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USE OF IMMUNOBLOTTING ASSAY IMPROVES THE SENSITIVITY OF PARACOCCIDIOIDOMYCOSIS DIAGNOSIS

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ABSTRACT: The purpose of this work was to evaluate two serological assays: double immunodiffusion (DI) and immunoblotting (IB) in immunodiagnosis of paracoccidioidomycosis (PCM). We evaluated by IB assay 23 sera samples from patients with clinical confirmation of PCM, all of them with negative DI results against culture filtrate from *Paracoccidioides brasiliensis* isolate 113. For IB, as well as for comparative DI assay, we employed soluble components of the cell wall outer surface (SCCWOS) from *P. brasiliensis* isolate 113 cultivated at 36°C in Fava-Neto's agar medium for 5 and 10 days. Among the 20 sera samples analyzed by DI, 13 (65%) were negative and 7 (35%) were positive against SCCWOS obtained on the 5^{th} and 10^{th} days. By IB assay, 95.4% and 100% of sera reacted against gp43 and gp70 present in SCCWOS from the 5^{th} day and 95.6% recognized these fractions when evaluated against SCCWOS from the 10^{th} day. Our results demonstrated that the use of an immunoenzymatic assay significantly improves the sensitivity of PCM immunodiagnosis and also suggests that at least two serological tests for antibody detection should be adopted in cases of questionable diagnosis.

KEY WORDS: paracoccidioidomycosis, immunodiffusion, immunoblotting.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Paracoccidioidomycosis (PCM) is a systemic, chronic, granulomatous, progressive disease caused by the dimorphic fungus *Paracoccidioides brasiliensis* (5). The definitive diagnosis of PCM is usually based on the demonstration of multibudding yeast cells in different biological specimens and/or the isolation of the fungus by culturing; however, the former is insensitive, and the latter is time-consuming (5). Consequently, serological techniques are an important tool not only for disease diagnosis but also for monitoring the patients' response to treatment (6, 8). Circulating antibodies to *P. brasiliensis* can be detected in the patients' sera by serological assays such as DI, counterimmunoelectrophoresis and immunoenzymatic assays (6, 20). DI test is routinely used by clinical laboratories due to its easy procedure, low cost involved in its execution as well as its high specificity (about 100%) and sensitivity (65%–90%) (6, 8). The use of undefined antigens has imposed important limitations on DI test; cross-reactivity has been a problem, as has the absence of antigen standardization (6, 8). Variation in antigen production can arise from differences in the used strains, in the chosen fungus growth phase, in the incubation time, and in the employed culture media (6, 8). Among the antigens used in serological approaches, gp43 is the most frequently studied *P. brasiliensis* component (6, 8, 11). Del Negro *et al*. (9) described that rarely a patient presents negative DI results. However, when further analyzed by an immunoenzymatic assay such as IB, these sera demonstrate anti-gp43 antibodies, probably due to the relatively low sensitivity of precipitation assays. The purpose of this work was to evaluate two serological tests: DI and IB in immunodiagnosis of PCM.

MATERIALS AND METHODS

Paracoccidioides brasiliensis Isolates

Paracoccidioides brasiliensis isolate 113, kindly provided by Professor Dr. Carlos da Silva Lacaz-Culture Collection of the São Paulo Institute of Tropical Medicine [Instituto de Medicina Tropical de São Paulo], Brazil, was used. The isolate was maintained in Potato agar medium at 25^{o}C and subcultured every three months at the Laboratory of Immunodiagnosis in Mycosis, Adolfo Lutz Institute, São Paulo, Brazil.

Paracoccidioides brasiliensis Antigens

Two types of antigens were used in this study. The first was a 20-day culture filtrate (Ag CF) obtained according to Garcia *et al*. (10) from the yeast phase of *P. brasiliensis*. Briefly, the fungus was cultured in NGTA liquid medium for 20 days at 36^{o}C with shaking. After incubation time, the culture was treated with borate-thimerosal solution (1:5,000), filtered, divided into small volumes and stored at 4^{o}C until use. The second antigen employed was *P. brasiliensis* SCCWOS obtained 15 years ago by Assis (1–4). Yeast cells were grown in Fava Netto's agar at 36^{o}C. The cells were collected on days 5 and 10, suspended in 0.85% NaCl solution and pelleted by centrifugation. Supernatants, treated with borate-thimerosal solution (1:5,000) and containing the soluble components, were divided into small volumes and stored at 4^{o}C until use.

