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Short communication

Interpretation and clinical correlation of serological tests in paracoccidioidomycosis

A. C. F. DO VALLE*, R. L. B. COSTA*, P. C. FIALHO MONTEIRO†, J. VON HELDER†, M. M. MUNIZ† & R. M. ZANCOPÉ-OLIVEIRA†

**Department of Infectious Diseases, Evandro Chagas Research Center, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil; †Mycology Section, Department of Microbiology, Immunology and Parasitology (DMIP) Evandro Chagas Research Center, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil*

In order to correlate the findings of two serological tests, double immunodiffusion (IDD) and immunoblotting (IB), with the clinical diagnosis and follow-up of paracoccidioidomycosis (PCM), 325 serum samples from PCM patients were tested at the beginning of specific therapy and after its completion. Group I included 245 PCM patients at the onset of symptoms without treatment. In 221 cases (90·2%) the IDD showed positive reactions and in 24 (9·8%) the results were negative. Of the 24 IDD negative samples, 23 were investigated by IB and were positive. Group II included 80 PCM patients under follow-up after treatment. There were four cases of relapse in which the IDD and IB tests were positive (100%). Among the 76 cases with inactive mycotic infection, the IDD was negative in 71·2% and positive in 28·8%; the IB was positive in all cases (100%). The control group (Group III) included 27 samples from patients with other mycoses, tuberculosis and from healthy individuals. All showed negative IDD tests but positive reactions with IB, which could be abolished by serum dilutions without altering the PCM reactivity. Therefore, the utilization of the IB, an immunoenzymatic method for the diagnosis of PCM, raised the sensitivity to 100%.

Keywords immunology, *Paracoccidioides brasiliensis*, paracoccidioidomycosis, serological tests

Introduction

Paracoccidioidomycosis (PCM), caused by *Paracoccidioides brasiliensis* (Pb), is the most prevalent systemic mycosis in Latin America. Serologic evidence is the prime diagnostic indicator of PCM and is essential mainly in cases with cryptic lesions in internal organs. Various serological tests such as complement fixation, double immunodiffusion in agar gel (IDD), indirect immunofluorescence, immunoelectrophoresis, counter-immunoelectrophoresis and immunoblotting (IB) have been used [1]. Antibody detection of Pb antigens has also been employed in the follow-up of antifungal drug responses [2–5]. The first test widely used in the diagnosis and follow-up of PCM patients was complement fixation but, because of its low specificity and the technical difficulty involved, it is now seldom used. IDD is used routinely by clinical laboratories, due to its easy procedures, its high specificity and its 65–100% sensitivity [2]. More recently, the IB test has been used in the evaluation of diagnosis and cure of PCM [6–8].

The sensitivity and specificity of serological reactions are dependent on the test and the antigen used. Several authors suggest the use of a species-specific 43 kDa glycoprotein (gp43) as a diagnostic antigen [9,10]. The detection of circulating Pb antigen is another highly sensitive method for diagnosis and follow-up of PCM [11–14].

Correspondence: Antonio C. Francesconi do Valle, Centro de Pesquisas Hospital Evandro Chagas, FIOCRUZ, Avenida Brasil, 4365, Manguinhos, CEP 21045-900, Rio de Janeiro, RJ, Brazil. Tel.: +55 21 5984263; fax: +55 21 5909988; e-mail: afvalle@fiocruz.br

In this paper, we report both the correlation between IDD, IB and the extent of PCM as evaluated by clinical, radiological and mycological data, in order to facilitate the interpretation of these tests in the diagnosis and follow up of PCM.

Materials and methods

Study population

We used serum from 325 patients with PCM before, during and after therapy, at the Centro de Pesquisas Hospital Evandro Chagas (CPqHEC). Diagnoses of PCM were confirmed by demonstrating Pb in direct examination, culture or histopathology.

Two groups of patients were defined. Group I comprised patients who had not received antimycotic therapy: 245 such patients were subdivided into 217 with the adult type PCM, and 28 with the juvenile type. Group II included 80 patients being followed up after treatment for 8 months to 17 years. One of these patients had juvenile PCM and 79 had the adult type. Seventy-one patients had been treated with sulfonamides (sulfadiazine, sulfamethoxipiridazine or sulfamethoxazole plus trimethoprim) for 2 years; the remaining patients received ketoconazole (five patients) or amphotericin B (four patients) after therapeutic failure or development of hypersensitivity to sulfonamides.

For monitoring of the severity of disease, patients were given a complete physical examination, a complete and differential blood count, blood and urine analysis, parasite stool tests, chest radiography, endoscopy of upper digestive and respiratory tract (UADT), lesion biopsy, paracoccidioidin skin test, IDD and IB, and adrenal evaluation by comparing basal plasmatic cortisol levels with those after adrenocorticotropic hormone (ACTH) stimulus.

