the fungus, for successful testing in CF. Since this study, a number of different techniques for the use and preparation of antigens have been described. However, in 1955, Fava Netto [2] standardized a polysaccharide-rich *P. brasiliensis* antigen to use in CF tests. With this test and this kind of antigen, the serology for PCM achieved a 90% sensitivity, thus demonstrating the usefulness of the technique not only in diagnosis but also in monitoring the course of the disease.

Double immunodiffusion (ID), that involves agar or agarose gel support, is the most used test in immunodiagnosis of PCM. The preparation of culture filtrate antigens and their use for immunodiagnosis of PCM by ID were pioneered by Restrepo [3] and Negroni [4], who observed a level of sensitivity close to 90%. During many years their antigens were kindly supplied by them and used by almost all laboratories working in this area.

For several years, many different antigenic preparations have been used for the serodiagnosis of PCM by ID. These various antigens lacked a standardized preparation from one laboratory to another, and included sonic extracts from the yeast forms, concentrated filtrates, and lyophilized to cite just a few approaches. Moreover, each antigen was prepared from cells grown in different culture media and under different growth conditions (such as incubation time, growth temperature, size of initial inoculum, and shaken or stationary cultures). It is possible for different antigenic preparations to vary considerably in activity and quality as a result of lack of proper lot-to-lot production standards even within the same laboratory. With this variety of problems in producing a proper *P. brasiliensis* antigen, it is not surprising that there is considerable disagreement regarding the sensitivity of the ID test for the serodiagnosis of PCM [5–11].

In 1988, we reported that a diagnostically useful exocellular antigen preparation, Ag7, could reproducibly be produced from yeast forms of *P. brasiliensis* [12]. We suggested that this could be used as a standard method in ID tests. The method required, first, the determination of the number of viable cells in a 3-day old pre-inoculum, the transfer of this growth to Fernbach flask culture for 7 days, the pooling of six lots consisting of three flasks each in a total of 9.9 l medium, dialysis of this filtrate, concentration by vacuum evaporation, new dialysis, and finally lyophilization. The successful use of this Ag7 is based on the presence of abundant amounts of gp43, a 43,000-Da protein that is the immunodominant antigen of the system and that is considered specific for *P. brasiliensis* in ID tests. Since many steps are necessary to obtain the final antigen preparation, efforts were made to simplify the methodology in order to circumvent the material and economic difficulties found in most Latin American laboratories.

Since 1993, a simplified Ag7 antigen preparation technique as described here has been reproduced monthly in our laboratory with very good results. The fungus was initially grown on Sabouraud medium (SAB) slant tubes for 3 days at 35°C. At that time, growth (consisting entirely of yeast cells) was collected from at least 10 tubes, yielding an inoculum of approximately  $2 \times 10^6$  cells. These cells were inoculated into 500-ml Erlenmeyer flasks containing 100 ml yeast extract-peptone-dextrose (YPD) broth (Difco). This culture was incubated for 3 days at 35°C on a rotatory incubator at 50 r.p.m. The growth obtained was transferred to 1,800-ml Fernbach flasks containing 500 ml YPD broth. The flasks were further incubated as above for 7 days. Supernatant fluids were collected following paper filtration, concentrated under vacuum at 45°C, and dialyzed against distilled water. The presence of gp43 was monitored by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining. After this step, the antigen was tested by ID. If positive reactions were obtained with the control sera, the antigen was aliquoted and maintained at 5°C. If the reactions were negative, the antigen was concentrated again and retested. In general, concentration of the filtrate 10- to 15-fold is necessary.

This simplified protocol for Ag7 production has shown a high degree of sensitivity, specificity, and

reproducibility in the serodiagnosis of PCM. We believe this procedure can be performed by most laboratories, even those with relatively few resources. Figure 1 shows the antigenic profile of Ag7.

### Antibody detection

During the process of infection, the antigens from *P. brasiliensis* can activate B lymphocytes which main function is to produce immunoglobulins (Ig). These proteins play a crucial role in defense against a variety of infectious microorganisms; they are present in high concentrations in body fluids and in stable form. These properties led to their relative ease of detection and quantitation, providing major criteria for the presence and activity of the disease (serodiagnosis), and for monitoring both evolution of the disease and response to treatment.

In the management of a suspected PCM infection, no diagnostic test is superior to the isolation of the causative agent from a relevant clinical specimen, or to its unequivocal physical identification in clinical specimens and/or in histopathological setting of

tissue invasion. However, these ideal situations are not always possible, and so one must often employ diagnostic approaches that are based on serologic testing. In the case of PCM, serologic testing, such as immunodiffusion, is so reliable that a positive test, even if the titer is low, is indicative of infection.

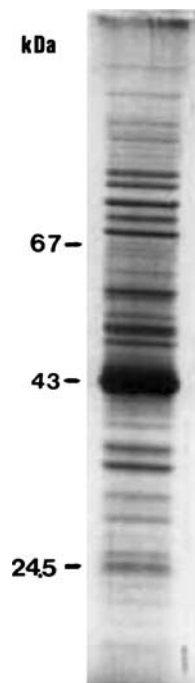
The last decades show a rapidly expanding field of immunology permitting the development of several new techniques, which were gradually being adopted by researchers interested in the diagnosis of fungal diseases, and several methods were currently being used as possible aids in diagnosing some mycotic infections, mainly PCM. Much of those serologic assays utilize polyclonal/monoclonal antibodies or antigens which can be easily quantified with high sensitivity and have resulted in methods with more sensitivity and specificity.