Sera Samples

Twenty-three sera samples from patients with proven cases of active PCM (13 sera obtained from patients with chronic and 10 from patients with acute form of the disease), but non-reactive, with culture filtrate or SCCWOS antigens by DI assay, were analyzed. The diagnosis was established by histological or direct microscopic examination and/or by isolation of *P. brasiliensis.*

Double Immunodifusion Assay

Reactions were performed according to the modified method of Ouchterlony (18). Glass slides were covered with 3.0ml of a gel composed of 1% agarose type II medium (Sigma Chemical Co. St. Louis, Mo, USA) in a buffered saline (pH 6.9) containing 0.4% sodium citrate and 7.5% glycine. Antigen (12μl) was placed in the central well, reference and patients' sera (12μl) in the surrounding wells. The slides were incubated in a humid chamber at room temperature for 48h. They were then washed in saline with several changes over a 24h period. The gels were dried and stained with 0.4% Coomassie Brilliant Blue R-250 (Sigma Chemical Co. St. Louis, Mo, USA) in an ethanol-acetic acid-water mixture as the solvent.

SDS-PAGE and Immunoblotting Assay

For sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), *P. brasiliensis* antigens were diluted in a buffer containing 62mM Tris-HCl (pH 6.8); 2% (wt/vol) SDS, 50mM 2-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue

were boiled for 3min and centrifuged before application to gels. Cell wall antigens were then subjected to electrophoresis (20mA at room temperature) on a 10% discontinuous SDS buffer system (13) in a Mini-protean II, Electrophoresis Cell (Bio Rad Laboratories, Richmond, CA, USA), and the molecular mass was determined by the use of a 6.5–175kDa standard prestained protein marker (New England BioLabs). Immunobloting assays were conducted as previously described (19). Proteins from SDS-PAGE were electrotransferred onto 0.20μm nitrocellulose membranes (Sigma Chemical Co. St. Louis, Mo, USA) in a Mini Trans-Blot Cell (Bio Rad Laboratories, Richmond, CA, USA), with 25mM Tris, 192mM glycine, pH 8.3, and methanol 20% (v:v). The nitrocellulose membranes containing electrophoresed antigens were blocked with 5% non-fat dry milk in PBS for 1h at room temperature. Membranes were incubated for 2h at room temperature with human sera diluted 1:40 in PBS containing 0.05% Tween-20 (PBS-T), then washed six times with PBS-T and developed with peroxidase-conjugated goat anti-human IgG antibody (Sigma Chemical Co. St. Louis, Mo, USA) for 2h at room temperature. The reactions were visualized with 4-chloro-1-naphtol substrate (Sigma Chemical Co. St. Louis, Mo, USA).

RESULTS

Table 1 summarizes the DI results. Among the 23 sera samples analyzed, 100% showed negative DI results against culture filtrate antigen; 13 (65%) were negative and 7 (35%) were positive against SCCWOS antigens obtained on the 5^{th} and 10^{th} days of culture. However, when these negative sera were further analyzed by IB assay, we observed that 95.4% and 100% of sera reacted against gp43 and gp70 present in SCCWOS from 5 days, and 95.6% recognized these fractions when evaluated against SCCWOS from 10 days. (Figure 1)

Table 1. Reactivity pattern of paracoccidioidiomycosis sera samples by double immunodiffusion and immunoblotting assays employing culture filtrate antigen (Ag CF) and soluble components of the cell wall outer surface (SCCWOS) of *Paracoccidioides brasiliensis* antigens obtained from isolate113.