A control group (Group III) of serum samples was obtained from patients with histoplasmosis, coccidioidomycosis, aspergillosis, coccidioidomycosis, cryptococcosis and tuberculosis, as well as healthy individuals.

Antigens

Yeast antigen was prepared from a pool of Pb strains (18, SM, 192 and 265) grown in synthetic broth as previously described [15]. After 30 days incubation, the cells were killed with an aqueous thimerosal solution (1:10 000), and left to stand for 48 h at 4 °C. The supernatant was filtered through paper Whatman No. 1 centrifuged at 1050 *g* for 10 min, concentrated 20-fold by pervaporation, and dialyzed against phosphate-buffered saline (PBS) (0·01 M; pH 7·2). The protein concentration was measured with a dye-binding assay in comparison with an albumin-globulin standard [16].

Immunodiffusion

The reactions were performed in 1% agar noble (Difco Laboratories, Detroit, MI, USA) according to the modified method of Ouchterlony [17]. All control serum samples were checked by immunodiffusion (ID) against *Histoplasma capsulatum*, Pb and *Aspergillus fumigatus* exocellullar antigens produced in the Mycology Section, Department of Microbiology, Immunology and Parasitology, CPq-HEC, using standard procedures [18].

Sodium docecyl sulfate-polyacrylamide gel electrophoresis

Antigen (87·5 µg final concentration) was denatured at 100 °C for 5 min in 0·125 M Tris-HCl, pH 6·8, containing 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol and 0·025% bromophenol blue. SDS-polyacrylamide gel electrophoresis (PAGE) was performed with 10% resolving gels and 4% stacking gels in a Mini-Protean II Electrophoresis Cell (BioRad Laboratories, Richmond, CA, USA). The electrophoresis conditions were 10 mA of constant current for stacking and 30 mA for separation of the proteins.

Immunoblotting

Gels were electrotransferred to nitrocellulose membranes in a Mini Trans-Blot cell (BioRad) containing transfer buffer (25 mM Tris-HCl, 192 mM glycine and methanol [20% v/v], pH 8·3) operated at 400 mA for 1 h. Free binding sites in the membranes were blocked by incubation for 30 min in 5% (wt/vol) non-fat dry milk in PBS containing 0·3% Tween 20 (PBS-T). Membranes were sliced vertically and strips were incubated for 60 min at room temperature with serum specimens diluted 1/100 in PBS-T containing 5% nonfat milk. Strips were washed in PBS four times for 20 min each; goat anti-human immunoglobulin (Ig)G-horseradish peroxidase conjugate (Sigma Chemical Co., St. Louis, MO, USA) diluted in PBS-T was then added and incubated as described above. Blot strips then were washed and incubated with substrate solution consisting of 0·5 mg ml^{-1} of 3,3'-diaminobenzidine tetrahydrochloride (Sigma) plus 5 µl of H_2O_2 (30% vol/vol) per 50 µl. After color development, strips were rinsed exhaustively in distilled water [19].

Results

Table 1 summarizes and compares results obtained in the two PCM groups tested by IDD and IB. Among the 245

Table 1 Comparison of double immunodiffusion and immunoblotting tests to detect antibodies against *P. brasiliensis* filtrate antigen in serum samples from paracoccidioidomycosis patients

Source of human serum samples	Immunodiffusion		Immunoblotting	
	No. positive/*n**	%	No. positive/*n**	%
Group I	221/245	90·2	23/23†	100
Adult type	201/217	92·6	15/23	100
Juvenile type‡	20/28	71·5	8/8	100
Group II	26/80	32·5	80/80	100
Adult type	26/79	32·9	79/79	100
Juvenile type	0	–	1/1	100
Total	247/325	76	103/103	100

*, Total number tested;
†, immunoblotting was performed only in 23 of the 24 cases, which were nonreactive in IDD;
‡, negative IDD tests were more frequent in the juvenile type ($\chi^2 = 12{\cdot}17$ [$P < 0{\cdot}001$]).

Group I serum samples obtained during active disease before treatment and tested by IDD, 221 (90·2%) samples reacted positively and 24 (9·8%) were negative, especially samples from patients with juvenile PCM ($P < 0{\cdot}001$). Among these 24 negative samples, 23 were tested by IB and all were positive. In Group II, consisting of 80 treated PCM patients, relapse of the disease was verified in four cases; the IDD and IB results were positive in all of them. In 76 Group II cases without clinical signs of disease, the IDD was negative in 71% and positive in 29%. However, the IB was positive in all of them (Table 2). Among 27 patients tested by IDD and followed from 8 to 24 months after treatment, 12 (44%) serum samples were nonreactive and 15 (56%) were positive. Among 49 patients with follow-up longer than 25 months, 42 (86%) showed negative and 7 (14%) positive reactions, although in the follow-up of 27 patients for up to 24 months, 15 showed positive and 12 negative results in this reaction, suggesting a tendency to become negative over the years. All samples tested by IB reacted strongly with gp43, while 47·4% reacted to the 70 kDa component (gp70), and 66·6% reacted with the 21 kDa protein (Fig. 1). Control group (Group III) sera were negative in IDD, but reactivity was sometimes observed in IB, mainly with serum samples from patients with other systemic mycoses. Although these unspecific reactions involved the major Pb antigens, they were weaker than those seen with the PCM serum samples. This reactivity was abolished when the dilution of heterologous serum samples was increased to 1:400 without affecting the reactivity of PCM serum samples.