The successful application of serological tests for diagnosing mycotic infections is very important because the incidence and mortality of the mycoses, particularly those caused by opportunistic fungi have shown a marked increase in recent years, especially in patients whose immune defense mechanisms have been compromised by severe underlying diseases such as AIDS, cancer, and hematologic disorders. This emphasizes the utility of standardized techniques and antigens as diagnostic tools in the clinical and laboratory evaluation of patients with suspected fungal infections. Fungal antigens are of great interest not only for the study of their fundamental properties, but also because of their use as reagents for the diagnosis of fungal diseases.

The scientific literature on the immunology of the mycoses for the last years shows an increasing number of papers dealing with serological testing and different antigenic preparations. In the early 1980s, Restrepo [13] reviewed the extensive literature about the serodiagnosis of PCM, and in the early 1990s, Mendes-Giannini et al. [14] wrote an excellent chapter about the same subject. However, in the early 1970s, Fava Netto [15] and Negróni [16] had also documented the problems about antigens for PCM diagnosis.

As there is extensive and diverse literature about serology in PCM, in this article we will mainly discuss our laboratorial experience, and the most important advances in the PCM serology, in order to show the current knowledge about the immunodiagnosis of PCM.

**Fig. 1** SDS-PAGE showing the antigenic profile of a *P. brasiliensis* exoantigen used in immunodiffusion test. Gp43 is the predominant antigen

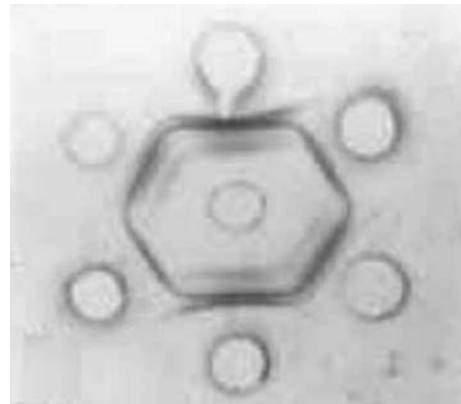


## Immunodiffusion

During the last years, immunodiffusion has been the test of choice for the initial diagnosis of suspected patients of PCM. The ID test has high specificity and sensitivity may vary from 65 to 100% depending on the kind of antigen used [14, 17]. Recently, the Ag7 was tested in ID by a multinational group of researchers, with the aim of providing regional laboratories, within the endemic areas, with an accurate yet simple test to promptly diagnose PCM [18]. The results showed that ID test was 84.3% sensitive and 98.9% specific, concluding that in those conditions the Ag7 can be regarded as an important diagnostic tool in PCM. However, in our laboratory false negative results have been occur in the order of 2–3%; these observations have also been noticed by others [19]. In these cases, the PCM patients have severe disease mainly with important pulmonary involvement. On the other hand, the diagnosis may be given by the finding of budding yeast cells in the sputum by direct examination with 30% KOH. It is our opinion that in these situations and in the moment of diagnosis, the immune system of the patient is depressed, and there is no sufficient antibodies to precipitate in ID test. The paracoccidioidin skin test is also negative. So the patient receives the initial treatment and after a period of 1–2 months, immunity is restored and ID test becomes positive (personal observations). Itano [20] has proposed that this problem may be due the presence of IgG asymmetric antibodies which possess a structure resulting from a predominant mannose-rich oligosaccharide moiety linked to the Fd part of only one of the Fab arms of the antibody molecule. As a consequence they are functionally univalent, non precipitating. On the other hand, Neves et al. [21] showed that the lack of reactivity of sera from PCM patients in ID tests may be related to the production of low-avidity IgG2 antibodies directed against carbohydrate epitopes. Figure 2 shows a typical ID test for diagnosis of PCM.

## Counter-immunoelectrophoresis (CIE)

Counter-immunoelectrophoresis is another test used to provide early diagnosis of PCM. Like in ID test, the antigens used varied from laboratory to



**Fig. 2** Immunodiffusion test for paracoccidioidomycosis. In the well center, Ag7 antigen; in the outer wells serum of PCM patients

laboratory, and they are extracts from sonicated yeast cell suspensions, exocellular antigens from yeast or mycelial cultures, cell-free antigens obtained from yeast cells, and cross-reactions are due to the use of unfractionated antigens. Counter-immunoelectrophoresis is regarded as having a sensitivity equal to or slightly greater than that provided by ID [22]. Some authors related sensitivity between 77% and 97% and specificity at least 95%. Some laboratories suggest CIE as the first routine screening of sera from suspected PCM patients, because the speed in which results can be obtained. In our experience, the time results in CIE is practically the same as in ID test. After electrophoresis, the glass slide need to be washed for 24 h, dried for further 24 h, and stained. This is the same time necessary for ID test. On the other hand, in CIE, it is necessary to use buffered agarose, buffer and a power supply. These conditions are not always accessible for laboratories in endemic regions of PCM, in underdeveloped countries.

