

## Case Report: Misleading Serological Diagnosis of Paracoccidioidomycosis in a Young Patient with the Acute Form Disease: *Paracoccidioides brasiliensis* or *Paracoccidioides lutzii*?

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**Abstract.** Negative results in serological routine screening of patients with microbiologically proven Paracoccidioidomycosis (PCM) are occasionally reported. Failure in detecting anti-*Paracoccidioides* antibodies has been ascribed to factors either related to serological techniques or to the status of the host immune reactivity. Recently, this issue has been renewed by the recognition that the *Paracoccidioides* genera comprises two species, *lutzii* and *brasiliensis*, which have distinct antigenic profiles and, therefore, may elicit different host antibody responses. We describe a patient with the acute form PCM due to *Paracoccidioides brasiliensis* with negative results on two reference centers' routine screening for *P. brasiliensis* antibodies, but positive results with *Paracoccidioides lutzii* antigens. The present case report suggests that antibodies elicited during *P. brasiliensis* infection recognize antigenic fractions shared by both species, highlighting the difficulties in distinguishing the two infections by means of the currently available routine serological assays.

### INTRODUCTION

Gold standard diagnosis of Paracoccidioidomycosis (PCM) is based on the observation of the characteristic yeast cells in patients' specimens (sputum, biopsy specimens, or scrapings of lesions) or cultures. However, as these specimens are not always readily available, requiring invasive procedures, serological assays are considered an important tool and have been widely adopted for PCM diagnosis and for patient follow-up.<sup>1</sup> On the other hand, patients with microbiologically proven PCM can occasionally present negative results on routine serological screening.<sup>2–5</sup> Several factors were described that could account for the failure in detecting anti-*Paracoccidioides* antibodies in these cases. Immunoprecipitation assays such as double immunodiffusion (DID) or counterimmunoelectrophoresis (CIE), which are low-cost techniques most commonly used in reference centers, have been reported to exhibit limited sensitivity, missing the diagnosis in more than 10% of cases.<sup>3,6,7</sup>

Lack of standardization may contribute to this limitation; most reference centers use in-house techniques with different antigen preparations and lack external controls, resulting in significant interlaboratory variability.<sup>1</sup> Another reason for false-negative results is the low anti-*Paracoccidioides* antibody levels generated by some patients, particularly those with a very localized chronic form disease.<sup>7,8</sup> Other potential causes for false-negative reactions are the prozone effect,<sup>3</sup> immune complex formation,<sup>3</sup> impaired humoral immunity<sup>9,10</sup> and production of low-avidity immunoglobulin (Ig) G2 antibodies directed against carbohydrate epitopes by patients with the chronic pulmonary form of the disease.<sup>4</sup>

Recently, this issue has been renewed by the recognition that the *Paracoccidioides* genera comprises two species, *brasiliensis* and *lutzii*, which have distinct antigenic profiles and, therefore, may elicit different host's antibody responses.<sup>11</sup> However, serological diagnosis of PCM due to *Paracoccidioides lutzii* has not yet been standardized in clinical practice.

We describe a boy with the acute form PCM (AF PCM) due to *Paracoccidioides brasiliensis* with negative results by two reference centers' routine screening for *P. brasiliensis* antibodies, but positive results with *P. lutzii* antigens, highlighting the difficulty in identifying the infecting species using serological methods.

### CASE REPORT

In February 2015, a previously healthy 16-year-old boy presented with a 4-month history of fever; cervical, axillary, and inguinal lymph node enlargements; asthenia; and a 10% weight loss. Physical examination disclosed generalized lymph node enlargement. Laboratory tests showed anemia (hemoglobin: 8.9 g/dL), leukocytosis (20,900 cells/mm<sup>3</sup>), and mild eosinophilia (418 cells/mm<sup>3</sup>), typical of the AF PCM. A Thoracic computed tomography (CT) scan showed normal pulmonary parenchyma but lymph node enlargements in several chains. An abdominal CT scan also revealed severe lymph node enlargement resulting in conglomerates with heterogenous enhancement delimiting central areas of necrosis. Histopathology of a cervical lymph node biopsy showed a granulomatous process with caseous necrosis and multiple budding yeast cells characteristic of *Paracoccidioides* spp. (Figure 1). Unfortunately, no microbiological cultures of the biopsy were done. The patient was treated for severe AF PCM with amphotericin B initially (21 days), followed by itraconazole with marked clinical improvement. The patient was still on itraconazole therapy on his last outpatient visit and was doing well.

