Development and Evaluation of a Latex Agglutination Test for the serodiagnosis of Paracoccidioidomycosis.

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ABSTRACT

Paracoccidioidomycosis (PCM) is the most prevalent systemic mycosis in Latin America. It is caused by the dimorphic fungus Paracoccidioides brasiliensis. The immunodiffusion (ID) test is one of the most widely used techniques for PCM serologic diagnosis due to the simplicity and low costs of its execution. However, it requires trained and qualified people to execute it. The purpose of this study was to evaluate a latex agglutination (LA) test for the detection of anti-\textit{P. brasiliensis} antibodies by using pooled crude exoantigens from the fungus. Fifty-one (51) serum samples of patients with PCM were tested. Positivity was observed in 84\% (43/51), and the agglutination patterns varied from 1+ to 4+. The antibody titer reactivity ranged from 1:2 to 1:64. Cross-reactivity was observed in sera from patients with aspergillosis, histoplasmosis and non-fungal disease. Serum samples obtained from healthy donors were not reactive. The sensitivity and specificity of the LA test was 84\% and 81\%, respectively. When comparing LA test with the double immunodiffusion test we found an agreement of 92\%. Further work is needed to improve on the performances of the LA assay, before this can be proposed as a reliable diagnostic tool, mainly in laboratories with little infrastructure.

KEY WORKS: Paracoccidioidomycosis; crude exoantigen; latex agglutination; antibody.
INTRODUCTION

*Paracoccidioides brasiliensis* is the etiologic agent of paracoccidioidomycosis (PCM), a thermally-dimorphic fungus that grows as a mycelial form at room temperature and as a budding yeast form at 35-37°C in host tissue or when cultured at 37°C (3). The disease is limited to Latin American countries, and the most important endemic regions are found in Brazil, Colombia, and Venezuela (14). In Brazil, PCM is considered the eighth most common cause of death among infectious and parasitic chronic diseases, overcoming leishmaniasis, with a mortality rate of 1.45 per million population (6).

Conidia of *P. brasiliensis* act as the infectious propagules which are inhaled and led to the lungs where transformation to the pathogenic yeast form occurs (10). PCM exhibits a wide spectrum of clinical and pathological manifestations, ranging from benign and localized forms to severe disseminated disease (11). In most cases, the infection is restricted primarily to the lungs but can spread to other organs (15). The disease presents two major clinical forms: (a) the acute or subacute form (juvenile type), with severe involvement of the mononuclear phagocyte system and (b) the chronic form (adult type), with slow evolution and involvement of one or more organs, usually the lungs (7).

The definitive diagnosis of PCM is usually made by visualization or isolation of the fungus from the lesions. Serologic tests appear to offer a means to achieve an early diagnosis of disease (1,4). Detection of antibodies in serum has been one of the main tools for the diagnosis of PCM and may be useful to monitor its evolution and its
response to treatment. Among the different serologic techniques, double
immunodiffusion (ID) test is most commonly used and presents sensitivity values that
vary from 80 to 95% (12). However, this technique is only feasible in reference
laboratories due to the necessity of especial reagents and experienced people to
count the test. The development of a simpler and less expensive methodology is
greatly requested by laboratories with little infrastructure, especially in poor countries,
where there are endemic areas of the disease. Such test could contribute to the
diagnosis and the screening of PCM, mainly in hospital and public health laboratories,
therefore, improving the diagnostic coverage of this infection which affects individuals
in their most productive period of life, especially male adults who live in rural areas.

Investigators have shown the latex particle agglutination (LA) test to be a
valuable tool for the diagnosis of various diseases, including fungal infections (9,16).
In 1978, Restrepo & Moncada (16), using LA test with a crude exoantigen prepared
from a pool of three strains of \textit{P. brasiliensis} (B 339, B 341,C 81) for the detection of
antibodies, showed that the maximum sensitivity value of the assay was 69.5%, while
specificity value varied from 18.5% to 46.8%.

In order to improve the serological parameters of the agglutination method for
the diagnosis of the PCM, the present study has the objective of producing a rapid LA
test using pooled crude exoantigen of \textit{P. brasiliensis} and to evaluate its possible use
in the serological diagnosis of this mycosis, as well as to compare results with the ID
test which is already established as a diagnosis tool for PCM.