## ELISA

Solid-phase immunoassays, especially enzyme immunoassays (ELISA), are popular for the quantitation of antibodies both in research and clinical medicine. Enzyme immunoassays employ antibodies or antigens conjugated to enzymes in such a way that the immunological and enzymatic moiety are preserved. These assays give reliable and reproducible results and are extremely sensitive. The basic principle of the

method is the use of detector antibody reagents bound to an enzyme indicator. The detector antibody is chosen to react specifically with an antigen or antibody component of interest. The amount of detector bound to an antigen or antibody can then be estimated by quantifying the enzyme activity that remains associated with the system after the unbound reagent is washed off. Since a single enzyme molecule can catalyze the conversion of millions of substrate molecules, the “signal” of bound detector molecules is therefore greatly amplified, giving the ELISA system a very high sensitivity. ELISA is relatively simple to perform, gives quantitative results, is highly sensitive and the reagents are long-lived, can be safely shipped and conveniently stored. Furthermore, different classes of immunoglobulins can be determined thus allowing a more precise evaluation of the humoral immune response.

Pons et al. [23] were the first to introduce ELISA for detection of anti-*P. brasiliensis* antibodies. ELISA has since been the basis of many other publications for detection of serum antibodies to *P. brasiliensis* [24–27], and has been used with a range of different antigens, since crude mixtures, partially purified and purified molecule as gp43, and generally have high sensitivity, but not necessarily been coupled with high specificity. Mendes-Giannini et al. [24] and Camargo et al. [25] give a good account to the development of the methodology of ELISA for anti-*P. brasiliensis* antibodies. Mendes-Giannini et al. [24] standardized ELISA using a *P. brasiliensis* yeast filtrate as an antigen, and found 100% sensitivity and 88% specificity, considering a cutoff of 1:40. However, cross-reactions were observed with sera from patients with histoplasmosis and Jorge Lobo’s disease. In order to overcome this cross-reactivity, the authors standardized an ELISA-abs test, in which the serum sample is first absorbed with *Histoplasma capsulatum* yeast cells and also with a mycelial filtrate. Camargo et al. [25] when evaluating the serological response of PCM patients’ sera by ELISA, and using *P. brasiliensis* yeast filtrate verified that the previous absorption of the sera with dead cells of *Candida albicans* was necessary to eliminate the cross-reactivity with heterologous sera. The level of sensitivity and specificity achieved with ELISA was 95% and 93.4%, respectively, considering a cutoff titer of 1:400. These filtrates are constituted mainly by the glycoprotein gp43, the

immunodominant antigen. This molecule has also been tested in ELISA, since when it was known as the E<sub>2</sub> fraction [14]. Mendes-Giannini [26] verified that E<sub>2</sub> (gp43) antigen did not cross-react, in ELISA, with histoplasmosis, aspergillosis, Jorge Lobo’s disease, sporotrichosis, tuberculosis, leishmaniasis sera when previously adsorbed with a filtrate of *H. capsulatum*. Puccia and Travassos [27] also tested gp43 in ELISA and immunoprecipitation with <sup>125</sup>I-labeled antigen, and verified that most of the PCM sera recognized primarily peptide epitopes of the gp43; however some PCM sera were able to recognize carbohydrate epitopes and accounted for up to 45% of the total reactivity. 53% of the histoplasmosis sera reacted within the same range of the PCM sera. Camargo et al. [28], testing gp43 in antibody capture ELISA, were able to discriminate among PCM and heterologous sera (histoplasmosis, Jorge Lobo’s disease, candidiasis, aspergillosis). In this study, the minimal titer obtained with PCM sera was 1:51,200 and the maximal titer was 1:819,200; the arithmetical mean titer was 1:549,093. Only one serum from Jorge Lobo’s disease and one serum from aspergillosis had maximal titer of 1:51,200. The sensitivity of the capture ELISA was 100% and the specificity was 96.7%, considering the end point of reactivity. However, if the analysis is made according to the O.D., e.g., at the 1:800, 1:1,600, 1:3,200, etc., serum dilutions, there occur enormous differences among homologous and heterologous sera. At these dilutions, the capture ELISA is 100% specific. In the same study [28], gp43 was also analyzed by conventional ELISA, with PCM and heterologous sera, showing that heterologous sera reacted in the range of PCM sera, mainly histoplasmosis and Jorge Lobo’s disease sera. These contradictory results can be explained by how gp43 is presented to be recognized by antibodies in different tests. When gp43 is fixed onto solid substrates, as in ELISA plates, made of plastic, there are conformational changes in the molecule, that cause the exposition of carbohydrate epitopes recognized by heterologous sera; when the molecule is in solution, as in capture ELISA (gp43 is fixed on MAb anti-gp43), gp43 assumes different conformation, presenting epitopes more reactive with specific antibodies, those directed against protein epitopes. Deglycosylation of gp43 frequently abolishes the reaction of the heterologous sera with this antigen [29]. The antibody capture ELISA represents



an advance on the detection of specific antibodies, once it uses specific monoclonal antibody against gp43, which is fixed on the plates; however the users depend on this MAb. Classical ELISA is really an excellent format for the detection of antibody response in PCM for many laboratories. As seen in various reports, gp43 is not a totally specific molecule, since its carbohydrate composition contains epitopes recognized by heterologous sera, mainly by histoplasmosis. However, one can perfectly separate the heterologous sera, mainly histoplasmosis sera, by the OD differences in each serum dilution. The gp43 may be considered a specific diagnostic antigen for PCM, provided the presentation in classical ELISA when the molecule is fixed directly on the plastic, or in capture ELISA when it is fixed on the Mab anti-gp43, and also how we interpret the results, considering the end point of reactivity or the OD in each serum dilution.

