

Effect of serum sample inactivation on the performance of latex agglutination test for paracoccidioidomycosis serodiagnosis

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Paracoccidioidomycosis is diagnosed from the direct observation of the causative agent, but serology can facilitate and decrease the time required for diagnosis. The objective of this study was to determine the influence of serum sample inactivation on the performance of the latex agglutination test (LAT) for detecting antibodies against Paracoccidioides brasiliensis. The sensitivity of LAT from inactivated or non-inactivated samples was 73% and 83%, respectively and the LAT selectivity was 79% and 90%, respectively. The LAT evaluated here was no more specific than the double-immunodiffusion assay. We suggest the investigation of other methods for improving the LAT, such as the use of deglycosylated antigen.

Key words: paracoccidioidomycosis - serodiagnosis - latex agglutination test - inactivation of samples

Endemic to Latin America, paracoccidioidomycosis (PCM) is a human disease with high incidence in Brazil, Venezuela, Colombia and Argentina (Blotta et al. 1999). PCM is caused by the thermo-regulated dimorphic fungus *Paracoccidioides brasiliensis* (Manns et al. 1996). The definitive diagnosis of PCM is made by identifying budding yeast in biopsies or other pathological material. However, the fungus is difficult to observe in many cases; diagnosis is often performed through serological assays (Mendes-Gianinni et al. 1994). Several serological assays have been used for the early diagnosis of PCM. However, a standard diagnostic method has been difficult to establish because of variation in assay sensitivity, specificity and reproducibility (Camargo 2008).

Double immunodiffusion (ID) is the most frequently used test for the immunodiagnosis of PCM and the use of Ag7 as an antigenic preparation standard is believed to be necessary for a successful diagnosis with this methodology (Camargo et al. 2003). Despite its low complexity, few laboratories can use ID for routine diagnosis. Nevertheless, countries and endemic areas with poor infrastructure require more executable methods at the clinical level. The latex agglutination test (LAT), a slide agglutination test for the serodiagnosis of PCM, was described primarily in the 1970s, but the results obtained with this assay were inconclusive (Restrepo & Moncada 1978). Recently, our laboratory evaluated LAT to detect antibodies against *P. brasiliensis* in the sera of patients with both clinical forms of PCM (Silveira-Gomes et al. 2011) and the sensitivity and specificity rates obtained

were 84% and 81%, respectively. Cross-reactivity was observed with pulmonary fungal [aspergillosis (ASP) and histoplasmosis (HP)] and bacterial infections.

The serodiagnosis of cryptococcosis led to the use of LAT in the immunological diagnosis of fungal infections (Bloomfield et al. 1963). However, this accomplishment was only possible because interfering factors could be detected and then corrected for, which allowed for high performance of the assay. Several studies use different methods to minimize the effect of interfering factors while performing these tests. The mostly widely used technique is the prior inactivation of serum to avoid the interference of complement and other serum proteins in the assay (Stockman & Roberts 1983). The inactivation of samples prior to testing is critical for complement fixation (Almeida & Cunha 1981), but it is still unclear how inactivation influences the efficacy of LAT to detect antibodies against *P. brasiliensis*. In our previous study, all serum samples were tested in their fresh forms, which is the same procedure used in the gold standard test (ID test). The aim of the current study was to describe the influence of prior the inactivation of serum samples on the performance of LAT for detecting antibodies against PCM.

A total of 30 PCM patients with diagnoses established by either direct examination (n = 4) or positive serological ID tests (n = 26) were included in this study. This population was composed of 26 males and four females aged 22-75 years. Approximately 10% of the patients presented with the acute form of the disease, while 90% had the chronic form. Heterologous serum samples of patients with HP (n = 6), ASP (n = 5) and non-fungal disease (NFD) (patients presenting bacterial infections; n = 49) were used. Eleven serum samples from healthy individuals with no history of pulmonary disease were also studied as negative controls and were termed normal human sera (NHS). All serum specimens were divided into aliquots and were stored at -20°C. Serum samples were inactivated by incubation at 56°C for 30 min prior to testing.

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P. brasiliensis strains (Pb01-like and B339) and isolates (PbIOC, Pb113, Pb34, Pb30, PbWT, Pb101) were used to produce exoantigen as previously described (Camargo et al. 2003). The exoantigen pool was coupled to latex particles. The protein concentration was measured by the Bradford method (Bradford 1976) and the electrophoretic pattern was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Laemmli 1970) followed by silver nitrate staining. Immunodiffusion was performed with serum samples as described previously (Camargo et al. 1988). Each PCM, HP, ASP, NFD and NHS serum sample was tested against the antigenic preparation of *P. brasiliensis* and titrated.

The LAT was performed as previously described (Silveira-Gomes et al. 2011). The latex particles (1% suspension) were coated with the antigenic preparation (400 µg/mL) in carbonate-bicarbonate-buffered saline solution (pH 9.2) overnight before the addition of bovine serum albumin to 0.1%. The optimal solution used produced a clear agglutination with the serum positive control (pool of PCM sera). The test was performed by mixing 25 µL of latex suspension with 25 µL of serum test sample on a dark slide and a result was considered positive for *P. brasiliensis* antibodies when agglutination (clumping of 1+ to 4+) was observed. After testing undiluted serum samples, if agglutinations were graded 1+ or higher, the reactions were titrated (1:2 through 1:1024). All samples were tested without and with prior inactivation. Sensitivity and specificity values, as well as receiving operating characteristics (ROC) curve analysis (to determine the positive cut-off that would give optimal performance of the LAT), were obtained using BioEstat (version 5.0).

