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Immunoenzymatic Absorption Test for Serodiagnosis of Paracoccidioidomycosis

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A sensitive enzyme-linked immunosorbent assay is described for paracoccidioidomycosis serodiagnosis, with antigen represented by filtrates from *Paracoccidioides brasiliensis* cultures. Cross-reactivities were, however, observed with sera from patients with other mycoses such as histoplasmosis, lobomycosis, cryptococcosis, candidiasis, and sporotrichosis. These cross-reactions did not occur when we used as antigen Yarzabal E_2 component prepared by affinity fractionation of the culture filtrates. Specific results could also be obtained with the whole filtrate antigen when sera were absorbed with *Histoplasma capsulatum* yeast and mycelial components. Besides a very high sensitivity (100% of the 69 cases of paracoccidioidomycosis tested), this assay showed no false-positive results for the 206 non-paracoccidioidomycosis sera studied, including those from patients with other mycotic diseases.

Human infections with *Paracoccidioides brasiliensis* are observed in tropical and subtropical regions of Latin America, from Mexico to Argentina. Cases are seen mainly in Brazil, Venezuela, Colombia, and Guatemala and present a variety of clinical forms such as mucocutaneous, lymphonodular, or visceral. Paracoccidioidomycosis is usually diagnosed through microscopic identification of the fungus in clinical material such as sputum, lesion scrapings or exudates, biopsy specimens, etc. Since in many cases such demonstration of the fungus is difficult, diagnosis must be based on serological tests, which are also valuable in evaluating therapeutic results and controlling the cure of the disease. Although complement fixation (CF) tests are most commonly used for routine purposes, other tests have been described such as immunofluorescence (9), precipitation in tubes (3), agar-gel immunodiffusion (8), counter-immunoelectrophoresis (A. M. de Siqueira, Thesis, Instituto de Ciências Biomédicas da Universidade de São Paulo, São Paulo, Brazil, 1982), and agglutination (11). In CF tests, either a polysaccharide fraction (3) or whole culture filtrates of yeasts, as well as mycelial-phase culture filtrates, have been used as antigen. Although tests with filtrate antigens have been found to be more specific, false-positive results are still frequently observed for other mycotic diseases such as histoplasmosis, cryptococcosis, sporotrichosis, and candidiasis. More specific results have been described for the immunodiffusion test, by identifying a precipitation line corresponding to a specific antigen referred to as E_2 by Yarzabal et al. (15) and as fraction 1 by Restrepo and Moncada (10).

The only immunoenzymatic assay described for paracoccidioidomycosis is performed with *P. brasiliensis* yeast cells fixed on microscopic slides, with cross-reactions observed for histoplasmosis and candidiasis (6).

Here we describe a very sensitive and specific absorption enzyme-linked immunosorbent assay (ABS-ELISA) for paracoccidioidomycosis, which was developed by using *P. brasiliensis* culture filtrate sera previously absorbed with *Histoplasma capsulatum* antigens.

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MATERIALS AND METHODS

Serum samples. Samples from 119 patients with well-established clinical and laboratory diagnoses of mycotic diseases were obtained from the collection of the Mycology Laboratory, Instituto de Medicina Tropical de São Paulo (IMTSP). These samples had been stored at −20°C for various periods up to several months. There were 69 sera from cases of paracoccidioidomycosis, 20 of histoplasmosis, nine of aspergillosis, nine of lobomycosis, five of candidiasis, four of cryptococcosis, and three of sporotrichosis. Serum samples from 34 patients with other diseases, from the Serum Bank, Immunology and Seroepidemiology Laboratory, IMTSP, were also included. These had been stored for different periods, and were fluid at −20°C since they were preserved with analytical grade, recently distilled glycerin. Eight sera were from cases of mucocutaneous leishmaniasis, four of chronic Chagas' disease, five of malaria, seven of recent syphilis, seven of acute toxoplasmosis, and three of active tuberculosis. Serum samples from 122 clinically normal individuals were obtained from blood donors (Hospital das Clínicas, Universidade de São Paulo) and from individuals who submitted to clinical and laboratory examinations.

