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Diagnosis of Paracoccidioidomycosis by Dot Immunobinding Assay for Antibody Detection Using the Purified and Specific Antigen gp43

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The dot immunobinding assay, a rapid, visually read test, was adapted for serodiagnosis and follow-up of paracoccidioidomycosis (PCM). Purified gp43 antigen was tested before and after sodium metaperiodate treatment. To evaluate the assay, it was tested with sera from PCM, histoplasmosis, Jorge Lobo's disease, aspergillosis, candidiasis, and cryptococcosis patients and healthy subjects. Native gp43 gave positive results with all sera from PCM patients and weakly positive results with sera from Jorge Lobo's disease patients (31.3%). No false-positive results were obtained when periodate-treated gp43 was used as the antigen. These results indicate that the dot immunobinding test is sensitive, specific, economical, and fast for serodiagnosis and follow-up studies of PCM.

Paracoccidioidomycosis (PCM), caused by *Paracoccidioides brasiliensis*, is a fungal disease that affects many individuals in Latin America. Since it is not classified as a notifiable disease, the exact number of patients is unknown. Diagnosis can be obtained by direct visualization of the fungus in biologic materials, by culture, or indirectly by various serologic tests. Although there are serologic procedures that provide diagnostic information, many are time-consuming and lack specificity. In the case of PCM, most serologic diagnostic assays use crude antigenic preparations, which give cross-reactions. Therefore, there is a need for more rapid, sensitive, and specific procedures for the diagnosis of fungal diseases. Such a need stems from the fact that a definitive diagnosis is helpful in order to initiate appropriate management as well as to evaluate the use of new therapeutics.

Dot immunobinding assay is a simple and rapid technique based on the principle of enzyme immunoassays, which when used with purified antigens can provide an efficient diagnosis for PCM. A 43-kDa glycoprotein (gp43) of *P. brasiliensis* secreted in the supernatant of cultures has proven to be a specific antigen for the serodiagnosis of PCM in immunodiffusion tests (4). Dot immunobinding assay has been used with success for serodiagnosis of human hydatidosis, toxoplasmosis, visceral leishmaniasis, and other diseases (2, 7, 12).

This article describes a rapid and accurate dot immunobinding assay for the detection of antibodies to *P. brasiliensis* gp43 antigen and the titration of the specific antibodies in the patient's sera during antimycotic therapy.

Sera were obtained from 50 PCM patients with active disease, 25 with the acute form and 25 with the chronic form. All sera were taken before antimycotic therapy. Ten PCM patients were serologically monitored during the course of 2 years of therapy (a total of 30 serum samples). All PCM sera were titrated for immunoglobulin G (IgG) anti-gp43. Sera from individuals with systemic candidiasis (n = 16), aspergillosis (n = 16), Jorge Lobo's disease (n = 16), histoplasmosis (n = 8), and cryptococcosis (n = 8) and from healthy subjects (n = 50) were also tested as controls.

Concentrated antigen was prepared from a 7-day-old yeast phase culture of *P. brasiliensis* B-339, according to methods described in our previous paper (4), and used to purify the gp43 antigen.

Purified gp43 was obtained by affinity column chromatography of the crude antigen of *P. brasiliensis* B-339 on rabbit IgG anti-gp43 linked to Sepharose 4B (8). The gp43 eluted from this column with 0.1 M HCl-glycine buffer, pH 2.8, was immediately neutralized with 2 M Tris, pH 9.0, and concentrated by ultrafiltration with a PM 10 membrane (Amicon). The purity of the gp43 was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5). Protein content was measured by the Bradford method (1).

One hundred microliters of gp43 (400 ng) was applied to a nitrocellulose membrane (Millipore, São Paulo, Brazil) in each well of a 96-well microfiltration apparatus (Bio-Rad, Richmond, Calif.). To enhance binding of the gp43 to the membrane, gravity filtration was used. The membrane was blocked with 200 μl of 20 mM Tris-HCl, pH 7.5–500 mM NaCl (TBS) plus 5% nonfat dry milk (TBS-M) per well for 1 h to prevent nonspecific binding. After sensitization of the membrane with gp43, this molecule was oxidized with 10 mM sodium metaperiodate (Merck) in 50 mM acetate buffer, pH 4.5, for 30 min at 28°C in the dark in order to eliminate carbohydrate epitopes sensitive to periodate oxidation. The reaction was interrupted by adding 0.5% glycerol for 10 min at room temperature. Aldehyde groups were then blocked with 1% (final concentration) glycine in 0.1 M Tris-HCl buffer, pH 7.4, containing 0.2% bovine serum albumin for 1 h at room temperature. The membrane was washed with 200 μl of 0.05% Tween 20-TBS (TTBS) per well, and the serum diluted 1:100 in TBS-M was applied and allowed to filter through by gravity. The membrane was washed again, and 100 μl of a 1:500 dilution of secondary antibody labelled with goat IgG anti-human IgG-peroxidase (Sigma) per well was applied and allowed to filter by gravity, and the membrane was then washed. Each gravity filtration step took 45 to 60 min. Then, the membrane was immersed in a fresh mixture of 5 mg of 3,3'-diaminobenzidine-4HCl (Sigma) in 50 ml of 0.1 M Tris buffer, pH 7.5, plus 5 μl of 30% H_2O_2. The