Sera Samples	**Double immunodiffusion**			**Immunoblotting**			
	***P. brasiliensis* Antigens**						
	Ag CF 20 days	**SCCWOS 5 days**	**SCCWOS 10 days**	**SCCWOS 5 days**		**SCCWOS 10 days**	
				gp 43	**gp70**	**gp 43**	**gp70**
01	-	-	+	*	*	+	+
02	-	-	-	-	*	-	+
03	-	+	+	+	+	+	+
04	-	-	-	*	*	*	+
05	-	-	-	*	*	*	+
06	-	-	-	*	*	*	*
07	-	-	-	*	*	*	*
08	-	+	+	+	+	+	+
09	-	+	+	+	+	+	+
10	-	+	+	+	+	+	+
11	-	+	+	+	+	+	+
12	-	-	-	+	+	*	+
13	-	+	-	+	+	+	+
14	-	-	-	+	+	*	+
15	-	-	-	+	+	*	+
16	-	-	-	*	+	*	*
17	-	-	-	*	+	+	+
18	-	-	-	*	+	+	+
19	-	-	-	*	+	*	+
20	-	-	-	*	+	*	+
21	-	...	...	+	+	*	-
22	-	...	...	+	+	*	*
23	-	...	...	...	...	*	*

*: Weakly visualized
...: Not performed
+: positive
-: negative

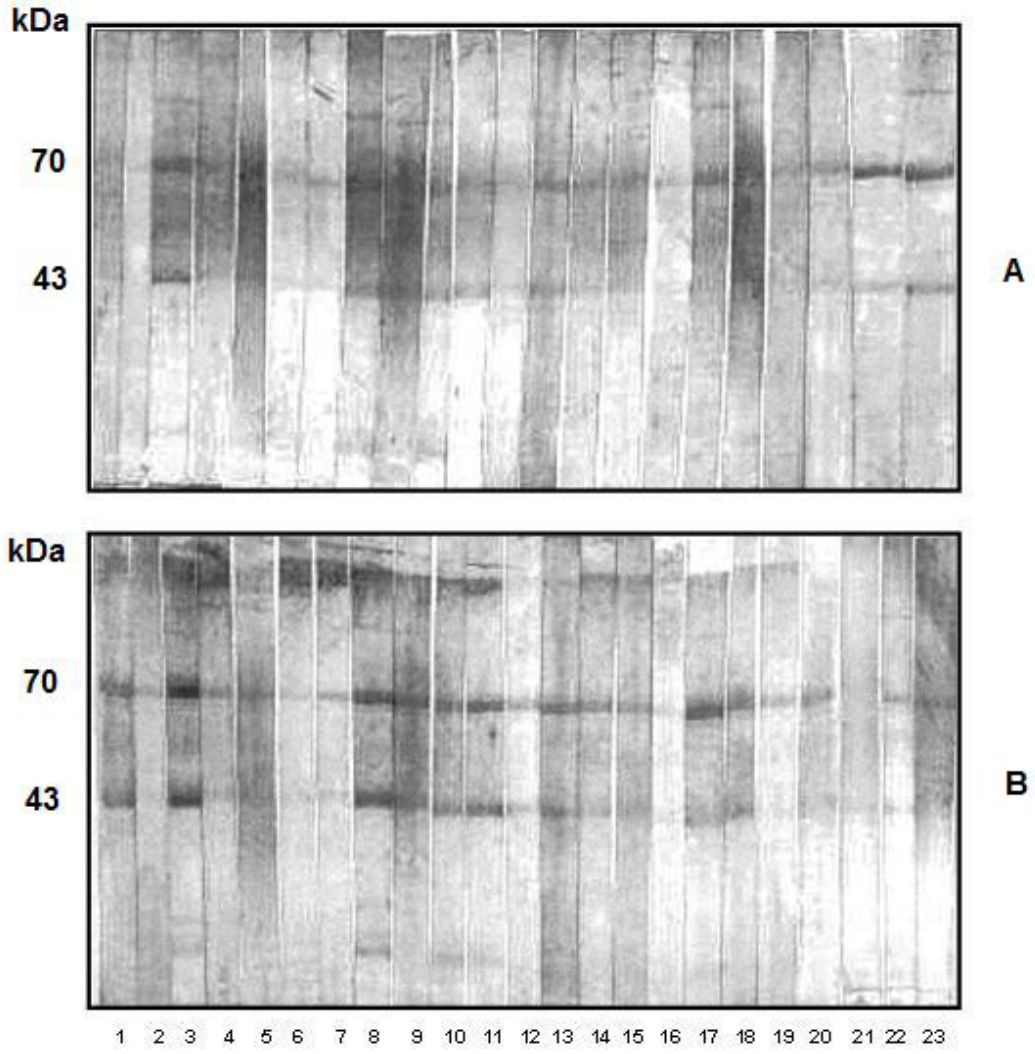

Figure 1. Antibody response to a soluble component of the cell wall outer surface of *Paracoccidioides brasiliensis* obtained on the 5th (Panel A) and 10th (Panel B) days of culture probed with 23 negative double immunodiffusion paracoccidioidomycosis sera samples (Lanes 1 to 23). Goat anti-human IgG serum conjugated with peroxidase was used as the second antibody.

DISCUSSION

For several years, many different antigenic preparations have been used for the immunodiagnosis of PCM by DI assay. However, these various antigenic preparations lacked a standardized preparation from one laboratory to another and included cytoplasmatic extracts from the yeast forms, concentrated filtrates, lyophilized filtrates, and cell wall components to cite a few approaches (7). Moreover, these antigens were prepared from different *P. brasiliensis* isolates grown in different culture media as well as under different growth conditions (6, 12, 16). Distinct *P.*

brasiliensis isolates, on the other hand, can produce remarkably different qualities and quantities of antigens, a response that is also influenced by the culture medium (6, 12, 16, 17). With all these variations in *P. brasiliensis* antigen production, it is not surprising that there is considerable disagreement regarding the sensitivity and specificity in DI assay (6, 12, 16, 17). There are reports of DI false negative results in PCM patients with active infection (9, 14, 16, 18, 21). Do Valle *et al*. (20) reported that the negative DI tests were more frequent in the acute compared with the