Antigens from *P. brasiliensis*. Three strains of *P. brasiliensis* from the IMTSP collection (strains 113, 150, and 666) were used for preparing the antigens.

The polysaccharide antigen was produced by the method of Fava Netto (3). Culture filtrates were prepared by growing the fungus in the yeast phase in different media, which had been previously obtained from proteose-peptone agar medium cultures kept at 35°C for 8 to 10 days. The yeasts were then grown in stationary phase for 3 months at 35°C, in 200-ml volumes of the following media: proteose-peptone broth (3), Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) (8), Sabouraud-dextrose broth (14), and Shadomy medium modified by the method of Casals (2), which we modified by substituting casein hydrolysate for asparagine and adding 10 μg of thiamine per ml. After adding Thimerosal (Eli Lilly & Co., Indianapolis, Ind.) for a final dilution of 1:10,000, the medium was centrifuged at 400 × *g* for 30 min, and the supernatant was dialyzed against distilled water for 48 h at 4°C and then 10× concentrated by pervaporation. After centrifugation at 400 × *g* for 10 min the

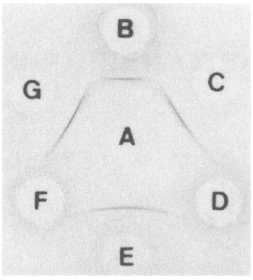

FIG. 1. *P. brasiliensis* antigen (A); Yarzabal anti-E_2 immunoserum (B, E); IMTSP anti-E_2 immunoserum (C, G); and histoplasmosis serum (D, F).

amount of protein in the filtrate was determined (5), and the filtrate was divided into portions and stored at −20°C. Because of the much better reactivity found for the Casals medium filtrate, only this was used in the immunoenzymatic tests. Protein concentration in the different filtrate batches varied from 4.5 to 6.0 mg/ml.

The E_2 antigen used in the tests was prepared by affinity fractionation of culture filtrates with anti-E_2 rabbit globulins polymerized as described by Avrameas and Ternynck (1). Initially, an anti-E_2 immunoserum kindly furnished by L. Yarzabal (CEPIALET, Caracas, Venezuela) was used, but later it was prepared in our laboratory. For this purpose the E_2 fraction was identified and removed from about 60 immunoelectrophoresis preparations of culture filtrates against rabbit anti-*P. brasiliensis* immunoserum. The corresponding area in the agar was cut from slides previously kept in saline solution for 48 h, emulsified with complete Freund adjuvant, and used to immunize rabbits. Each animal was injected intradermally with a total of 2 ml of the mixture once a week for periods of 7 weeks and bled when a titer of at least 1:32 against culture filtrates was found by the agar-gel diffusion test. An identity precipitation line was observed between the anti-E_2 antiserum prepared by Yarzabal and the immunoserum obtained in this laboratory (Fig. 1). Globulins were fractionated from the serum by adding an equal volume of 1.56 M ammonium sulfate (pH 6.5). For polymerization, 0.3 ml of a 2.5% glutaraldehyde solution in distilled water was added to 30 mg of globulins in 1 ml of 0.2 M acetate buffer (pH 5.0), and the mixture was kept for 3 h at room temperature. After being washed in phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M phosphates, pH 7.2), the precipitate was kept in this solution with 0.01% sodium azide.

To prepare the E_2 antigen, this immunosorbent precipitate was added to 0.5 ml of culture filtrate which contained about 30 mg of proteins. After 1 h at room temperature the suspension was centrifuged, washed three times with saline solution, and suspended in 1.0 ml of 0.1 M glycine-HCl buffer (pH 2.8) for 5 min at room temperature. After centrifugation, the supernatant was neutralized with 0.05 M NaOH, dialyzed for 18 h at 4°C against PBS, and stored at −20°C. The protein concentration of different batches varied from 500 to 640 μg/ml.

Antigens from *H. capsulatum*. Culture filtrates of mycelial-phase *H. capsulatum* were prepared from different strains (387 and 391) kept at IMTSP. The fungus was grown at room temperature for 6 months in Smith medium (12). The same procedure was followed as that described for *P. brasiliensis* culture filtrates.

