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# Detection of Anti-*Paracoccidioides brasiliensis* antibodies in suspected tuberculosis patients

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**ABSTRACT.** Paracoccidioidomycosis (PCM) is an important systemic mycosis in Latin America that occurs as active disease in 1-2% of *Paracoccidioides brasiliensis* infected people. Like PCM, tuberculosis (TB) affects mainly the lungs and the clinical and radiological aspects do not always allow differentiation between them. The aim of this study was to carry out serological investigation for detecting anti-*P. brasiliensis* antibodies, by three serological methods, in patients with symptoms suggestive of pulmonary TB. From August 2005 to September 2006, 76 patients with pulmonary symptoms suspected for TB were attended at the Regional Specialties Center Laboratory in the city of Paranavaí, Paraná, Brazil and submitted to microbiological TB research, ELISA, immunodiffusion and immunoblotting for PCM. Of all the individuals, 21 (27.63%) were reactive to *P. brasiliensis* by ELISA and 11 (14.47%) showed a laboratory diagnosis of pulmonary TB. Of all the individuals serologically reactive to *P. brasiliensis*, by ELISA, none had positive results by immunodiffusion and one reacted with antigen 43 kDa when Immunobloting was carried out. Our results lead us to reflect a necessity to obtain a more specific serologic test for diagnosis of PCM disease in patients with respiratory symptoms considering the high number of individuals reactive to *P. brasiliensis* especially in endemic areas.

**Key words:** paracoccidioidomycosis, tuberculosis, serology, ELISA, immunobloting.

**RESUMO. Detecção de anticorpos anti-*Paracoccidioides brasiliensis* em pacientes suspeitos de tuberculose.** Paracoccidioidomicose (PCM) é importante micose sistêmica na América Latina, que ocorre como doença ativa em 1-2% dos indivíduos infectados com *Paracoccidioides brasiliensis*. Assim como a PCM, a tuberculose (TB) afeta principalmente os pulmões, porém os aspectos clínicos e radiológicos nem sempre permitem a diferenciação entre essas doenças. O objetivo deste estudo foi realizar um inquérito sorológico para a detecção de anticorpos anti-*P. brasiliensis*, utilizando três métodos sorológicos, em pacientes com sintomas sugestivos de tuberculose pulmonar. De agosto de 2005 a setembro de 2006, 76 pacientes sintomáticos foram atendidos no Laboratório do Centro Regional de Especialidades de Paranavaí, Paraná, Brasil e submetidos à investigação microbiológica para TB e de anticorpos por ELISA, imunodifusão e immunobloting para PCM. Destes, 21 (27,63%) foram reativos para *P. brasiliensis* por ELISA e 11 (14,47%) apresentaram diagnóstico laboratorial de tuberculose pulmonar. Dos indivíduos sorologicamente reativos para *P. brasiliensis*, por ELISA, nenhum apresentou resultado positivo pela técnica de imunodifusão e um reagiu com antígeno de 43 kDa quando do uso de immunobloting. Os resultados obtidos nos levam a refletir da necessidade de se obter um teste sorológico mais específico para o diagnóstico de PCM doença em pacientes com sintomas respiratórios, considerando o elevado número de indivíduos reativos para *P. brasiliensis* principalmente em áreas endêmicas.

**Palavras-chave:** paracoccidioidomicose, tuberculose, sorologia, ELISA, Immunobloting.

## Introduction

Paracoccidioidomycosis (PCM), an important systemic mycosis in Latin America, is the eighth highest cause of mortality among infectious and parasitic diseases in Brazil, and the fifth highest in the state of Paraná (BITTENCOURT et al., 2005). PCM occurs as active disease in 1-2% of *Paracoccidioides brasiliensis* infected peoples, whose number is estimated to be 10 million in Latin American endemic areas (RESTREPO-MORENO, 1994).

Like PCM, tuberculosis (TB) mainly affects the lungs, accompanied by coughing and weight loss.

The radiological images are generally exuberant, however, chest X-rays do not always allow differentiation between TB and PCM (QUAGLIATO JUNIOR et al., 2007). Coexistence of TB and PCM was previously observed by Valle et al. (1992) in Rio de Janeiro, at rates up to 15% and by Paniago et al. (2003) in 5.5% of the patients in a study carried out in Mato Grosso do Sul State, Brazil.