\textbf{MATERIALS AND METHODS}
Sera and Patients. Fifty-one serum samples obtained from patients with active PCM
(47 males and 04 females ranging from 15 to 75 years of age) were included in the
study. Six of them presented the acute form and 45 the chronic form of the disease.
All of them exhibited clinical and laboratory signs of the disease, such as nervous
central and pulmonary systems involvement, mucosal or mucocutaneous lesions and
increased specific antibody levels. The diagnosis was confirmed either by direct
examination of biological fluids (n=4), serological immunodiffusion tests (n=25) or by
both tests (n=22). Eleven serum samples from patients with histoplasmosis (HP), 15
from patients with aspergillosis (ASP), 49 from patients with non-fungal disease
(patients presenting bacterial infections) were used. On the other hand, 20 serum
specimens from healthy individuals with no history of pulmonary disease were also
studied. These sera were termed normal human sera (NHS) and were individually
tested and used as negative controls. All serum specimens were divided into aliquots
and were stored at -20°C.

Fungal strains and crude exoantigen production. Three isolates of *P. brasiliensis*
(Pb34, Pb113 and PbIOC), obtained from the culture collection of Evandro Chagas
Institute were selected for this study. The isolates were initially grown on Sabouraud
medium slant tubes for 3 days at 35°C. The growth (consisting entirely of yeast cells)
was collected from at least 10 tubes, yielding an inoculum of approximately 2x10⁶
cells. These cells were inoculated into 500 ml-Erlenmeyer flasks containing 100 ml of
yeast extract – peptone- dextrose (YPD) broth (DIFCO, Sparks, MD, USA). This
culture was incubated for 3 days at 35°C on a gyratory shaker at 50 r.p.m. (Fanem,
SP, BR). The growth obtained was transferred to 1800 ml-Fernbach flasks containing
500 ml of YPD broth. The flasks were then incubated as above described for more 7 days. The culture was killed with Thimerosal (0.2 g/l). Supernatant fluids were collected following paper filtration, concentrated under vacuum at 45°C, and dialyzed against distilled water. After dialysis, the solution was concentrated again (5).

The protein content was measured by the Bradford method (2) and the electrophoretic pattern was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7) and followed by silver nitrate staining.

**ID test.** Immunodiffusion was performed with pooled crude exoantigen of *P. brasiliensis* as described previously (4). Each PCM, HP, ASP and NHS serum was individually tested against an antigenic preparation from *P. brasiliensis* strains (Pb34, Pb113 and Pb IOC) and the titer of each serum sample was determined.

**Preparation of the LA test.** The LA test antigen consisted of a pool of six lots of crude exoantigen and adjusted to a concentration of 400 µg/ml and used to coat the test latex particles. A 1% suspension of latex polystyrene beads (diameter, 0.8 µm; SIGMA, St Louis, MO, USA) in carbonate/bicarbonate-buffered saline solution (pH 9.2) was sensitized by mixing the beads with pooled crude exoantigen of *P. brasiliensis* at 4°C overnight before the addition of bovine serum albumin (BSA) to a final concentration of 0.1% (wt/vol). The optimal solution for use was the one that produced a clear agglutination with the serum of animals experimentally infected and with positive control sera from patients with PCM. The latex particles (1% suspension) were coated primarily with pooled crude exoantigen (400 µg/ml). The LA test was performed by placing 25 µl of latex suspension on a dark slide. A total of 25
µl of serum was added to the latex particles. After the reagents were mixed, the slide was gently agitated in a Kline agitator for 5 minutes. Negative control latex consisted of a 25 µl of latex solution added to 25 µl of saline solution. Samples were considered positive for P. brasiliensis antibodies when agglutination (clumping) was observed. The grading of agglutination was defined as negative, when suspension had a fine granular background or a milky suspension aspect with the absence of agglutination; One plus (1+), suspension had small clumping against a cloudy background; Two plus (2+), suspension had small to moderate clumping against a slightly cloudy background; Three plus (3+), suspension presented moderate and large clumps against a clear background; Four plus (4+), suspension had large clumps with a very clear background. After the screening test on the undiluted sera samples, if grading agglutination were 1+ or higher, the reactions were titrated (1:2 through 1:1024).

**Statistical analysis.** ROC curve analysis was performed to determine the cutoff for positivity that would give the optimal sensitivity and specificity. Agreement was calculated by the following formula: 
\[
\frac{A+D}{A+B+C+D},
\]
A is a specimen with a positive result by both LA and the ID. B is a specimen with a positive result by LA but negative results by the ID. C is a specimen with a negative result by LA but positive results by the ID. D is a specimen with negative results by both assays.