Yeast-phase cell suspensions of *H. capsulatum* were obtained by growing the fungus in brain-heart infusion medium with 0.1% cysteine (7) for 4 to 5 days at 37°C. Cells removed by centrifugation of the cultures were washed twice and suspended in PBS with a 1:10,000 dilution of Thimerosal. Suspensions were standardized either by counting cells in a Neubauer counting chamber or by optical density (OD) determination at 550 nm.

Immunoenzymatic conjugate. An anti-human immunoglobulin G serum produced in sheep and rendered specific for

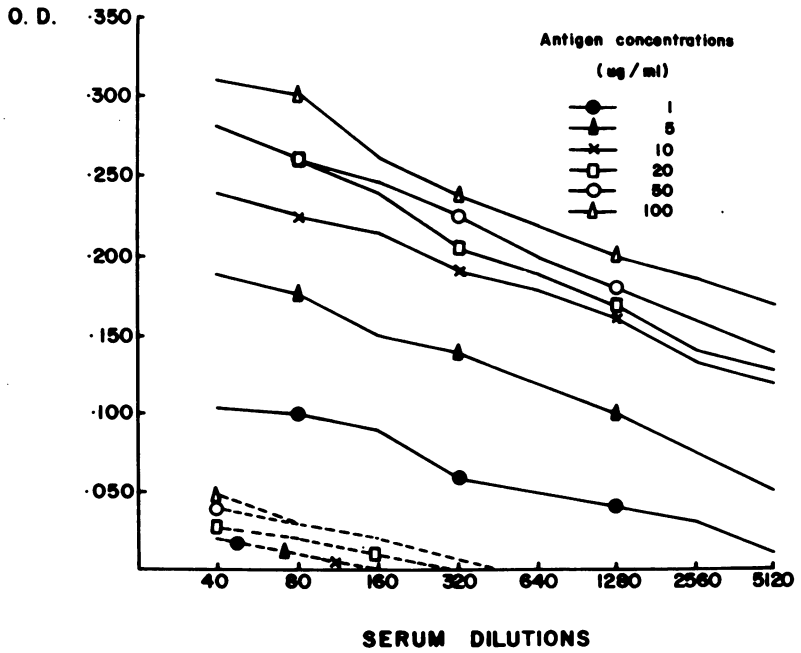

FIG. 2. Reactivity for standard positive and negative sera in the immunoenzymatic assay with different sensitizing culture filtrate antigen concentrations. Reactive serum (———) and nonreactive serum (- - - -).

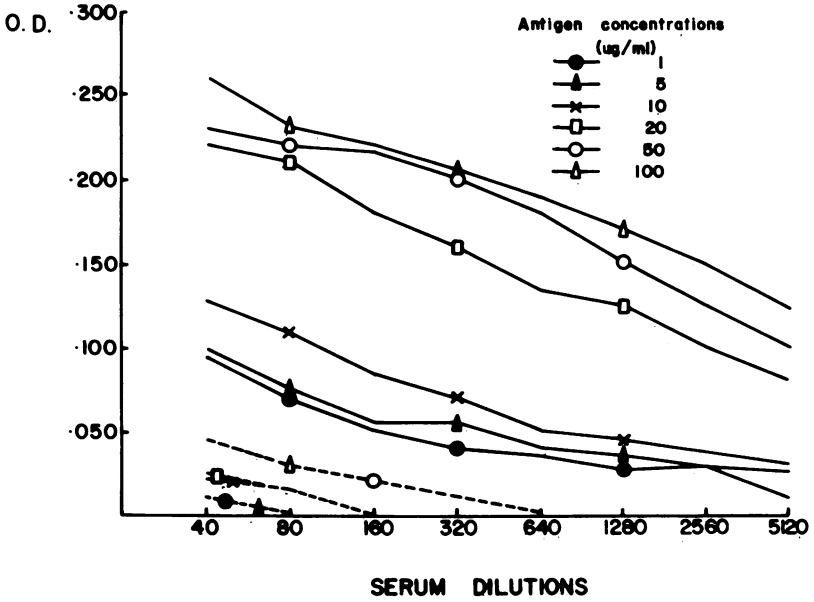

FIG. 3. Reactivity for standard positive and negative sera in the immunoenzymatic assay with different sensitizing E_2 fraction antigen concentrations. Reactive serum (——) and nonreactive serum (- - -).