Silver staining showed a profile of five proteins ranging from 20-180 kDa. The serologic reaction of this preparation presented three bands against control serum in the ID test, whose titre was 1:32. The LAT latex particle solution was reactive at 3+ with the control serum, whose titres were 1:16 and 1:8 in samples without and with inactivation, respectively.

The sensitivity and specificity of LAT for the samples without and with inactivation was obtained by ROC curve analysis. The sensitivity values from ID test, LAT without and with inactivation were 87%, 73% and 83%,

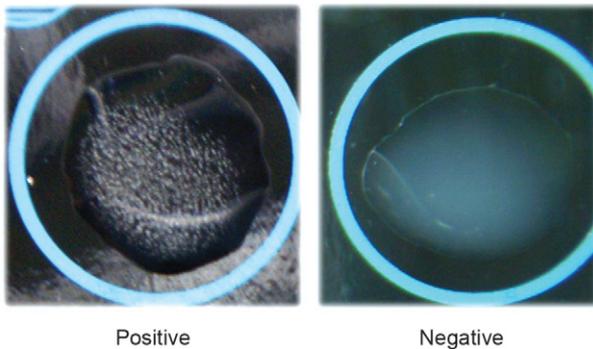


Fig. 1: all results obtained with paracoccidioidomycosis samples using double immunodiffusion test and latex agglutination test without and with inactivated samples.

respectively; the specificity values were 100%, 79% and 90%, respectively. It was predicted that a cut-off of 1+ with non-diluted samples would produce the best sensitivity and specificity values. Although the ROC curve analysis showed improvements in the sensitivity and specificity values, the majority of weak reactions (1+) were still retained. This result indicated that there were agglutinating antibodies with little avidity in the samples. In a previous study (Silveira-Gomes et al. 2011), LAT was evaluated for serodiagnosis of PCM, but using fresh samples resulted in a large number of non-specific reactions. Here, the sensitivity and specificity were improved by 10% and 11%, respectively, after inactivation of serum samples. These results were possibly due to the reduction of heterologous serum sample reactions, suggesting that serum macroglobulins interfere in the reactions of non-inactivated samples.

Four samples from PCM patients were negative with ID test and the titres of antibodies in the positive samples (26/30) ranged from pure (without dilution) to 1:1204 (Fig. 1). None of the heterologous and control serum samples were reactive for this test. In the LAT for non-inactivated samples of PCM patients, 22 samples were positive and possessed agglutination patterns varying

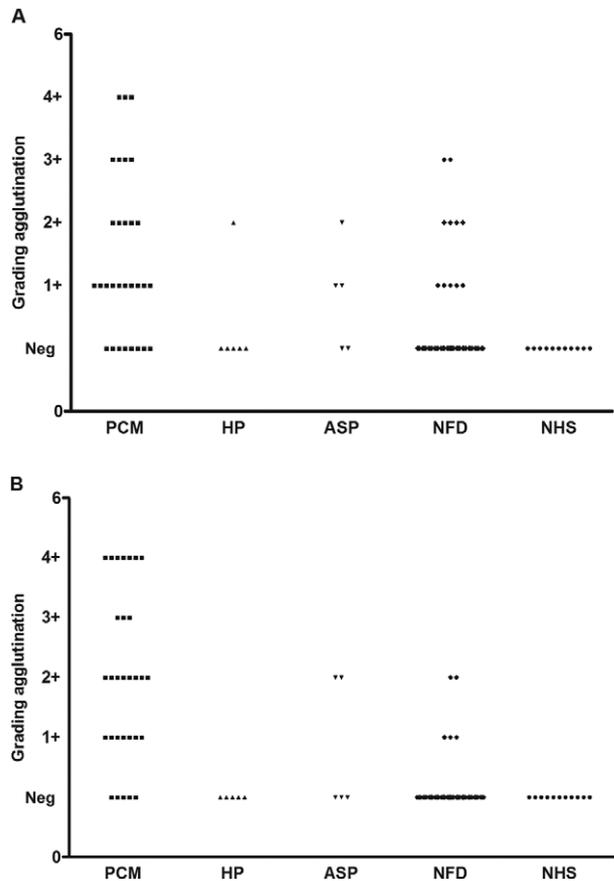


Fig. 2: results of agglutinations from paracoccidioidomycosis (PCM), histoplasmosis (HP), aspergillosis (ASP), non-fungal diseases (NFD) and normal human sera (NHS) in the latex agglutination test (LAT) without (A) and with (B) inactivated samples.

from 1+ to 4+ (Fig. 2A) and titres ranging from pure to 1:64 (Fig. 1). Eight samples tested negative and heterologous sera presented cross-reactivity, but no reaction was observed in NHS (Fig. 2A). After inactivation, five samples from PCM patients were negative and the positive samples showed reactions ranging from 1+ to 4+ (Fig. 2B) and titres ranging from 1:4-1:256 (Fig. 1). Only one PCM patient positive by direct examination tested negative by LAT with prior inactivation; three samples tested were negative by LAT without prior inactivation. Two ASP and five NFD samples were reactive after inactivation and agglutinations with human normal sera were not observed (Fig. 2B). Prozone effect was observed in two PCM samples tested by LAT with inactivation, which shows the need for dilution of samples during this test.

In summary, we observed the reduction, but not the elimination, of cross-reactivity and false-negative results. Serum sample inactivation takes an additional 30 min, but without the requirement of a complex experimental setup for this procedure. However, it will be necessary to discover other ways to make the LAT more sensitive and specific, such as using antigens of *P. brasiliensis* in their deglycosylated form for coupling particles (Puccia & Travassos 1991) or treating samples with 2- β -mercaptoethanol and pronase (Engler & Shea 1994, Whittier et al. 1994).

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