chronic form. In contrast, Vidal *et al*. (21) reported that negative DI in acute form is rarely observed, and the few negative results are most exclusively related to the localized chronic form of PCM. In our study, we observed that 100% of DI negative PCM sera samples were positive in IB assay showing strongly reactivity with 43 and 70kDa antigenic fractions. The gp43 is the immunodominant and species-specific component of *P. brasiliensis*, being recognized by virtually all patients' sera employing different serological tests (6–8). Another important *P. brasiliensis* antigen is gp70, recognized by 96% PCM sera (6). Neves *et al*. (14) analyzed 46 sera samples of patients with proven PCM and with DI negative versus DI positive results, showing by immunoblotting that both DI negative and positive sera recognized predominantly the gp43 fraction of *P. brasiliensis.* Takahachi *et al*. (18) evaluated by immunoblotting 78 PCM sera samples with DI negative results, showing 100% of positivity. Some hypotheses could explain the negative DI results such as: prozone effect (20), production of low-avidity IgG2 antibodies directed against carbohydrate epitopes (14, 20). In conclusion, our results demonstrated that the use of an immunoenzymatic assay significantly improves the sensitivity of PCM immunodiagnosis and also suggest that at least two serological tests for antibody detection should be adopted in cases of questionable diagnosis.

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REFERENCES

1 ASSIS CM. *Antígenos de* Paracoocidioides brasiliensis *solúveis em NaCl 0,85%*. São Paulo: Universidade de São Paulo, 1990. 96p. [PhD Thesis].

2 ASSIS CM., MENDES-GIANNINI MJ., DEL NEGRO GMB., GAMBALE W. *Paracoccidioides brasiliensis* cell wall antigens soluble in NaCl 0,85*%. Arq. Biol. Tecol.,* 1989, 32, 101.

3 ASSIS CM., MENDES-GIANNINI MJ., DEL NEGRO GMB., GAMBALE W., LACAZ CS. Isolation of immunoreactive outer cell wall proteins produced by *P. brasiliensis*. *Rev. Inst. Med. Trop. São Paulo*, 1993, 35, 72.

4 ASSIS CM., MENDES-GIANNINI MJ., GAMBALE W. Isolation and morphology of an immunoreactive outer cell wall protein produced by *P. brasiliensis* 113. In: *Pan American Association of Biochemical Societies*, 6, São Paulo, 1990. Anais… São Paulo: [s.n], 1990: 163.