gamma heavy chain by absorptions was kindly furnished by H. Rangel (Immunology Department, UNICAMP, Campinas, Brazil). The serum globulin fraction which salted out with 1.56 M ammonium sulfate was labeled with horseradish peroxidase (type VI; Sigma Chemical Co., St. Louis, Mo.) by the method of Wilson and Nakane (13). For use, the conjugate was diluted to the highest dilution which gave maximal reactivity in the test.

Immunoenzymatic tests. Tests were carried out in flexible plastic polyvinyl plates with U-shaped wells (Plásticos Ampla, São Paulo, Brazil). For sensitizing, plates were incubated for 3 h at 37°C and for 18 h at 4°C with antigen dilutions in 0.06 M carbonate buffer (pH 9.6), washed with PBS with 0.05% Tween 20 (PBS-T), and stored at 4°C for use.

For the tests, sensitized plates were incubated for 1 h at 37°C successively with doubling serum dilutions in PBS-T and with conjugate diluted in PBS-T according to titer. After each incubation, plates were washed three times for 5 min each in PBS-T. Substrate with 0.005% hydrogen peroxide and 0.08% 5-aminosalicylic acid was then added to each well, the plates were incubated for 1 h at room temperature, and the reaction was stopped with 1 drop of 1 M NaOH. The OD at 450 nm was then determined for each well.

For the immunoenzymatic absorption test, serum samples were previously absorbed with *H. capsulatum* antigens. Different sorbents were tested and made up of a cell suspension (with either 6×10^5 or 1×10^6 cells per ml), a culture filtrate (with either 25 or 50 μg of protein per ml), or a cell suspension (6×10^5 cells per ml) in culture filtrate (50 μg of proteins per ml). For absorption, an equal volume of antigen was added to a 1:20 serum dilution, and the mixture was incubated for 18 h at 4°C and for 1 h at 37°C and centrifuged at 400 × *g* for 10 min. Tests were performed with the supernatant, which corresponded to a 1:40 serum dilution, and with successive doubling dilutions when necessary.

CF test. The microtechnique CF test recommended by the Pan-American Health Organization was employed (4), using *P. brasiliensis* culture filtrates as antigen.

RESULTS

Standardization of the immunoenzymatic tests. Plates were sensitized with *P. brasiliensis* culture filtrate or E_2 fraction dilutions with protein concentrations from 1 to 100 μg/ml, and tests were performed with a positive serum (counter-immunoelectrophoresis test titer, 1:512; complement fixation test titer; 1:2,048; with *P. brasiliensis* antigens) and a negative serum. From results shown in Fig. 2, culture filtrate dilutions with 10 μg/ml were chosen as antigen for the test. For the E_2 fraction, best results were obtained with the 20-μg/ml dilution (Fig. 3). Maximal reactivities were obtained with the anti-immunoglobulin G conjugate diluted at 1:6,000 for tests with culture filtrate and at 1:2,000 for tests with E_2 antigen.

As a cutoff point between negative and positive results, an

TABLE 1. Reactivity for non-paracoccidioidomycosis sera in ELISA with *P. brasilienisis* culture filtrate antigen

Serum sample	No.	No. with an ELISA titer of:				
		<40	40	80	160	320
Normal	122	122				
Nonmycotic diseases						
Syphilis	7	6	1			
Tuberculosis	3	2	1			
Malaria	5	4	1			
Leishmaniasis	8	7	1			
Chagas' disease	4	4				
Toxoplasmosis	7	7				
Mycotic diseases						
Histoplasmosis	20	9	2	4	2	3
Lobomycosis	9	4	1	2	1	1
Aspergillosis	9	7	2			
Cryptococcosis	4	2	2			
Candidiasis	5	4	1			
Sporotrichosis	3	3				

OD of 0.050 was chosen, and endpoint titers were taken as the highest serum dilution showing a positive test. This cutoff was chosen by the mean ± 2 standard deviations.