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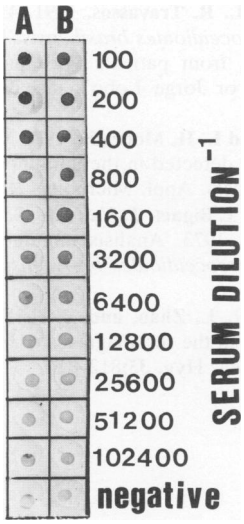

FIG. 1. Titration of PCM patient serum with a titer of 1/102,400. A, chronic form PCM patient serum; B, acute form.

specific positive reactions were visible as a brown precipitate. In the quantitative determinations, the sera were diluted from 1/100 to 1/102,400, and the same protocol was followed. For the follow-up study, the sequential sera were diluted at 1:100. Control reactions followed the same protocol except that periodate was not added.

Typical PCM titrations in serum (IgG antibodies) obtained by dot immunobinding assay are shown in Fig. 1. The titers of the PCM sera varied from 1/800 to 1/102,400 (data not shown).

All patients undergoing antimycotic treatment showed a decreased titer for antibodies in their sera. Typical anti-gp43 IgG antibody reactions obtained by dot immunobinding assay of serum from PCM patients undergoing treatment are shown in Fig. 2. Similar results were obtained with the other patients (data not shown), indicating that the response to gp43 is a good indication of clinical improvement.

The results of the present study show that dot immunobinding assay with purified *P. brasiliensis* gp43 can be successfully adapted to the serological diagnosis of human PCM and for use in the follow-up of patients undergoing antimycotic therapy. In our series of 50 cases of PCM, 100% of the assays gave positive results. The rate of false-positive reactions was 4.3% for all non-PCM cases, including healthy

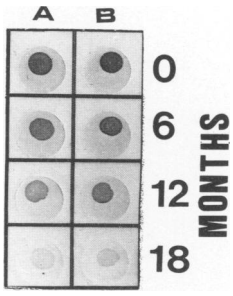

FIG. 2. Serological follow-up of two PCM patients during antimycotic therapy. A, chronic form; B, acute form. Serum dilution was 1:100 on each dot. The number of months of antimycotic treatment is indicated. Month 0, before treatment.

subjects with native gp43. However, no false-positive reactions were obtained for assays in which we used gp43 treated with sodium metaperiodate which showed a sensitivity and specificity of 100%.

The immunodiffusion test which has been the test of choice in the diagnosis of PCM has a sensitivity and specificity that vary from 94 to 100%, according to some authors (4, 10, 11). Furthermore, the nonspecific reactions reported may be related to the use of crude antigens. The enzyme-linked immunosorbent assay (ELISA) has also been employed for the serological diagnosis of PCM, but its specificity depends on the previous adsorption of sera with *Candida albicans* or *Histoplasma capsulatum* antigens (3, 6).

In PCM, Puccia and Travassos (9) investigated the role of carbohydrate epitopes responsible for the cross-reactions in ELISAs, using native and deglycosylated gp43. Cross-reactions with histoplasmosis and Jorge Lobo's disease sera in ELISA were predominantly attributed to periodate-sensitive epitopes containing galactosyl residues. They have also found that >85% of the reactions of PCM sera with gp43 involve peptide epitopes. In our dot assay, the specificity of the test was obtained after oxidation of periodate-sensitive carbohydrate epitopes of gp43, which seems to be responsible for the cross-reaction with Jorge Lobo's disease, confirming a previous report (9).

Our study showed that the dot immunobinding assay was useful in the analysis of humoral response (IgG anti-gp43) in PCM. As seen in Fig. 2, the level of anti-gp43 antibodies decreased during regular antimycotic treatment. Similar results were observed for the other patients studied (data not shown).

In conclusion, dot blot assay using gp43 has great potential for use in serodiagnosis of PCM and follow-up studies. This method is readily adaptable to seroepidemiologic investigations in the field to rapidly screen a large number of serum samples at a single dilution. Moreover, the dot blot uses minute amounts of antigen per test, making this assay specially useful in cases with only limited amounts of antigens available. Positive results are easily interpreted visually as colored dots on the white nitrocellulose membrane. The protocol is very simple, requires no special instruments for evaluation of results, and can be completed within 3 h, while an immunodiffusion test requries at least 48 h. The nitrocellulose spotted with periodate-treated gp43 can be conserved at −20°C for long periods before being used. Such a stable, rapid, and inexpensive method can be of great benefit in developing countries where expenditures for health care are low and expensive equipment for state-of-the-art diagnostic procedures is cost prohibitive.

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