Effects of Pretreating Serum Samples on the Performance of a Latex Agglutination Test for Serodiagnosis of Paracoccidioidomycosis

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Paracoccidioidomycosis (PCM) is a fungal disease caused by *Paracoccidioides brasiliensis*, and Brazil is one of the principal countries where it is endemic. Diagnosis is based on the observation of budding *P. brasiliensis* yeast in clinical specimens from patients; however, the sensitivity of the visualization of fungi is low, indicating that serological tests are used for early diagnosis. The double-immunodiffusion test (ID) is the "gold standard" test for serology in PCM, although the execution of this test requires the availability of laboratory infrastructure. We report the improved performance of a latex agglutination test (LAT) by pretreating 30 serum samples from PCM patients and 71 controls (histoplasmosis and aspergillosis patients, patients with bacterial infections, and normal human sera) with a dilution buffer incubated at 37°C for 30 min. The sensitivity and specificity of the LAT test in the nonpretreated samples were 73% and 79%, respectively. However, when samples were pretreated, the sensitivity and specificity of the test increased to 90%. In this study, we did not observe cross-reactivity with histoplasmosis patient sera, but some reactions to sera from patients with aspergillosis and bacterial infections were noted. Normal human sera were not reactive in our tests. These results indicate the need for the elimination of heterologous reactions so that we can adequately use this method for screening cases of PCM.

Paracoccidioidomycosis (PCM), caused by *Paracoccidioides brasiliensis*, is the major systemic mycosis in Latin America; areas of endemicity extend from Argentina to Central America. In Brazil, PCM is the eighth leading cause of death because of chronic infectious disease (9). The usual route of infection is the inhalation of conidia of the fungus, which are converted into yeasts in the host tissues. The disease presents a wide spectrum of clinical manifestations ranging from benign and localized forms to severe and disseminated forms. It is more frequent in males than in females, with an overall ratio of approximately 15:1 in areas of endemicity (20). Patients that present clinical manifestations give rise to the acute/subacute (juvenile-type) and chronic (adult-type) clinical forms (16). Patients with benign PCM (subclinical and chronic unifocal presentations) present low levels of specific antibodies, whereas those with the disseminated form typically show high levels of specific antibodies (5).

Laboratory diagnosis of PCM is largely based on the visualization of the fungus in clinical specimens and its isolation by culture. However, early diagnosis is also possible by the detection of antibodies against *P. brasiliensis* in patient sera through serological techniques, such as immunodiffusion (ID) and immunoenzymatic assays (4). ID is the "gold standard" methodology used for PCM serodiagnosis because of its high sensitivity (91.3%) and specificity (100%) rates and its higher efficiency values than other assays (10). Nevertheless, few laboratories located in countries where PCM is endemic can perform ID as a routine test.

The latex agglutination test (LAT) presents advantages such as a simple and fast execution and the obtainment of results for visual reading. This methodology is based on the detection of circulating antigens or antibodies in serum, which is a rapid means of diagnosis. In fungal infections, such as candidiasis and aspergillosis (ASP), LAT can be employed to detect antigens in the serum of patients. For example, it is possible to obtain sensitivity and specificity of 100% and 80%, respectively, for candidiasis (15). LAT is thus a fundamental tool for the diagnosis of cryptococcosis (2).

The use of LAT for diagnosis of PCM was previously reported by two groups, in 1978 (18) and 2011 (21), who observed crucial differences in sensitivity and specificity; this discrepancy was the driving factor for our study of alternative methods that might improve the technique. One of the principal aspects that can increase the sensitivity and specificity values of PCM diagnostic tools is the treatment of the samples, such as for the elimination of immune complexes, before they are tested (22). Pretreatment has most improved the detection of antigens (antigenemia); however, some studies have also observed its importance for detecting antibodies (23).

We have shown that antibodies against *P. brasiliensis* can be detected by LAT in the serum of PCM patients; however, cross-reactivity was obtained (21). The objective of this study was to determine the influence of the pretreatment of serum samples on the performance of LAT as an immunodiagnostic assay for detection of antibodies in PCM patients.