Discussion

Our study demonstrated that the use of an immunoenzymatic test, IB, significantly improved the sensitivity of PCM immunodiagnosis. Although cross-reactions with serum samples from other mycoses could be observed in this test, the reactions were less intense than those with serum from PCM patients, and could be minimized by progressive dilutions of the specimens, as previously demonstrated by Camargo *et al.* [18]. Our IB results also confirm the suggestion that at least two serological tests for PCM antibody detection should be adopted in the cases of questionable diagnosis, as has been previously recommended [20].

Table 2 Detection of *P. brasiliensis* circulating antibodies by double immunodiffusion and immunoblotting in 80 after specific therapy

		Immunodiffusion		Immunoblotting	
	Patients *n* (%)	Positive (%)	Negative (%)	Positive (%)	Negative (%)
Disease status					
Active	4 (5)	4 (100)	0 (0)	4 (100)	0 (–)
Inactive	76 (95)	22 (29)	54 (71)	76 (100)	0 (–)
Total	80 (100)	26 (32·5)	54 (67·5)	80 (100)	

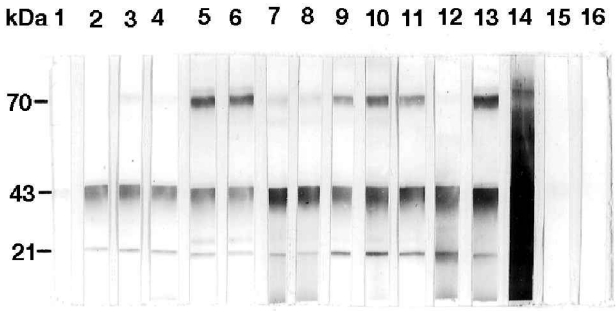

Fig. 1 Representative immunoblot reactions for *P. brasiliensis* filtrate antigen probed with serum samples from patients with paracoccidioidomycosis.

The combination of IDD and IB appeared to be very useful in the diagnosis and monitoring of therapeutic response in patients with active disease, especially in those with juvenile PCM. Overall, in patients with active disease (Group I) the IDD had a high degree of sensitivity (90·2%). Similar results were previously obtained in a multicenter study, where 841 serum samples from PCM patients with progressive disease tested by IDD showed 84·3% sensitivity and 98·9% specificity [21]. Even with the use of purified antigen fractions, such as gp43, there are reports of IDD false negative results in PCM patients with active mycosis [22]. In our study, the negative IDD tests were more frequent in juvenile PCM (28·5%) when compared with the adult chronic form (7·3%). Several causes for these false negative reactions may be suggested: (i) the prozone effect due the excess of antigens because the extensive dissemination of the disease; (ii) the formation of immune complexes with occluded epitopes; (iii) presence of asymmetric antibodies which inhibit secondary binding in precipitation reactions [23]; and (iv) antibody levels below the sensitivity of the method. We felt that the application of an immunoenzymatic assay could abolish some of these phenomena. When our IDD false negative serum samples were probed by IB, all of them showed reactivity, corroborating our hypothesis.

Our results with treated patients (Group II) were similar with those reported previously [2,4,5]. Several hypotheses have been suggested to explain the persistent serological positivity in some patients after clinical cure, including a new exposure to Pb, the persistence of antibodies in the absence of active disease, and maintenance of subclinical infection [5]. In agreement with previous authors [5], we can add that the majority of such patients showed chronic obstructive pulmonary disease.

IB showed reactivity in all serum samples that were included in Group II, and the utilization of IB in the monitoring of patients' response to therapy, in terms of the intensity or number of bands in serum samples, should be further evaluated [24]. Camargo *et al.* [18] have demonstrated that a group of PCM patients under therapy between 1 and 24 months showed a reduction in the reactivity with gp70 antigen followed by a decrease in the antibody response to the gp43 antigen.

In conclusion, we have demonstrated that serological methods for antibody detection, despite their limitations, are important in the diagnosis and monitoring of PCM mainly when a very sensitive method, such as IB is used with combination with a very specific test as such as IDD. IB should be applied for initial diagnosis of PCM, and IDD should be chosen for the follow-up of disease, considering that the antibodies disappear, or remain at low levels after cure of the mycosis. We suggest that the application of the IDD and IB for antibody detection, in combination with methods that detect circulating antigen, should be utilized in the diagnosis of PCM, and adopted as a guidance for cure control of PCM.

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