For the differential diagnosis, in addition to clinical and radiological triage, the laboratory data with microbiological findings contribute significantly. The Ministry of Health's protocol (MINISTÉRIO DA SAÚDE, 2002) mandates that every clinical suspected case of pulmonary TB be confirmed by acid fast bacilli (AFB) smear. However, as the incidence of TB in Brazil is high, it directs that even without definite laboratory confirmation, patients with characteristic signs and symptoms be treated as TB. Some of these patients do not respond to specific anti-TB chemotherapy and only had clinical signs improved when PCM treatment was introduced.

Confirmation of a PCM case is accomplished by demonstrating the presence of the fungus, in biological samples such as sputum, by direct observation, histopathology, or culturing. However, it is not routinely performed in patients attended at basic health clinics due the time and material required to perform these techniques (QUAGLIATO JUNIOR et al., 2007).

Cellular immunity is protective against PCM, but some studies have demonstrated that high antibody titers against fungal antigens suggest heavy fungal loads during severe stages of disease (CARVALHO et al., 2008). At present, serology is not used for diagnostic purpose in investigating PCM (CARVALHO et al., 2008; MAMONI et al., 2001), although it may be employed in post-treatment follow up (RESTREPO, 1985; SANTOS et al., 2003). Among the immunological methods, the immunodifusion (ID) test is the method of choice for antibody detection. However, false negative results are not rare due to its sensitivity (85 – 90%) (DEL NEGRO et al., 1991). Immunoblotting (IB), an immunoenzymatic method that seems to be useful for PCM diagnosis, raises the sensitivity to 100% (VALLE et al., 2001). The *enzyme linked immunosorbent assay* (ELISA) has been shown to be a sensitive method (approximately 100%) (ALBUQUERQUE et al., 2005; DEL NEGRO et al., 2000) and it is appropriate for testing large numbers of samples in epidemiological studies (MALUF et al., 2003) considering that it is less cumbersome and time-consuming procedure. However, cross-reaction with other diseases may occur in histoplasmosis, blastomycosis and coccidioidomycosis that are caused by dimorphic ascomycetes genetically close to *P. brasiliensis* (SHIKANAI-YASUDA et al., 2006; RAPPLEYE; GOLDMAN, 2006). Despite the limitation of serological tests Immunoblotting (IB) is a very sensitive method for PCM diagnosis and monitoring especially in combination with ID (VALLE et al., 2001).

The detection of serum anti-*P. brasiliensis* antibodies in previous studies (MALUF et al., 2003; FORNAJEIRO et al., 2005) had been carried out in our laboratory using ELISA and has contributed to knowledge of PCM epidemiology in Paraná State. The large number of individuals reactive to *P. brasiliensis* antigens detected in these studies suggests the region is endemic for the fungus, which appears to affect a large number of people. The aim of the present study was to carry out a serological investigation by ELISA, ID and IB to detect anti-*P. brasiliensis* antibodies, in patients with symptoms suggestive of pulmonary TB.

## Material and methods

From August 2005 to September 2006, 76 patients with symptoms suggestive of pulmonary TB that were attended at the Central Specialty Laboratory (Laboratório Central de Especialidades) in the city of Paranavaí, state of Paraná for TB laboratorial diagnosis, were submitted to research for anti *P. brasiliensis* antibodies.

The study was approved by the Ethics Committee of the State University of Maringá, Paraná State (protocol No. 218/2005). All participants approved the research protocol and signed the informed consent. A structuralized and standardized questionnaire was applied to all the participants including questions on age, sex, place of residence, type of work, as well as the use of tobacco, alcohol and illicit drugs (marijuana, crack and cocaine).

A total of 189 sputa samples (1 to 3 from each individual) obtained by spontaneous expectoration, and 76 sera samples were subjected to mycobacteria research and determination of anti-*P. brasiliensis* antibodies, respectively.