However, serological diagnosis of the patient done in parallel was inconclusive. Routine anti-*P. brasiliensis* serology testing carried out at the Immunology Center of the Institute Adolfo Lutz, the reference laboratory of the Health Department of the State of São Paulo, before starting antifungal treatment was negative. This test was repeated with a new serum sample collected 2 weeks later and confirmed the lack of reactivity against Pb113-exoantigen (AgPb113); however, the DID carried out at the same time with cell-free antigens from *P. lutzii*-208 (AgPI208),<sup>11</sup> was strongly positive (up to 1:256 dilution) (Figure 2). The patient had no epidemiologic history

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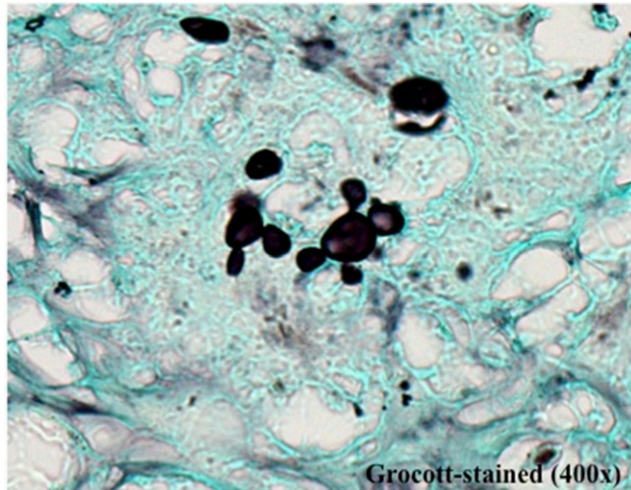


FIGURE 1. Methamine silver staining of a section of a cervical lymph node biopsy showing multiple budding yeast cells characteristic of *Paracoccidioides* spp. This figure appears in color at [www.ajtmh.org](http://www.ajtmh.org).

suggestive of *P. lutzii* infection; he was born and lived in a rural area close to São Paulo city (Itapeverica da Serra) except for the last year when he moved to the city of São Paulo. He had traveled only once in his life, to Saquarema, a nonendemic city in the state of Rio de Janeiro.

With these conflicting results, additional serological assays were performed at the Institute Adolfo Lutz laboratory and at another reference center, the Medical Mycology Laboratory of the University of São Paulo, to clarify whether the PCM was due to *P. brasiliensis* or *P. lutzii*. First, immunoblotting (IB) of all three serum samples using the Pb113-exoantigen (AgPb113) showed the presence of anti-gp43 and anti-70 kDa antibodies, as expected for *P. brasiliensis*-infected patients (Figure 3A).<sup>12-14</sup> Two additional exoantigens were used, from *P. brasiliensis*339 (AgPb339) and from *P. lutzii*66 (AgPl66) for assaying immunoprecipitating antibodies using CIE. Consistent with the previous DID assays, the patient's sample #2 was negative with AgPb339 but strongly positive (1:64 dilution) with AgPl66. Third, IB of the three serum samples against AgPl208 used in the DID showed reactivity against several fractions with molecular masses of approximately 115, 70, 64, 49, and 26 kDa (Figure 3B).

As the serological survey was not able to discriminate the *Paracoccidioides* species, molecular studies were carried out.

DNA from the lymph node biopsy was extracted using the QIAamp DNA tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. A seminested polymerase chain reaction (PCR) assay was performed using primers previously designed from the *P. brasiliensis* gp43 sequence.<sup>15</sup> DNA samples from both *P. brasiliensis* 339 (Pb339) and *P. lutzii* 66 (Pl66) isolates were amplified in parallel. The PCR products of approximately 300 bp were obtained from both the patient's and the Pb339 DNA samples, although the amplification was negative for the Pl66 DNA sample, as expected for *P. lutzii* isolates (Figure 4).<sup>16</sup> Identification of the species as *brasiliensis* was further confirmed by sequencing the products of amplification (ABI 3500 DNA Analyzer; Thermo Fisher, Carlsbad, CA). The patient's sequence showed 98% identity when compared with the gp43 sequences of *P. brasiliensis* available in GenBank.