Materials and Methods

**Serum samples.** Thirty serum samples obtained from patients with active PCM (26 males and 4 females ranging from 22 to 75 years of age; 3 with the acute form and 27 with the chronic form of the disease) were included in this study. The diagnosis was confirmed by the presence of *P. brasiliensis* in biological fluids (4/30) and the ID test (26/30). Six serum samples from patients with histoplasmosis (HP), 5 from patients with acute invasive ASP, 49 from patients with nonfungal disease (NFD; bacterial infections),...
and 11 from healthy individuals (normal human serum [NHS]) were used as controls (non-PCM patients). The positive control for both ID and LAT tests was produced by mixing six serum samples from confirmed PCM patients. All serum samples were divided into aliquots and stored at -20°C.

**Pretreatment of serum samples.** The pretreatment of all serum samples was conducted as follows: a serum sample was mixed with an equal volume of dilution buffer (2.05% glucose, 0.8% trisodium citrate, 0.42% sodium chloride, 0.1% Tween 20 in water, pH 6.4) and incubated for 30 min at 37°C before testing (23). The pretreatment step was originally developed to test previously stored serum samples and to minimize cross-reactivity. The assay is considered qualitative because only nondiluted serum samples were used.

**Fungal strains and antigen preparation.** Six isolates of *P. brasiliensis* (PhIOC, Pb34, Pb30, PbWT, Pb113, Pb101) and reference strains (B339A[TCC 32069] and Pb01-like [ATCC MYA826]), obtained from the culture collection of the Instituto Evandro Chagas, were selected for this study. All isolates were initially grown in Sabouraud medium (Difco, Sparks, MD). These cultures were then subcultured on 1,800-ml Erlenmeyer flasks containing 100 ml of yeast extract-peptone-dextrose (YPD) broth (Difco, Sparks, MD). These cultures were then transferred to 500-ml Erlenmeyer flasks containing 50 ml of YPD broth. The flasks were then incubated with shaker at 30 rpm (Panem, Sao Paulo, Brazil). The cultures obtained were transferred to 1,800-ml Fernbach flasks containing 500 ml of YPD broth. The flask was then incubated as described above for 7 more days. The culture was killed with thimerosal (0.2 g/liter). Supernatants were collected following paper filtration, concentrated under vacuum at 45°C, and dialyzed against distilled water. After dialysis, the solutions were concentrated again (7). The antigenic preparations obtained from each isolate were used to produce a pool of antigens and employed in this study through the LA procedure and LAT.

**Analysis of antigenic preparation and LA solution.** The pool of exoantigens derived from *P. brasiliensis* isolates was reactive in the ID test (1:32) with the positive serum control. Exoantigen profile showed 5 components ranging from 22 to 180 kDa, as shown by SDS-PAGE. Two dominant antigens of the fungus (gp43 and gp70) were observed. The LA solution was reactive to a level of 3+ with both nonpretreated and pretreated positive serum controls.

**Detection of antibodies in serum by ID test and LAT.** In the ID test, four (4/30; 13%) samples from PCM patients were negative. All control samples (from HP, ASP, and NFD patients and NHS) were negative in this test. The precipitating reactions were observed in both clinical forms of PCM, with titers ranging from 1:2 to 1:024. Among the four negative patients, three presented with the chronic unifocal form of PCM and one had the chronic multifocal form.

The LAT of nonpretreated samples produced false-negative results in eight of the PCM samples (8/30). Cross-reactivity was observed in samples from HP (1/6), ASP (3/5), and NFD (11/49) patients, and no reaction was obtained with NHS (Table 1). On the other hand, results from the LAT of PCM patients using pretreated samples showed only three negative results (3/30). Some samples used as controls (non-PCM patients) presented cross-reactivity: 2/5 for ASP and 5/49 for NFD. No reaction was obtained with HP or NHS samples (Table 1).