### Ziehl-Neelsen and culture for mycobacteria

Sputa samples were processed for direct research of acid fast bacilli (AFB) by microscopy (Ziehl-Neelsen) and cultured by the Petroff Sodium Hydroxide method in Difco™ Lowenstein-Jensen Medium Base (L-J) (Becton, Dickinson and

Company, Sparks, MD, USA). Colonies positive for AFB on L-J were confirmed as *M. tuberculosis* by biochemical methods (KENT; KUBICA, 1985). The cultures that showed no AFB growth after 2 months at 35ºC were considered negative for mycobacteria.

### Anti-*P. brasiliensis* antibodies detection

The yeast antigen used in the immunological tests was obtained as described by Camargo et al. (1988) using the *P. brasiliensis* B-339 and was supplied by the Centro de Pesquisa e Produção de Imunobiológicos (CPPI), of the Health Secretariat of Paraná State.

ELISA tests were carried out in all the patients as described in elsewhere (MALUF et al., 2003). Briefly, 100 $\mu$L exoantigen (500 ng of protein), diluted in 0.06 M carbonate-bicarbonate buffer (pH 9.6) was added to each well of polystyrene microtiter plates (NUNC$^{TM}$ Brand products, Switzerland) and incubated at 37ºC for 2 hours and after 18 hours at 4ºC. The plates were washed three times with phosphate-buffered saline (PBS) containing Tween 20 (0.05%) and 200 $\mu$L 5% dried skin milk in PBS-Tween 20 were added to the wells and incubated at 37ºC for 1 hour to block free sites. The plates were washed three times with PBS-Tween 20. One hundred microlitres of each serum sample previously diluted (1:200) in 0.1 M PBS, pH 7.2 were added to each well. The plates were incubated at 37ºC for 1 hour and washed three times with PBS-Tween 20. One hundred microlitres of peroxidase labeled anti-human immunoglobulin G (1:1000) (Sigma, St. Louis, Missouri, USA) were added to the wells and the plates incubated at 37ºC for 1 hour. After three washes with PBS-Tween 20, 100 $\mu$L of o-phenylenediamine (0.2 mg mL$^{-1}$) (Sigma, St. Louis, Missouri, USA) plus 0.1 M citrate buffer were added to each well and incubated for 5-10 minutes at room temperature. The reaction was stopped with 50 $\mu$L of 4 N $H_2SO_4$. Serum optical density (OD) was measured at 492 nm in an Anthos 2010 ELISA microplate reader (Labtec). The reactions were run in fourfold. A positive and negative serum and non specific reaction (white) controls were added in each assay. Sera with two fold or more the absorbance of the negative control were considered positive in agreement with previously established criteria (MALUF et al., 2003). Sera samples with OD values > 1.0 were considered reactor and OD ≤ 1.0 not reactors for the *P. brasiliensis* B339 exoantigen.

Immunodiffusion tests were performed in 1% agar noble (Difco Laboratories, Detroit, MI, USA) according to the modified Ouchterlony method as described previously (CAMARGO et al., 1988) in 21 patients with ELISA OD values > 1.0.

Sodium Dodecyl Sulfate polyacrilamide gel electrophoresis (SDS-PAGE) and Immunobloting analysis were performed according to Valle et al. (2001) in the 21 patients with ELISA OD values > 1.0. Firstly, exoantigen (87.5 $\mu$g final concentrations) was denatured at 100°C for 5 min. in 0.125 M Tris-HCl, pH 6.8, containing 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol and 0.04% bromophenol blue. SDS - polyacrylamide gel electrophoresis (PAGE) was performed with 12% resolving gels and 4% stacking gels in a Mini-Electrophoresis Cell (Biosystems, Curitiba, Paraná State, Brazil). The electrophoresis conditions were 20 mA of constant current for stacking and 25 mA for protein separation. Gels were electrotransferred to nitrocellulose membranes in a Mini Trans-Blot cell (Biosystems, Curitiba, Paraná State, Brazil) containing transfer buffer (25 mM Tris-base, 192 mM glycine and methanol [20% v v$^{-1}$] pH 7.4) operated at 14 Volts overnight and checked by Ponceau S to determine equal loading. Free binding sites in the membranes were blocked by incubation for 2h in 5% (wt vol$^{-1}$) dried skin milk in Tris buffer containing 0.05% Tween 20 (Tris-T). Membranes were sliced vertically and strips were incubated for 2h at room temperature with serum sample diluted 1/400 in Tris-T containing 1% dried skin milk Strips were washed in Tris-T three times for 10 min. each. Goat anti-human immunoglobulin G (IgG) horseradish peroxidase conjugate (Sigma Chemical Co., St. Louis, MO, USA) diluted 1:1000 in Tris-T was added and incubated for 60 min. at room temperature. Blot strips were washed in Tris-T and incubated with 0.5 mg mL$^{-1}$ of 3,3'-diaminobenzidine tetrahydrochloride (Sigma) plus 4 $\mu$L $H_20_2$ (30% vol vol$^{-1}$). After color development, the strips were rinsed exhaustively in distilled water.