**RESULTS**

Immunodiffusion and SDS – PAGE analysis of pooled crude exoantigen.
As shown by the immunodiffusion (ID) test, the *P. brasiliensis* pooled crude exoantigen reacted positively up to the dilution 1:16. Exoantigen SDS-PAGE showed at least 12 components (15 to 180 kDa) following silver staining. The main *P. brasiliensis* antigenic determinants gp 43 and gp 70 were observed.

**Detection of antibodies by ID and LA tests.**

Serum specimens from 51 cases of PCM and 95 controls including 26 cases with fungal infection (HP, ASP), 20 cases of normal human sera and 49 of non-fungal diseases were tested simultaneously by ID and LA using a pooled crude exoantigen (Table 1). Among these 51 PCM sera, 47 (92%) were positive and 4 (8%) were not reactive by the ID test. The serum titers obtained by the ID test ranged from 1:2 to 1:1024 (Table 2). Five PCM sera reacted only with non diluted serum. ROC analysis identified the optimal cutoff for positivity to be the agglutination pattern equivalent to one plus (1+). The sensitivity and specificity of the ID test were of 92% and 100%, respectively. The LA test sensitivity was 84%, the specificity was 81%, and the area under the curve was 0.841 (Figure 1). The LA and ID tests presented agreement of 92%.

In the LA test, the cut off value was determined to one plus (1+) and anti-*P. brasiliensis* antibodies were detected in 43 out of 51 (84%) serum samples; false negative results were observed in 8 (16%) samples. Figure 2 shows the results obtained when patients’ sera were classified according to the screening and to the titration procedures. Figure 2 A shows the results ranging from 1+ to 4+ in non-dilated sera. Figure 2 B shows antibody titers ranging from 1:2 to 1:64 after titration.
The ID and LA tests were able to detect anti-\textit{P. brasiliensis} antibodies in the PCM acute form. Of the sera collected from patients with the chronic form, four presented negative results (1 chronic multifocal form; 3 chronic unifocal form), whereas in the LA test, eight serum samples presented negative results (3 chronic multifocal; 5 chronic unifocal) (Table 1).

In the ID test, serum samples from patients with acute form showed antibody titers ranging from 1:4 to 1:1024. On the other hand, in the LA test antibody levels ranged from 1:8 to 1:32. Considering the chronic form, antibody titers varied from 1:2 to 1:128 by ID test and from 1:2 to 1:64 by LA test. Figure 4 shows the results obtained when patients' sera were separated according to the different clinical forms. Four negative sera in the ID test were also negative in the LA test. The antibody titers observed in the ID test were higher than those observed in the LA test (Figure 3).

A total of 95 heterologous serum samples were tested by ID and LA tests, including samples from patients with histoplasmosis (\(n = 11\)), patients with aspergillosis (\(n = 15\)), healthy donors (\(n = 20\)), and patients with non-fungal disease (\(n = 49\)). In the ID test, all non-PCM serum samples were negative. However, in the LA test, cross-reactivity was obtained with sera from patients with histoplasmosis (27%; 3/11), aspergillosis (27%; 4/15) and serum samples from non-fungal disease (22%; 11/49). None of these samples reacted at a titer rate higher than 1:4. No positive reaction was observed in the sera of healthy donors (Table 1; Figure 2A and 2B). There were statistically significant differences when the positivity reaction in the LA test serum samples from patients with PCM was compared with those in NHS.
(p<0.0001) (Table 1), which indicates that the test is able to discriminate patients with disease from those without the disease.

**DISCUSSION**

Serological tests are useful tools for the diagnosis of systemic fungal infections. These tests are based either on the detection of serum antibodies against fungal components or on the detection of products of the fungus itself. Their effectiveness depend on the reagents, the antigenic preparation and the methods used, and also on the comprehension of their limitations and precise interpretation of the results obtained. However, they are not available in the routine of most public health services. Only a few research laboratories located in areas considered endemic for systemic mycosis perform them. Paracoccidioidomycosis is endemic in Brazil. However, it is not a notifiable disease and its real prevalence and incidence can not be calculated, which makes it difficult to establish its impact on the different Brazilian states.