## DISCUSSION

Negative results in serological routine screening of patients with microbiologically proven PCM is occasionally reported.<sup>2-5</sup> Failure in detecting anti-*Paracoccidioides* antibodies has been ascribed to factors either related to serological techniques or to the status of the host immune reactivity.<sup>3,4,9,10,17,18</sup> Recently, this issue has been renewed by the recognition that the *Paracoccidioides* genus comprises two species with distinct antigenic profiles and geographical distribution. Whereas PCM due to *P. brasiliensis* appears widespread in Brazil and other South American countries, PCM due to *P. lutzii* appears restricted to central areas of Brazil.<sup>19</sup>

The immunodominant antigen of *P. brasiliensis* is a 43 kDa glycoprotein that, although with a yet unknown biological function in the fungus physiology/metabolism, is highly expressed by yeast cells. Previous studies showed that removal of gp43 from *P. brasiliensis* antigenic preparation strongly reduced patients' serological reactivity.<sup>20</sup> *Paracoccidioides lutzii*, on the other hand, expresses small amounts of a 43 kDa glycoprotein that has glucanase activity and antigenic properties distinct from *P. brasiliensis*' gp43.<sup>21,22</sup> In fact, several mutations distinguish the gp43 gene of the two species.<sup>16,22</sup> This and other antigenic differences may explain the early observations made by some clinicians about the high rate of negative results with gp43-based routine serological screening of PCM patients from the central areas of Brazil (C. J. Fontes, personal communication), as well as the studies

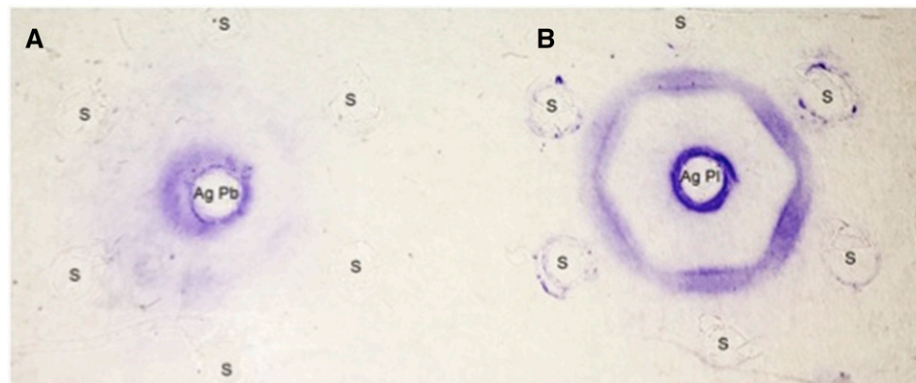


FIGURE 2. Immunodiffusion test showing reactivity of the patient's serum with antigens from *Paracoccidioides*. The center well contains AgPb113 (A) and AgPl208 (B). This figure appears in color at [www.ajtmh.org](http://www.ajtmh.org).

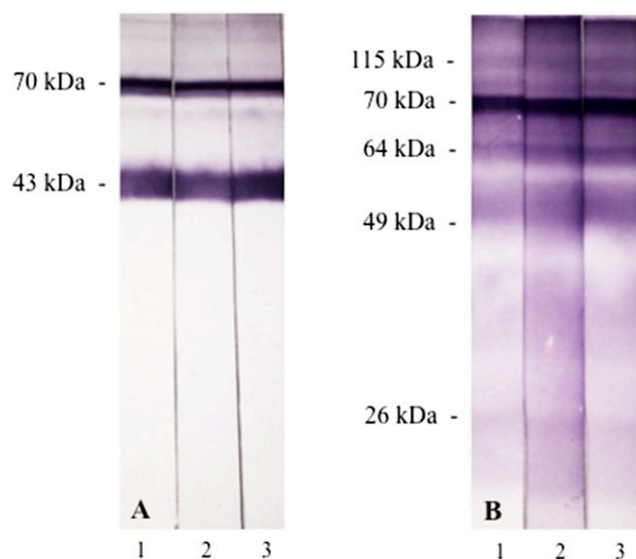


FIGURE 3. Immunoblotting of the patient's serum response to antigens from *Paracoccidioides*. (A) AgPb113 and (B) AgPI208. Antigens were transferred electrophoretically to nitrocellulose membranes and incubated with sequential patient's serum samples (1, 2, and 3) and peroxidase-conjugated antihuman immunoglobulin G. Reactions were developed with 4-chloro-1-naphthol. This figure appears in color at [www.ajtmh.org](http://www.ajtmh.org).

reporting unexpected negative serological results in patients from these areas.<sup>5,23-25</sup>