5 BRUMER E., CASTANEDA E., RESTREPO A. Paracoccidioidomycosis: an update. *Clin. Microbiol. Rev.,* 1993, 6, 89-117.

6 CAMARGO ZP., FRANCO MF. Current knowledge on pathogenesis and immunodiagnosis of paracoccidioidomycosis. *Rev. Iberoam. Micol*., 2000, 17, 41-8.

7 CAMARGO ZP., UNTERKIRCHER C., CAMPOY SP., TRAVASSOS LR. Production of *Paracoccidioides brasiliensis* exoantigens for immunodiffusion tests. *J. Clin. Microbiol.*, 1988, 26, 2147-51.

8 COSTA MRE., LACAZ CS., KAWASAKI M., CAMARGO ZP. Conventional versus molecular diagnostic tests. *Med. Mycol.*,2000, 38,139-45.

9 DEL NEGRO GMB., BENARD G., ASSIS CM., VIDAL MSM., GARCIA NM., OTANI C., SHIKANAI-YASYDA MA., LACAZ CS. Lack of reactivity of paracoccidioidomycosis sera in double immunodiffusion test with the gp43 antigen: report of two cases. *J. Med. Vet. Mycol.,* 1995, 33, 111-16.

10 GARCIA NM., ASSIS CM., DEL NEGRO GMB., AGUIAR MSMV., LACAZ CS. Análise imunoquímica de antígeno metabólico de *P. brasiliensis*. *Rev. Inst. Med. Trop. São Paulo,* 1993, 35, 70.

11 HAMILTON AJ. Serodiagnosis of histoplasmosis, paracoccidioidomycosis and penicilliosis marneffei; current status and future trends. *Med. Mycol.,* 1998, 36, 351-64.

12 KLOTH VR., ASSIS CM., SILVA DF., ZAMBONI IM., FAZIOLI RA., MATANO G., VICENTINI AP. Production and characterization of *Paracoccidioides brasiliensis* antigens. *Rev. Soc. Bras. Med. Trop.,* 2001, 34,179.

13 LAEMMLI UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 1970, 227, 680-5.

14 NEVES AR., MAMINI RL., ROSSI CL., CAMARGO ZP., BLOTTA MHS. Negative immunodiffusion test results obtained with sera of paracoccidioidomycosis patients may be related to low-affinity immunoglobulin G2 antibodies directed against carbohydrate epitopes. *Clin. Diag. Lab. Immunol*., 2003, 10, 802-7.

15 OUCHTERLONY O. Antigen-antibody reactions in gels. *Acta Pathol. Microbiol. Scand.*, 1949, 26, 507-17.

16 SILVA DF. *Análise da estabilidade de exoantígenos de* Paracoccidioides brasiliensis. São Paulo: Coordenadoria de Controle de Doenças, Secretaria de Estado da Saúde, 2005. 169p. [PhD Thesis]

17 SILVA DF., ASSIS CM., ZAMBONI IM., OLIVEIRA LE., BENARD G., PORTELA-LINDOSO AAA., VICENTINI AP. Evaluation of different antigens of *Paracoccidioides brasiliensis. Rev. Soc. Bras. Med. Trop*., 2003, 36, 211.

18 TAKAHACHI G., GUILHERMETTI E., SILVA JA., SVIDZINSKI TIE. Importância do western blott no diagnóstico seguro da Paracoccidioidomicose. In: *INTERNATIONAL MEETING IN PARACOCCIDOIDOMYCOSIS*, 7, Campos do Jordão, 1999. Proceedings… Campos do Jordão, 1999: B.87.

19 TOWBIN H., STAEHELIN T., GORDON J. Transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA*, 1979, 76, 4350-4.

20 VALLE ACF., COSTA RLB., MONTEIRO PCF., VON HELDER J., MUNIZ MM., ZANCOPE-OLIVEIRA RM. Interpretation and clinical correlation of serological tests in paracoccidioidomycosis. *Med. Mycol.* 2001, 39, 373-77.

21 VIDAL MSM., BENARD G., BRITO T., DANTAS KC., PEREIRA CN., FRANCA CO., SILVA AM., MARTINS JE. Atypical serological response marked by a lack of detectable anti-gp43 antibodies in a patient with disseminated paracoccidioidomycosis. *J. Clin. Microbiol.*, 2005, 43, 3014-6.