## Results

The ages of the 76 individuals included in the study ranged from 15 to 87 years (mean 47.43); 47 (61.84%) were male. Regarding their place of residence, 44 (57.89%) lived in the urban region; and 68 (89.47%) did not work at night. The use of tobacco was reported by 39 (51.32%), of whom 37 (94.87%) had smoked for longer than five years. Social or continuous use of alcohol was reported by 38 (50.00%); 69 (90.79%) denied the use of any type of illicit drugs (Table 1).

**Table 1.** Distribution of the 76 patients attended at the Regional Specialties Center Laboratory in Paranavaí, Paraná State, Brazil according to variables of interest for pulmonary tuberculosis and paracoccidioidomycosis, from August 2005 to September 2006.

| | Patients | | | | |
|---|---|---|---|---|---|
| Variables | n[a] | % | | n | % |
| Age (years) | | | | | |
| 30 or younger | 17 | 22.37 | | | |
| 31 to 60 | 38 | 50.00 | | | |
| 61 or older | 21 | 27.63 | | | |
| [b]Mean age = 47.43 | | | | | |
| Sex | | | | | |
| Female | 29 | 38.16 | | | |
| Male | 47 | 61.84 | | | |
| Place of Residence | | | | | |
| Rural | 32 | 42.11 | | | |
| Urban | 44 | 57.89 | | | |
| Night Work | | | | | |
| Yes | 8 | 10.53 | | | |
| No | 68 | 89.47 | | | |
| Smoker | | | | | |
| Yes | 39 | 51.32 | ≥5 years | 37 | 94.87 |
| | | | <5 years | 2 | 5.13 |
| No | 37 | 48.68 | | | |
| Alcohol Use | | | | | |
| Yes | 38 | 50.00 | | | |
| No | 38 | 50.00 | | | |
| Drug Use | | | | | |
| Yes | 7 | 9.21 | | | |
| No | 69 | 90.79 | | | |

[a]: number of patients; [b]: mean age of patients.

Pulmonary TB was bacteriologically confirmed in 11 (14.47%) individuals. In two cases, the direct AFB research was negative, and the bacteriological diagnosis was confirmed only by culturing.

According to the cutoff value established for the ELISA serological test for PCM, 21 (27.63%) individuals were reactive to *P. brasiliensis* (OD > 1.0). Of these, three individuals (14.29%) were among the 11 with pulmonary TB and 18 (85.71%) among those 65 individuals for whom the laboratory investigation for TB was negative (Table 2).

**Table 2.** Immunoblotting results from 21 individuals with suspected pulmonary tuberculosis with ELISA OD > 1.0 attended at the Regional Specialties Center Laboratory in Paranavaí, Paraná State, Brazil, from August 2005 to September 2006.

| | ELISA | | Immunobloting | | | |
|---|---|---|---|---|---|---|
| | (DO > 1.0) | | Positive | | Negative | |
| AFB results | n | % | n | % | n | % |
| Positive | 3 | 14.29 | 1 | 4.76 | 2 | 9.52 |
| Negative | 18 | 85.71 | 1 | 4.76 | 17 | 80.95 |
| Total | 21 | 100 | 2 | 9.52 | 19 | |

Among the 21 ELISA OD > 1.0 individuals, one (ELISA OD > 2.11) reacted with antigen of 43 kDa when Immunoblotting was performed. Meantime, all 76 sera samples subjected to ID showed negative results.