The unexpectedly very high titers in the DID and CIE techniques against *P. lutzii* antigen preparations supported the suspicion that the patient was indeed infected by *P. lutzii*. On the other hand, the IB showed marked reactivity against both gp43 and gp70, characteristic of *P. brasiliensis* infection. It remains to be determined why the anti-gp43 and anti-70 reactivity did not translate into positive reactions in the DID and CIE assays carried out at two reference centers with serum samples collected during the first 2 months of follow-up. Some hypotheses can be raised based on previous studies of both the host antibody response to *P. brasiliensis* and the differing characteristics of immunoprecipitating (DID and CIE) and IB techniques. One hypothesis is that, because the DID reactivity is based mainly on recognition of gp43 and because different isolates can produce different gp43 isoforms, these isoforms may not be recognized in serological assays that use soluble antigens, such as the DID, but they do not interfere in assays that use membrane-bound antigens, such as the IB.<sup>17</sup> Other hypothesis is that in patients, especially those with the acute form of the disease, the specific antibody response comprises mainly antibodies of the IgM class, which can fail to react or react weakly in DID/CIE but not in IB.<sup>26</sup>

On the other hand, IB of the three consecutive serum samples of the patient carried out with a *P. lutzii* antigen preparation consistently recognized four main fractions and other minor fractions. Paradoxically to the fact that the patient was infected with *P. brasiliensis*, as defined by its molecular characterization from a lymph node biopsy rich in fungal organisms, IB with *P. lutzii* antigen is consistent with the strong reactivity in the DID and CIE assays using antigen preparations from this species. Unfortunately, little is still known about the characteristics of the antibody response of patients with PCM due to *P. lutzii*. No immunodominant *P. lutzii*-specific

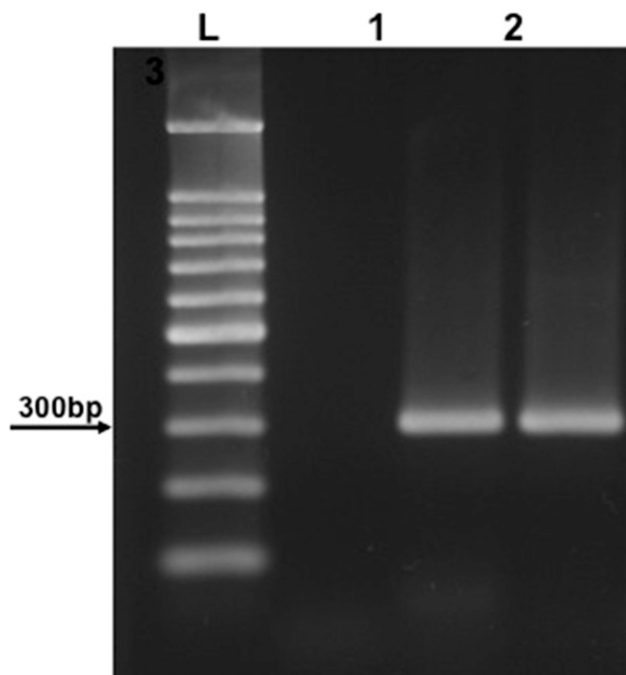


FIGURE 4. Agarose gel showing the seminested polymerase chain reaction products obtained from DNA samples after amplification with primers gp43.<sup>15</sup> L = mass ladder 100 bp; 1 = DNA sample from *Paracoccidioides lutzii* 66; 2 = DNA sample from the patient's biopsy; 3 = DNA sample from *Paracoccidioides brasiliensis* 339.

fraction has yet emerged in preliminary studies performed with five different isolates of this species (A. P. Vicentini, V. Morais, C. P. Taborda, unpublished data).

Previous studies reported that sera from patients with PCM due to *P. brasiliensis* do not recognize antigen fractions contained in the cell-free preparations of *P. lutzii*, unlike sera from patients with PCM due to *P. lutzii*, which were able to recognize antigenic fractions from both species.<sup>11</sup> However, other authors showed that 60% of a cohort of PCM patients living in the state of Paraná, in the southern region of Brazil and endemic for *P. brasiliensis*, yielded positive results in a DID assay with a *P. lutzii* antigen preparation.<sup>27</sup>

Altogether, our case reinforces the idea of an antigenic variability in the *P. brasiliensis* complex, which can in part explain some false-negative results in patients with active infection and the difficulty in adopting one single strain or molecule in the antigen preparation to diagnose a disease caused by two different species comprising isolates that may present distinctly unique antigen profiles. Our case report also suggests that antibodies elicited during *P. brasiliensis* infection recognize antigenic fractions shared by both species, highlighting the difficulties in distinguishing the two infections using the currently available routine serological assays.

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