### Immunoblotting

Immunoblotting is a procedure in which antigens separated by polyacrylamide gel electrophoresis are transferred (blotted) to nitrocellulose sheets, allowed to react with antisera, then sites where antibodies have bound to antigens are revealed by means of enzyme detector reagent and specific substrate. The procedure has been used to identify *P. brasiliensis* antigens that recognize antibodies in patient's sera [30–33].

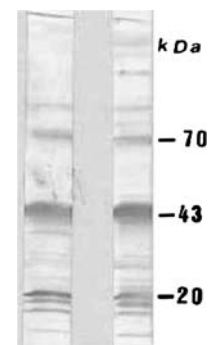
Camargo et al. [30], using a *P. brasiliensis* yeast exoantigen, standardized the Western blot for diagnosis and follow-up of PCM patients. Anti-*P. brasiliensis* IgG reacted with four major components of 70, 52, 43, and 20–21 kDa. The 43-kDa glycoprotein was the predominant IgG reactive antigen, recognized by 100% of the patient's sera; the 70-kDa antigen was recognized by 96% of the PCM sera. Cross-reactivity could be minimized by serum dilution. In this study the authors considered gp43 and gp70 as marker for human PCM. The authors studied 25 sera from patients prior antimycotic therapy and 72 sera of patients under therapy; the later patients presented an decrease of antibody titers against gp43 and gp70. In other study, Blotta and Camargo [33] studying 60 PCM patients' sera (30 acute form and 30 chronic form) found that gp43 was recognized by

100% of sera (IgG), corroborating previous findings. Mendes-Giannini et al. [32] studied the Western blotting assay based the profile showed by sera from patients with the distinct forms of PCM, both before and after therapy. The majority of the sera recognized gp43, but other molecules were also recognized. Although Western blotting is highly sensitive and an clear assay, it is not accessible for all laboratories in the PCM endemic regions of Latin America. Figure 3 shows a typical PCM immunoblot reaction. However, Taborda and Camargo [34], using an other format, a dot immunoassay, could provide a more user-friendly means of antigen detection (gp43) for PCM diagnosis. In this circumstance, they treated gp43 with sodium metaperiodate to abolish cross-reactivity with histoplasmosis sera, obtaining specific reactions.

### Antigen detection

In the past some investigators have tried to detect circulating antigen in PCM patients using polyvalent antigens or antibodies in different assays such as competition enzyme-linked immunosorbent assay (ELISA) [35] immunoradiometric assay [36], CIE [37], immunoelectrophoresis-immunodiffusion [38], passive hemagglutination inhibition [39], inverted linear immunoelectrophoresis [40], and immunoblotting [41]; but all of these assays presented low sensitivities. Gómez et al. [42] were the first to use monoclonal antibodies (Mabs) to detect the 87-kDa circulating antigen in PCM patients by inhibition ELISA (inh-ELISA), which showed 80.4% sensitivity. More recently, in our laboratory, the same technique was used to detect gp43 and gp70 in different specimens of PCM patients [43, 44].

**Fig. 3** Representative immunoblot of PCM patient's sera, showing the main reactive molecules



### Detection of antigen in serum (gp43 and gp70)

One of the main problems in the serodiagnosis of PCM based on antibody detection is the cross-reaction obtained with sera from patients with other mycoses, mainly histoplasmosis and lobomycosis. Tests based on ID are generally considered to have high specificity in the diagnosis of PCM, but lack in sensitivity. Estimates of the sensitivity of immunodiffusion-based tests vary from 65% to 100% [17] depending of the kind of antigen preparation used. On the other hand, tests with higher sensitivity, such as immunoenzymatic assays, present problems associated with specificity due to cross-reactivity with heterologous sera, i.e., patients with non-PCM fungal infections.

A more rational approach to the diagnosis of PCM may be the detection of *P. brasiliensis*-gp43 antigen in body fluids. The gp43 is a *P. brasiliensis* yeast-phase extracellular antigen and its detection in patients with proven PCM has been tested previously by many approaches. However, these tests varied in sensitivity and specificity, and in most of the studies only a small number of patients were analyzed.

Mendes-Giannini et al. [41] detected the gp43 antigen in pools of PCM patients' sera by Western blot assay, but no quantitation was attempted. Freitas-da-Silva and Roque-Barreira [35], using competitive ELISA, detected circulating antigens in 33.7% of 88 PCM patients' sera, whose antigen levels varied from 0.03 to 3.4 µg/ml. However, cross-reactions were obtained with heterologous sera, such as sera from patients with aspergillosis, cryptococcosis, and histoplasmosis.