Test results. Positive results were seen for the 69 sera from cases of paracoccidioidomycosis, with titers ranging from 1:80 to 1:5,120 in the immunoenzymatic test with culture filtrate antigen. Results obtained for normal individuals and cases of other diseases, mycotic or nonmycotic, are shown in Table 1. According to such results, a titer of 1:80 or higher was taken as a diagnostic value. However, cross-reactions were seen for a few cases of histoplasmosis and lobomycosis.

With the E_2 fraction antigen, results were very similar, with titers only somewhat lower for paracoccidioidomycosis sera, but positive results were also observed for a few cases of histoplasmosis. When the cutoff point was raised to an OD of 0.100, however, only one histoplasmosis serum was positive and no decrease in sensitivity occurred since all paracoccidioidomycosis sera were still positive.

Immunoenzymatic tests with culture filtrate antigen were then performed after treating sera with different *H. capsulatum* sorbents. Cross-reactions could be removed in different degrees according to the sorbent used. Only the suspension of 6×10^5 cells per ml in *H. capsulatum* culture filtrate with 50 μg of protein per ml could completely remove any cross-reactivity. In this way, with a 0.050 OD cutoff, titers lower than 1:40 were seen for all 206 sera of non-paracoccidioidomycosis cases studied, including normal sera and sera from patients with other diseases (mycotic and nonmycotic). A few examples are shown in Table 2.

No significant titer differences were observed after sorbing sera of the 69 cases of paracoccidioidomycosis; titers were within one dilution for all sera but one, which differed by two dilutions (Table 3).

In the CF test, 67 paracoccidioidomycosis sera were reactive, with titers from 1:8 to 1:2,048, and two were nonreactive (titer < 1:8) (Table 4). Negative results were seen for all normal sera and sera from cases of nonmycotic diseases for which a conclusive result could be obtained. Inconclusive results were caused by anticomplementarity of several sera. Positive results were seen in 9 of 43 cases of mycotic diseases other than paracoccidioidomycosis, with titers ranging from 1:8 to 1:64. These included cases of histoplasmosis, lobomycosis, cryptococcosis, and candidiasis.

TABLE 2. ELISA titers for cases of paracoccidioidomycosis and histoplasmosis before and after absorbing sera with different absorbents (ABS-ELISA)

Serum no.	Serum titer					
	ELISA	ABS-ELISA[a]				
		A	B	C	D	E
Paracoccidioidomycosis						
1	5,120	5,120	5,120	5,120	5,120	5,120
2	5,120	5,120	5,120	5,120	5,120	5,120
3	1,280	1,280	1,280	1,280	1,280	1,280
4	320	320	320	640	160	320
Histoplasmosis						
5	320	160	80	160	80	<40
6	320	160	80	160	40	<40
7	80	40	40	40	40	<40

[a] Sera were absorbed with: A, mycelial-phase culture filtrate diluted to 25 μg/ml; B, mycelial-phase culture filtrate diluted to 50 μg/ml; C, yeast-phase cell suspension, 6×10^5 cells per ml; D, yeast-phase cell suspension, 1×10^6 cells per ml; E, cell suspension (1×10^6 cells per ml) in culture filtrate (50 μg of protein per ml).

TABLE 3. Comparison of ELISA and ABS-ELISA titers of 69 sera from cases of paracoccidioidomycosis

ABS-ELISA titer	No. with an ELISA titer of:							
	<80	80	160	320	640	1,280	2,560	5,120
<80								
80		2	2					
160		2	5	4	1			
320				2	4			
640				1	9	3		
1,280					2	9	6	
2,560						2	7	7
5,120								1

Geometric mean titers for the 69 paracoccidioidomycosis sera were 20 for CF and 140 for ABS-ELISA. Between CF and ABS-ELISA no agreement of titers was seen, as shown by κ statistics. Different combinations of tests gave the following κ values: ELISA-CF, −0.071; ELISA-ABS-ELISA, 0.41 (significant at 5%); ABS-ELISA-CF, −0.037.