Our study evaluated the development of a serologic test using latex particles coated with pooled crude exoantigen of *P. brasiliensis*. *P. brasiliensis* is a fungus that grows appropriately in a variety of different media and we obtained a very good antigenic preparation from yeast filtrate culture grown in YPD medium broth (Yeast Extract, Peptone and Dextrose). This antigenic preparation has demonstrated a high degree of sensitivity, specificity, and reproducibility in the serodiagnosis of paracoccidioidomycosis by immunodiffusion in our laboratory. The pooled crude
exoantigen used was obtained after homogenization of equivalent amounts of each lot of antigen with molecular mass ranging from 15 to 180 kDa.

The sensitivity observed in the LA test was 84.31% (43/51), and the antibody titer ranged from 1:2 to 1:64. Eight patients presented false negative results in the LA test, while in the ID test only four serum samples were not reactive. This may be attributed to several factors, such as different immune responses by the host, since these results were found only in sera from patients presenting the chronic form of PCM, in which low antibody production in PCM unifocal forms is usually observed (12). On the other hand, it is also possible that the fungus strain infecting these patients had different virulence, which could have influenced the immune response. Another possibility may be the loss of conformational epitopes in the LA test using the pooled crude exoantigen of *P. brasiliensis*.

Restrepo and Moncada (16) tested four different crude antigenic preparations for PCM diagnosis by LA (mycelial and yeast phases, both treated and non-treated with ethanol). The crude yeast filtrate used to sensitize the particles was found to be better than the ethanol-treated yeast filtrate; however, sensitivity reached only 69.5% in the assay. A cause for difference observed between our study and Restrepo & Moncada (1978) concerning the sensitivity results, can be explained by the chemical nature of the substances present in yeast-phase culture filtrates, which are highly constituted of sugars, such as glucose, galactose, arabinose, and glucosamine (17). This antigenic preparation was employed by authors in 1978, however its composition has lower concentration of interest antigenic proteins, and it frequently produces high cross-reactivity with sera of patients with histoplasmosis and aspergillosis, influencing
on the specificity of the test. We utilized antigen standardized by Camargo et al (5),
whose specificity was 81.1%, whereas the assay of Restrepo & Moncada (1978)
showed maximum specificity of 46.8%, when compared with sera of patients with
histoplasmosis.

In our study, detection of antibodies by ID test reached sensitivity of 92.16%
(47/51) with antibody titer ranging from 1:2 to 1:1024. However, in the serum of four
PCM patients precipitating antibodies were not detected. A possible reason for these
false-negative results may be the presence of low-avidity IgG2 antibodies directed
against carbohydrate epitopes, which may interfere with the ID tests in PCM patients
(13).

Antibody reactivity observed both in ID and LA tests was higher in PCM
patients presenting the acute form of the disease than in those with the chronic form.
It is known that the acute form of PCM is generally more severe, presenting higher
titers of antibodies, while, in its chronic form, low antibody titers or occasionally titer
fluctuation may be detected (12). The lack of reactivity (negative and low
agglutination pattern {1+}) in the LA tests of sera from patients with proven PCM may
be related to the production of low titers of antibodies or to the intrinsic characteristic
of the patient or the infective strain. Although cross-reactions with serum samples
from other mycoses could be observed in this test (aspergillosis {4/15} and
histoplasmosis {2/11}), reactivities were less intense and occurred at lower titers (1:4)
than those with serum from PCM patients, and could be minimized by progressive
dilutions of the specimens. We believe that this cross-reactivity will not hinder the use
of this diagnostic tool in the detection of antibodies for PCM. On the other hand, the
fungus probably shares common antigenic determinants or similar determinants of related chemical structure that could explain cross-reaction. Travassos et al. (18) reported that, in general, specific reactions are obtained when the antigen is in a solution similar to that in the ID test, and that fixing antigen onto solid phase substrate increases the number of nonspecific reactions. Nevertheless, other assays using latex particles coated with pooled crude exoantigen pretreated with sodium metaperiodate (13) and sera samples pretreated with 2-β-mercaptoethanol must be conducted to eliminate cross-reactivity. Such a procedure will optimize the chances of accurately identifying true-positive LA test results in sera samples.

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 aspergillosis (12,16). On the other hand, lack of reactivity or low antibody titers has been a problem very common in our region. With the cutoff set at 1+ and the following titration of the samples, some patients with proven PCM were serologically classified as negative in the LA test. However, some of these patients also had negative results in ID test. We believe that the format of the test and the patient's immune status may have contributed to such results. However, it is possible that other factors interfere (prozone, presence of rheumatoid factor and other serum macroglobulins) with the performance of the test, which means that, new studies must be conducted in order to better evaluate this serological pattern.