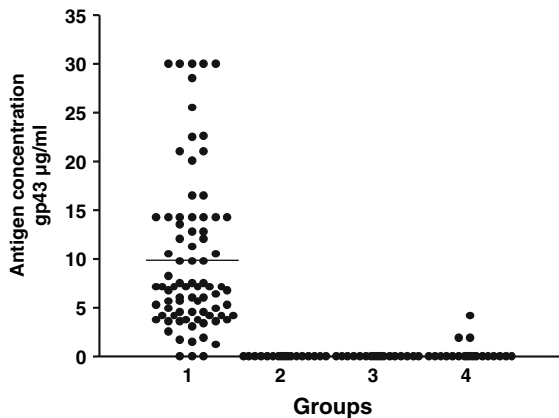
The most refined study of PCM antigenemia was conducted by Gómez et al. [42] using monoclonal antibodies directed against an 87-kDa antigen of Pb (gp87) in an inh-ELISA and detected the presence of antigen in cases of PCM. In this study antigen concentrations as low as 0.0058 µg/ml of serum could be detected in sera from 46 PCM patients with PCM, showing 80.4% sensitivity. Moreover, considering sera of patients with the acute form of the disease, the inh-ELISA exhibited 100% sensitivity, with the highest antigenemia (mean, 16.25 µg/ml), while 83.6% of the patients with the multifocal chronic form of PCM showed a mean antigenemia of 14.92 µg/ml and 60% of patients with the chronic unifocal form showed a mean antigen concentration

of 14.7 µg/ml. Cross-reactions with aspergillosis and histoplasmosis were also observed. Also, the authors observed cross-reactions with sera from patients with tuberculosis. They assumed that coinfection with tuberculosis and PCM was a possible explanation for these cross-reactions.

The inh-ELISA was tested in our laboratory and it was able to detect gp43 antigen in the concentration ranging from 0.0053 to 30 µg/ml of serum. The overall sensitivity of the test among 81 PCM patients was 95.1%, with a higher sensitivity in patients with the acute form of PCM (100%) with a mean antigen concentration of 18.23 µg/ml. Among the PCM patients with the chronic form, gp43 was detected in 95.71%, with mean concentration of 8.55 µg/ml. Our results showed that the gp43 detection assay can be used for both clinical forms of the disease. Consequently, the gp43 antigen detection test may be a valuable tool in PCM diagnosis. Gp43 detection by this inh-ELISA showed 95.1% sensitivity which was higher than the 80.4% sensitivity previously reported by Gómez et al. [42] to detect the 87-kDa antigen, and also much higher than the 33.7% sensitivity previously described by Freitas-da-Silva and Roque-Barreira [35]. The use of the 43-kDa antigen detection system provide a rapid, sensitive, and specific mean to diagnose and differentiate between acute and chronic disease. Moreover, this test system's true potential may lie in tracking the course of antimycotic therapy by monitoring the reduction in fungal load as evidenced by a sequential reduction in antigen detected [43]. Figure 4 shows the results of gp43 detection in PCM patient's sera, and in control groups.

Detection of low amounts of gp43 in some normal serum controls may represents PCM subclinical infection in normal people. PCM subclinical infection is defined as an asymptomatic infection caused by *P. brasiliensis* in normal individuals who live in an endemic area and prove reactive to the paracoccidiodin skin test [45]. Positive paracoccidiodin skin testing in health population may vary from 3.70% to 62.60%, depending on the kind of antigenic preparation [46]. In our previous study, using gp43 or a polysaccharide antigen (Fava Netto's antigen) as paracoccidiodin, 5% of healthy people reacted positively to both antigens [47].

Another special antigen searched in biological fluids of patients with PCM is the 70 kDa molecule.



**Fig. 4** Detection of circulating antigen (gp43) in sera from patients with PCM or with other mycoses and in normal human sera by inh-ELISA. Groups studied: 1, PCM; 2, cryptococcosis; 3, histoplasmosis; 4, normal human sera

In this sense, the overall sensitivity of gp70 detection among 81 PCM patients was 98.8% in the presence of a mean antigen concentration of 8.19 µg/ml and reached 100% in patients with acute form of the disease, with a mean antigen concentration of 11.86 µg/ml. Among patients with chronic form of PCM, antigenemia due to gp70 was observed in 98.43% of serum samples from patients with the multifocal form (mean antigen level, 7.87 µg/ml); on the other hand, the sera of 100% of the patients with the chronic unifocal form had gp70, with a mean antigen concentration of 4.83 µg/ml. No cross-reactions were observed with sera from patients with other mycosis [44].

#### Detection of antigen in the cerebrospinal fluid

The involvement of the central nervous system (CNS) in PCM, neuroparacoccidioidomycosis (neuroPCM), is secondary to the hematogenous dissemination of the fungus. NeuroPCM usually occurs as a manifestation of widely disseminated disease but, occasionally, CNS may be the sole location of the infection. Whenever the CNS is involved, neuro-radiological methods such as computerized tomography (CT) and magnetic resonance imaging (MRI) are needed for the identification of expansive lesions. NeuroPCM is usually represented by multiple or solitary parenchymal lesions that lead to sensory or motor deficits, seizures, changes in mental status,

and intracranial hypertension [48, 49]. However, the definitive diagnosis of NeuroPCM may only be obtained after visualization of the fungus in biopsy material, fungal isolation by culture or by serological methods. Considering the morbidity associated with the invasive neurological procedures, clinicians are not prone to indicate aspiration or biopsy of CNS lesions. Therefore, sensitive and specific immunodiagnostic assays to determine the presence of *P. brasiliensis* in cerebrospinal fluid (CSF) specimens are indispensable.