The LAT of nonpretreated samples showed reactions of 1+ (10/30), 2+ (5/30), 3+ (4/30), and 4+ (3/30). The cutoff indicated 1+ for the best sensitivity and specificity values. In heterologous serum samples from HP and ASP patients, identified as controls, we observed reactions ranging from 1+ to 2+. The samples of NFD showed weak reactions (1+ in 4 samples); however, strong reactions (2+ or 3+) were also observed, but only in the nondiluted serum samples. False-positive results were not observed with NHS samples (Fig. 1A). Comparison of antibody detection based on the clinical forms of PCM showed that in the

### TABLE 1 Results of detection of antibodies using ID test and LAT with and without pretreatment of sera

<table>
<thead>
<tr>
<th>Patient group (n)</th>
<th>ID test</th>
<th>LAT With serum pretreatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LAT Without serum pretreatment&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Paracoccidioidomycosis (30)</td>
<td>26 (87)</td>
<td>4 (13)</td>
<td>22 (73)</td>
</tr>
<tr>
<td>Histoplasmosis (6)</td>
<td>0</td>
<td>6 (100)</td>
<td>1 (17)</td>
</tr>
<tr>
<td>Aspergillosis (5)</td>
<td>0</td>
<td>5 (100)</td>
<td>3 (60)</td>
</tr>
<tr>
<td>Nonfungal infection (49)</td>
<td>0</td>
<td>49 (100)</td>
<td>11 (22)</td>
</tr>
<tr>
<td>Healthy individuals (11)</td>
<td>0</td>
<td>11 (100)</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> ID, immunodiffusion; LAT, latex agglutination test.
<sup>b</sup> Sensitivity, 90%; specificity, 90%.
<sup>c</sup> Sensitivity, 90%; specificity, 90%.

<sup>3</sup> Sensitivity, 73%; specificity, 79%.

<sup>4</sup> Sensitivity, 90%; specificity, 90%.

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Influence of Pretreating Serum on Diagnosis of PCM

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unifocal and multifocal chronic forms of the disease, patterns of lower levels of agglutination are obtained in the LAT from non-pretreated samples than pretreated samples (Fig. 2A and B).

In the LAT of pretreated PCM samples, the agglutination patterns were observed to range from 1+ to 4+. Additionally, weak agglutinations (1+ and 2+) were observed in the control samples (Fig. 1B). According to the clinical forms of PCM, the three samples from patients with the acute form of PCM showed negative results (1/3) or a 1+ result (2/3) in the LAT. The results from patients with the chronic form of PCM were diversified. Samples from patients with the chronic unifocal cases produced 1+ (7/17), 2+ (4/17), 3+ (2/17), and 4+ (2/17) agglutinations, and two samples were negative. Samples from patients with the chronic multifocal form showed agglutinations of 1+ (4/10), 2+ (3/10), 3+ (2/10), and 4+ (1/10) (Fig. 2B).

The sensitivity and specificity values obtained from the ID test were 87% and 100%, respectively; those of LAT of nonpretreated samples were 73% and 79%, respectively; and those of LAT of pretreated samples were 90% and 90%, respectively. The agreement between the ID test and LAT of pretreated samples was 83%.

DISCUSSION

The ID test is a serological tool whose results are expressed in antibody titers. However, it takes at least 3 days to produce results. In the present study, we evaluated the effects of the treatment of serum samples before the LAT in an effort aimed at improving the sensitivity and specificity of this test. We obtained important evidence of the efficacy of using a pretreatment tool before executing the LAT, based on the observation of higher sensitivity (17%) and specificity (11%) rates in the LAT developed in this study. After the development of a pretreatment step for the samples, the rheumatoid factor and immune complexes were discovered to be major interfering factors for the diagnosis of cryptococcosis through LAT. These interferences were either decreased or eliminated, which directly contributed to the performance of the test and has been essential for the current use of the methodology (11, 19). For the serodiagnosis of PCM based on LAT, such factors remain unclear. This was the stimulant to our study to outline a route for effective diagnosis.