The ID is still the gold standard serological test used in the diagnosis of PCM. It shows sensitivity and specificity of approximately 95-100% (4,13). It is a simple test, easy to perform and whose results can be obtained within 4 days, being used mainly...
in research institutes, because it requires equipments and qualified laboratory staff for
the production of antigens. These antigens show a great variability, making it very
difficult to standardize the diagnostic techniques on different laboratories.

The LA test used for other fungi pathologies has the advantage of being rapid
and simple, and it is not affected by anti-complement activity (9). Another important
advantage of this test is its stability. Sensitized latex beads were found to be able to
retain similar agglutination sensitivity after storage at 4 °C for at least 4 months (data
not shown). By following the precise procedure in the test, the assay can be useful as
a screening test in the hospital and public health laboratories, where the use of a
battery of tests or more complex procedures are not feasible. The rapid agglutination-
based latex particles assay developed in this study showed that it is crucial to
examine the effect of pretreatment of antigen and serum samples on the performance
of the assay to increase sensitivity and specificity, and to minimize or eliminate cross-
reactivity with sera of patients with other mycoses, before this assay can be
suggested as an additional reliable diagnostic tool in the qualitative presumptive
diagnostic of PCM and for the follow-up studies in endemic areas, mainly in
laboratories with poor infrastructure.

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REFERENCES


Table 1. Detection of anti-*P. brasiliensis* in the ID and LA in sera from patients with PCM or other mycoses and in sera from healthy individuals.

<table>
<thead>
<tr>
<th>Group</th>
<th>ID Positive (n)</th>
<th>ID Negative (n)</th>
<th>ID Total (n)</th>
<th>LA Positive (n)</th>
<th>LA Negative (n)</th>
<th>LA Total (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Chronic</td>
<td>41</td>
<td>4</td>
<td>45</td>
<td>37</td>
<td>8</td>
<td>45</td>
</tr>
<tr>
<td>Unifocal</td>
<td>19</td>
<td>3</td>
<td>22</td>
<td>17</td>
<td>5</td>
<td>22</td>
</tr>
<tr>
<td>Multifocal</td>
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<td>1</td>
<td>23</td>
<td>20</td>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td>Histoplasmosis</td>
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<td>11</td>
<td>11</td>
<td>3</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>Aspergilosis</td>
<td>0</td>
<td>15</td>
<td>15</td>
<td>4</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>Non-fungal diseases</td>
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<td>49</td>
<td>49</td>
<td>11</td>
<td>38</td>
<td>49</td>
</tr>
<tr>
<td>NHS*</td>
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<td>20</td>
<td>20</td>
<td>0</td>
<td>20</td>
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</tr>
</tbody>
</table>

* $\chi^2 = 42.759 \ p < 0.0001$, PCM and NHS
Table 2. Reactivity of 51 PCM patients serum samples by ID and LA tests.

<table>
<thead>
<tr>
<th>Sera</th>
<th>Test</th>
<th>ID</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Not diluted</td>
<td>5</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>1:2</td>
<td>10</td>
<td>19</td>
<td>5</td>
</tr>
<tr>
<td>1:4</td>
<td>13</td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td>1:8</td>
<td>7</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>1:16</td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>1:32</td>
<td>2</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>1:64</td>
<td>4</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>&gt;1:64*</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Nonreactive</td>
<td>4</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>51</td>
<td>100</td>
<td>51</td>
</tr>
</tbody>
</table>

*One sample corresponding at 1:128 and other at 1:1024.
FIGURE 1. ROC curve depicting assay sensitivity and specificity, based upon testing serum from 51 patients with PCM and 95 controls, included patients with histoplasmosis, aspergillosis, non-fungal diseases and healthy subjects. With a cutoff one plus (1+), the sensitivity is 84% and the specificity is 81%.
FIGURE 2. Detection of anti-*P. brasiliensis* antibodies by LA test in sera of 51 patients with PCM and 95 controls, included patients with histoplasmosis (HP), aspergillosis (ASP), non-fungal diseases (bacterial infection, BI) and healthy subjects (NHS). A, Screening test on the undiluted sera. B, Titer was reported as the highest dilution showing a 2+ or greatest reaction. P, non diluted sera; N, negative. The long, fine line represents the cutoff point (1+).
FIGURE 3. Comparative antibody titers obtained by ID and LA tests, according to PCM clinical forms. ID, immunodiffusion; LA, latex test; P, non diluted sera; N, negative.