Marques da Silva et al. [50] detected circulating gp43 and gp70 in all CSF samples from patients with NeuroPCM, with a mean antigen concentration of 19.3 and 6.8 µg/ml, respectively. Gp43 and gp70 were also detected in 10 (91%) serum samples out of 11 samples obtained among those 14 neuroPCM patients, with a mean antigen concentration of 4.6 and 4.0 µg/ml, respectively. Moreover, the antigen concentrations were found considerably higher in CSF than in sera, suggesting their local production, and the concentration of gp43 was always higher in relation to gp70 (19.3 vs. 6.8;  $P < 0.001$ ). These results suggest that monitoring specific antigens of *P. brasiliensis* may be helpful to define the diagnosis of NeuroPCM. On the other hand, antibody detection in CFS was negative when tested by ID but was positive against both antigens when tested by ELISA, despite at low titers. This data, from the limited number of patients studied, allow us to assume that the detection of antigen by inh-ELISA or the detection of specific antibody by conventional ELISA may be equally sensitive to identify NeuroPCM. Since antigen values for gp43 were always higher than those found for gp70, assaying only for gp43 may prove sufficient for that purpose.

#### Detection of antigen in bronchoalveolar lavage fluid

Also, the inh-ELISA was applied to detect gp43 and gp70 antigens in bronchoalveolar lavage fluids (BAL) [51]. In this purpose, PCM patients furnished BAL samples and gp43 and gp70 were detected in all samples with an average concentration of 9.38 and 4.37 µg/ml, respectively. BAL fluid samples from individuals with diseases other than PCM gave negative results. These results suggest that



monitoring specific antigens of *P. brasiliensis* in BAL samples may be helpful in determining a diagnosis of pulmonary PCM, particularly when the infection is in its initial stage. Since antigen values for gp43 were always higher than those found for gp70, assaying only for gp43 may prove sufficient for that purpose. No antibodies were detected in BAL fluids when tested by ID, whereas antibodies against both antigens were detected by ELISA, although the titers were low. These results point out that *P. brasiliensis* antigen detection in BAL fluid is a valuable tool for diagnosis of pulmonary PCM. Nevertheless, the limited clinical and radiological information available for the small number of patients enrolled in that study proscribed the possibility of establishing a correlation between antigen levels and the severity of the disease.

#### Detection of antigen in urine

Nowadays, few reports about the detection of *P. brasiliensis* in urine were related. The 43-kDa *P. brasiliensis* antigen has already been detected in the urine of patients with PCM, and this molecule was demonstrated earlier in urine of a patient with acute PCM [14]. Gómez et al. [42], using an inh-ELISA, reported the presence of an 87-kDa *P. brasiliensis* antigen in urine samples with low sensitivity in both reports. Salina et al. [52] used immunoblotting and competition enzyme immunoassay techniques for the detection of circulating antigen in urine samples. Both tests were specific, and gp43 remained present in the urine samples collected during the treatment period, with a significant decrease in reactivity in samples collected during clinical recovery and increased reactivity in samples collected during relapses.

In our laboratory, Marques da Silva [53] employing the inh-ELISA, reported the detection of gp43 (mean = 8.65 µg/ml) and gp70 (mean = 8.96 µg/ml) in the urine of PCM patients with 87.5% sensitivity and 100% specificity to both molecules. No cross-reaction was obtained when urine from patients with other mycosis (histoplasmosis, candidiasis, and cryptococcosis) besides normal human urine were tested. In this study, urine samples were taken before antifungal treatment. The inh-ELISA used in our laboratory allowed the detection of antigen

concentrations in urine ranged 0.5–25 µg/ml. Despite the high sensitivity, the presence of gp43 and gp70 antigens could not be detected in 12.5% of the urine samples tested.

#### Antigenemia during therapy

There is no consensus about the optimal duration of antifungal treatment for patients with PCM, but several publications have advocated that detection of circulating specific antibodies is an important parameter to define the time to stop antifungal treatment. However, PCM patients have a strong specific antibody response against gp43 that may persist throughout life. In contrast, some patients, depending on their immune status, are serologically negative at the time of diagnosis, with others showing low levels of specific antibodies for long periods of time. Consequently, sometimes it is very difficult to determine whether these patients are ever cured [54].

One of the main challenges in PCM is to establish when the clinician should stop the antimycotic therapy because there is no consensus about treatment options or about when the patient is really cured. Although healing of apparent lesions may occur within a short time after treatment is initiated, long therapeutic courses are desirable in order to prevent relapses. In this case, the host response must be monitored by indirect methods and, in this scenario, serology provides information about prognosis.

Due to these limitations of the antibody detection tests, some researchers have evaluated the performance of assays for the detection of circulating *P. brasiliensis* gp43 antigen for the diagnosis of PCM. Some tests have been used to detect gp43 circulating antigen with partial success in PCM [24, 35, 37–42].