The LAT is a methodology widely employed in the serodiagnosis of opportunistic fungal infections and works exceptionally well for early diagnosis (2). Nevertheless, for the immunodiagnosis of PCM, only two groups (18, 21) evaluated the performance of this test. These studies indicated that after the elimination/decrease of interfering factors, the LAT can be a diagnostic tool, principally in the regions of endemicity where the disease is highly neglected. In the present study, it was possible to correctly detect antibodies...
against *P. brasiliensis* in 27 out of 30 PCM patient serum samples using the LAT after pretreatment. We employed a pretreatment step which has been used by other authors to eliminate strong reactions from heterologous serum samples and to intensify weak reactions of samples that had been in long-term storage (22). The procedure is easy to execute routinely in the laboratory, with the only disadvantage being a 30-min increase in the waiting time before obtaining results. Corroborating the findings of Yu et al. (23), we obtained weak reactions with a few NFD posttreatment samples (5/49), which indicated a possibility for future improvement of this assay by determining an LAT cutoff based on previous dilutions and pretreatment of the samples.

The detection of specific antibodies against *P. brasiliensis* antigens in sera of patients suspected of having PCM is an additional tool for diagnosis of the disease. However, when the immunological tests, such as the ID test, are performed with a crude extract, there are some false-positive results due to cross-reactivity with other fungal diseases, including histoplasmosis and aspergillosis (14). Here, we observed that when using the same crude preparation, we can lower cross-reactivity with HP and ASP patient serum samples after the pretreatment of the samples (Table 1). Previous studies report the occurrence of cross-reactivity between serum samples from PCM and ASP patients (17), and cross-reactivity was observed in two pretreated samples from ASP patients in the present study, suggesting the presence of antibodies that can recognize antigenic fractions of immunogenic proteins of both fungal agents, *Aspergillus* and *P. brasiliensis*. These reactions also indicate the possibility of coupling deglycosylated antigenic preparations to the latex particle; this may also influence the performance of the test.

False-negative results were observed in samples tested before the pretreatment (Table 1), but these reactions were recovered after the pretreatment, suggesting the interference of both macro-globulins and immune complexes in the serum samples. Unfortunately, three pretreated samples from PCM patients did not react after the LAT, which suggested that this test may detect larger amounts of specific antibodies only in the serum of patients. Other studies stated the need to perform at least two serological techniques for the diagnosis of PCM, according to what had been reported before (8). Thus, the sensitivity and specificity values obtained in this study encourage the consideration of the LAT as a tool for screening patients in the regions where PCM is endemic but with an inadequate infrastructure for developing more specific assays.

On the basis of the clinical presentation of the disease, the agglutination pattern in the LAT before and after pretreatment of samples clearly showed the effect of treatment of the samples, providing effective results in comparison with the gold standard test, ID (Table 1). A previous study indicated the presence of elevated levels of IgM antibodies in acute clinical forms of PCM and trace levels of these antibodies in the other manifestations of the disease (12). This is a crucial parameter for the performance of a test based on agglutination of latex particles for the detection of antibodies and can cause the weak reactions (1 +) observed in our study (Fig. 1), suggesting that the LAT methodology will be important for diagnoses based on the detection of antibodies in acute cases of PCM. Consequently, further studies on using the LAT for the detection of *P. brasiliensis* antigens in serum samples from patients are needed to obtain conclusions upon the advantages or disadvantages of using the LAT as a tool for serodiagnosis of PCM in patients who present with one of the two clinical forms.

In conclusion, we describe the increase in sensitivity and specificity of the LAT for detecting antibodies in PCM patient serum samples by using an additional treatment before the test. We also showed the influence of using fresh serum on the performance of the test. However, the discovery of other interfering factors responsible for cross-reactivity with heterologous samples will be crucial to elevate the specificity and to suggest this methodology as an effective form of serodiagnosis of PCM.

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**REFERENCES**