## Discussion

Considering the overlap of clinical and radiological aspects of TB and PCM, all patients with respiratory symptoms were included for investigation of antibodies against *P. brasiliensis*. ELISA was used as a screening test to detect anti-*P. brasiliensis* antibodies, because of previous experience in epidemiological studies carried out in our laboratory (MALUF et al., 2003).

Among those individuals reactive to *P. brasiliensis* by ELISA, 15.00% had TB which led us to think of TB/PCM co-infection considering the climate and physiographic characteristics of Paranavaí city may propitiate the development of *P. brasiliensis* (RESTREPO, 1985). Although little is known about the natural habitat of the *P. brasiliensis* (SHIKANAI-YASUDA et al., 2006) there is a consensus that the regions where PCM is endemic are located in tropical and subtropical forests with a mean temperature between 14 and 20ºC, annual precipitation from 800 to 2,000 mm, and relatively high humidity (RESTREPO, 1985; RESTREPO et al., 2001).

Most of the individuals reactive to *P. brasiliensis* in the current study lived in the urban area. It must be considered that Paranavaí is a small municipality where rural and urban areas are not clearly defined. It is possible that some infections may have occurred in urban activities such as gardening, work at multiple construction and demolition that can provide conditions for *P. brasiliensis* infection (BLOTA et al., 1999). In this group of individuals (n = 21) the percentage of smokers (38.09%) and that had drinking habits (42.86%) were high that may be a predisposing factor to PCM (MARTINEZ; MOYA, 1992; SANTOS et al., 2003). On the other hand, we need to have in mind the possibility of cross-reaction with other mycotic diseases in serological tests (ALBUQUERQUE et al., 2005; SHIKANAI-YASUDA et al., 2006), leishmaniasis (SILVEIRA et al., 2006) and TB itself.

The result obtained in the current study shows the important issue of those individuals reactive to *P. brasiliensis* (85.71%) who were not diagnosed with pulmonary TB. In this group of individuals, nine had high ELISA OD values (OD > 2.16). In these individuals with symptoms such as cough, weight loss, and/or radiological images compatible with tuberculosis but without a conclusive diagnosis, PCM must be investigated considering the physiographic characteristics of Paranavaí. Although ELISA is used very frequently for serological diagnosis of many diseases, in our experience this serological technique is not still suitable for PCM

diagnosis as a unique toll. It is a technique with high sensibility, nevertheless variable specificity and reproducibility were observed and results can vary according to the individual characteristics of each serum, of the *P. brasiliensis* antigen, individual immune response against the specific strain, or even the presence of different antigenic epitopes (ALBUQUERQUE et al., 2005). Great care must be taken with the interpretation of the ELISA results especially when using crude antigenic preparations as in our work with positivity of 27.63%.

Although the ID showed negative results in all patients we cannot exclude PCM or exposure to *P. brasiliensis*. There are reports of ID false negative results in PCM patients with active mycosis. Some causes for ID false negative results are suggested as prozone effect, formation of immune complexes, low antibody level and presence of asymmetric antibodies that inhibit secondary binding in ID (VALLE et al., 2001).

Only one patient that showed ELISA OD > 2.1 (and was TB positive) reacted to 43 kDa antigen in IB. Considering IB is a very sensitive method to detect anti *P. brasiliensis* antibodies and the sera dilution (1:400) used in the test we can speculate the possibility of co-infection. Based in our experience in performing IB for PCM in TB patients (data not published) we had not detected cross-reaction with TB when performing the IB for PCM in sera diluted at 1:400. This patient is under observation with the objective of evaluating the progress of PCM or not.

## Conclusion

Our results showed a considerable number of individuals reactive to *P. brasiliensis* by ELISA, among individuals with respiratory symptoms that were sent for TB research. This kind of result leads us to reflect that a mycological test in clinical samples, to demonstrate *P. brasiliensis*, is mandatory. However this is not always easy to perform and sometimes needs several samples collected on different days. Then, the need to obtain a more specific serologic test or a more specific antigen for IB, ID and ELISA, for definitive diagnosis of PCM disease in patients with respiratory symptoms is imminent considering the high number of individuals reactive to *P. brasiliensis* especially in endemic areas.

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