Related to the detection of antigen during treatment, Mendes-Giannini et al. [41], by means of Western blot assay, and testing a pool of PCM sera, observed that gp43 started to disappear from the circulation after 10 months of chemotherapy and was undetectable after 2 years of treatment. No quantitative measurements were made in individual sera. More recently, the same group [52] determined the presence of *P. brasiliensis* antigens in the urine of patients by an indirect competition enzyme immunoassay (EIA-c) and an immunoblot test for monitoring

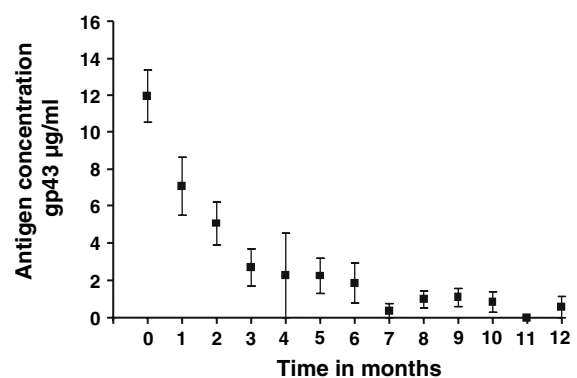
the response to therapy. By means of EIA-c, the presence of *P. brasiliensis* antigens could be detected in 75% of the urine samples tested.

Recently, we have studied the behavior of gp43 antigenemia during the treatment of PCM patients with Itraconazole (ITZ) until the apparent cure at the end of therapy (i.e., after 8–12 months) [55]. PCM is a chronic fungal infection that chiefly involves the reticulo-endothelial system (RES), as well as the lungs. The definition of cure or good clinical response is sometimes difficult to establish considering the low sensitivity of cultures, the lung sequels, and the limitations related to the evaluation of the infectious status at the RES. As a consequence, there is a consensus that nonculture methods are useful tool to help the clinician to better evaluate the therapy response. In this study, we evaluated the antigen levels of 23 PCM patients that were successfully treated with ITZ. The definition of a successful response to the treatment was based on the resolution of all signs and symptoms related to the infection, as well as improvement in the radiological findings. It is important to highlight that during the 12-month period of clinical follow-up, no single patient had any clinical or radiological evidence of relapse. Although we were not able to evaluate the behavior of the antigen titles during a relapse episode of the fungal infection, clinical improvement of all patients was followed by a decrease of the antigen levels to  $<5 \mu\text{g/ml}$ . This finding strongly suggests that gp43 antigenemia may be a useful tool for monitoring the therapeutic response to antifungal treatment.

In this study population, decreasing levels of *P. brasiliensis* gp43 were detected in patients during ITZ therapy. Of the 23 patients studied, 2 had antigenemia of  $<5 \mu\text{g/ml}$  after 2 months of treatment, 15 patients had antigenemia of  $<5 \mu\text{g/ml}$  after 3 months, and 5 patients had antigenemia of  $<5 \mu\text{g/ml}$  after 6 months of treatment. Only 4 patients presented negative levels of antigenemia at month 8. It was noted that, once decreased, the levels of gp43 in serum remained low until the end of therapy. On the other hand, in most cases, the antibody titers seemed to decrease a little later than this, i.e., after about 6 months of therapy. Some cases deserve particular attention. For example, one of the patients showed a decreased serum antigenemia after 8 months of treatment, but his anti-gp43 antibody titers remained high (1:16) and did not change

throughout the period of treatment. In contrast, another patient had undetectable antibodies (as determined by the ID test) from the time of diagnosis to month 11 of treatment but showed a substantial decrease in antigenemia levels during treatment. In conclusion, our results indicate that the detection and quantitation of the immunodominant 43-kDa *P. brasiliensis* antigen in sera by inh-ELISA is a sensitive method to be used for monitoring patients with PCM under treatment. Figure 5 shows the media of circulating antigen concentrations in sera from patients with PCM at the time of diagnosis and during treatment. Table 1 shows the characteristics of 23 PCM patients treated with ITZ during the period of treatment.

With respect to the follow-up of PCM patients based on gp70 antigenemia, Marques da Silva et al. [44] assessed the gp70-antigen clearance during antifungal treatment of PCM with ITZ, comparing serum samples collected at baseline and after 8–12 months of therapy. All 23 patients selected for the study showed a good clinical response to therapy, with no relapse of fungal disease documented over the follow-up period. Overall, decreasing levels of *P. brasiliensis* gp70 were detected in patients during successful antifungal therapy. In contrast, the ID titers of these patients varied over the follow-up period (1:16–1:2). At the time of diagnosis all patients showed detectable gp70 antigen and anti-*P. brasiliensis* antibodies (except for one patient with negative ID). A significant decrease of gp70 occurred during the first month of therapy in 22 patients; at the end of therapy, only 4 (17.39%) patients had



**Fig. 5** Media of circulating antigen concentrations in sera from patients with PCM at the time of diagnosis and during treatment. Error bars indicate the standard deviations

**Table 1** Characteristics of 23 PCM patients treated with ITZ in relation to antigen levels

Patient no. and local of lesions <sup>a</sup>	Age (year)	No. of samples tested	Time of follow-up (months)	Antigen level (µg/ml) at:		
				Diagnosis	First follow-up	End of treatment
1 (L, M, G)	67	8	11	10.5	8.25	0
2 (L, S, G)	40	7	9	12.75	4.36	0
3 (L, M, G)	51	7	9	7.12	2.85	0
4 (L, Of, G)	56	9	12	30.0	30.0	2.31
5 (L, S, M, G)	31	7	11	14.25	4.77	0
6 (L, M, G)	44	7	9	11.25	2.49	0
7 (L, G, Lx)	44	6	8	14.25	3.75	3.57
8 (L, M, G)	42	7	9	13.5	28.5	2.57
9 (L, M, G)	49	7	11	7.12	4.27	0
10 (L, M)	44	7	9	14.25	12.0	0
11 (L, M, G)	52	7	10	14.25	3.07	3.21
12 (L, M, G)	50	6	9	3.57	3.57	0
13 (L, M)	49	6	9	10.5	6.38	0
14 (L, M)	37	5	9	9.75	5.25	0
15 (L, M)	56	6	8	6.38	6.01	0
16 (L, M, G, Lx)	49	6	9	28.5	4.90	4.53
17 (L, M)	66	8	10	2.49	3.75	0
18 (L, M)	51	7	10	5.64	1.56	0
19 (L, Lx)	40	7	10	14.25	5.64	0
20 (L, M)	37	8	10	16.5	6.0	0
21 (L, CNS)	37	6	12	14.25	9.75	0
22 (L, M)	42	8	12	3.39	2.49	0
23 (L, M, G)	54	7	12	9.75	2.13	0

<sup>a</sup> L, lung; M, mucosa; G, ganglion; Lx, larynx; Of, oropharynx; S, skin; CNS, central nervous system

detectable gp70 antigen (antigen levels were <2.5 µg/ml) and positive titers by ID. By the end of the follow-up period, antibody titers were negative in three patients and one had undetectable antibodies (ID test) from the time of diagnosis to the end of treatment. However, the level of the antigen was also negative until the end of follow-up. Once decreased, the levels of gp70 in serum persisted low until the end of therapy. On the other hand, in various cases, antibody titers seemed to decrease a little later, after about 6 months of therapy. After the period of antifungal therapy (8–12 months) all PCM patients showed clinical improvement. In conclusion, our results indicate that the detection and quantification of *P. brasiliensis* gp70-antigen in serum by inh-ELISA is a sensitive method for the diagnosis of PCM and may be used to evaluate the clearance of fungal burden during treatment.

### Follow-up of PCM patients based on antibody detection

There is no consensus among the different groups about the “gold standard” method (based on antibody detection) for the follow-up of PCM patients. Some authors attempted to demonstrate that there was an effective relationship between the clinical and serological characteristics during the evolution of PCM [56, 57]. Campos et al. [56] compared various serological tests, such as CF, ID, CIE, and Magnetic-ELISA (MELISA) to find out the most effective tool for monitoring patients under antimycotic therapy. In their study, using semestral serology data, they showed that the clinical maintenance correlated with CIE and CF positive results; clinical remission was better determined when ID and MELISA furnished

negative results; also, they recommended the use of ID and MELISA for a more accurate correlation with clinical evolution.

Our experience with the follow-up of patients under treatment for PCM has shown that many times the antibody titers obtained by the ID test do not correlate with the clinical status of the patient. For example, in some cases elevated antibody titers (1:64) were observed until the end of treatment when the patients were clinically cured. On the other hand, although for most patients low antibody titers are related to the absence of clinical symptoms (1:2 or 1:4), in some cases low titers are present for months and in concert with clinical symptoms. Such discrepancy between the clinical status of the host and specific antibody detection is probably related to the fact that the cellular response and not the humoral response is the main immunologic mechanism able to contain *P. brasiliensis* in the infected organism. For these reasons, the use of serology as a single criterion of cure in PCM is controversial. In clinical practice, clinical, radiological, mycological and serological aspects must be evaluated over a long period of observation in order to assess the result of treatment.

Recently, in our laboratory, Alves [58] compared three methods (ID, ELISA, and capture-ELISA) in order to verify which one was better for the follow-up of PCM patients. In that study, serum samples were tested during the pretreatment period and after 6, 12, and 18 months of treatment. Immunodiffusion and capture-ELISA showed a significant decrease in titer after 6 months in good responder patients. ELISA detected decreasing titers only after 12 months of therapy. None of the methods were able to predict relapse of the disease prior to clinical observation, and increased titers could be observed only later, after the onset of relapse. Even with these limitations, that study indicated that the ID test seemed to be the best option for the follow-up of PCM patients compared to the other two tests.

For diagnostic purposes, tests based on ID are generally considered to have high specificity; however, false negative results may also occur. On the other hand, tests with higher sensitivity, such as immunoenzymatic assays, present problems associated with specificity due to cross-reactivity with heterologous sera.

## Conclusions

Our knowledge on the serology of paracoccidioidomycosis is almost ideal for diagnosing and monitoring the disease. Diagnostic approaches to PCM is well described and tests are reasonably accurate, and recognized deficiencies justify ongoing research. The laboratories in Latin America need to improve their conditions in order to offer a battery of tests for diagnosing and monitoring PCM patients.

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