For routine purposes, the ABS-ELISA could be made more practical by using only one serum dilution. Titers could then be expressed as the OD observed for a 1:100 serum dilution instead as of the endpoint titer obtained by testing a series of serum dilutions. The 1:100 serum dilution was prepared by diluting serum in the absorbent, represented by 10^6 *H. capsulatum* yeast cells per ml of *H. capsulatum* culture filtrate, with 50 μg of protein per ml.

After incubation overnight at 4°C and for 1 h at 37°C, this serum was centrifuged and the supernatant was tested. When comparing ABS-ELISA titers obtained by the endpoint titration or expressed as the OD of a 1:100 serum dilution, we observed a close relation (Fig. 4).

DISCUSSION

Among filtrates of *P. brasiliensis* cultures in different liquid media, those obtained in Casals modified medium showed the highest reactivity with paracoccidioidomycosis sera in the ELISA. Although no false-positive results were seen with normal sera and with sera from patients with different nonmycotic diseases, cross-reactions were frequent with sera from patients with other mycoses, such as histoplasmosis, lobomycosis, cryptococcosis, and candidiasis.

In a search for a better specificity in the ELISA we tried Yarzabal E_2 fraction as antigen. After producing a monospecific antiserum which reacted exclusively with this component in immunodiffusion and immunoelectrophoresis tests,

TABLE 4. Comparison of ELISA and CF titers of 69 sera from cases of paracoccidioidomycosis

CF titer	No. with an ELISA titer of:							
	<80	80	160	320	640	1,280	2,560	5,120
<8		1		1				
8		1		1		1		
16		1	4	3	1	1	2	
32		1	2	1	3	1		
64			1		3		2	1
128				1	6	5	5	1
256					3	4	2	1
512						1	1	2
1,024						1		1
2,048							1	2

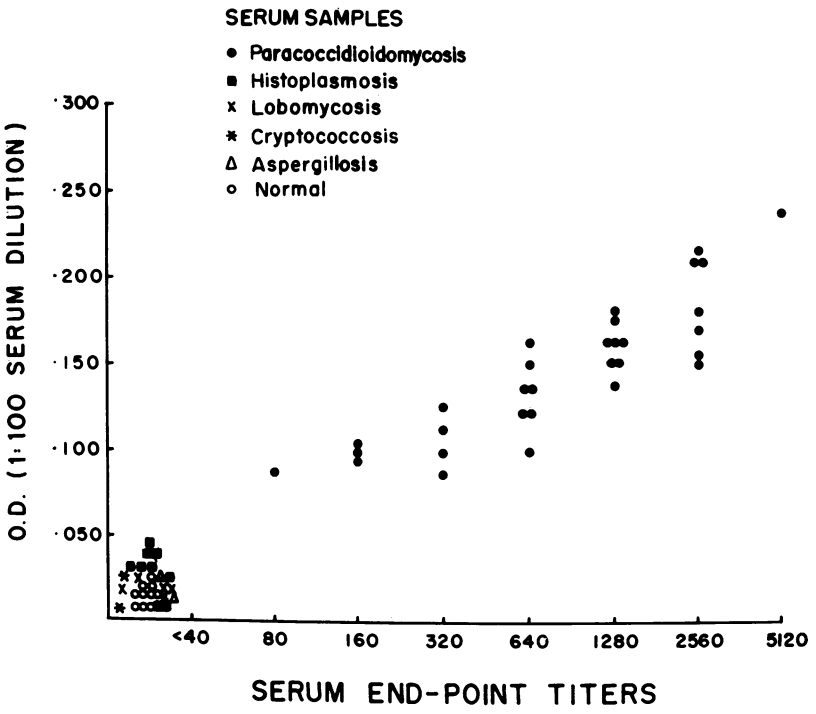

FIG. 4. Comparative results of ABS-ELISA expressed as OD for a 1:100 serum dilution or as endpoint titers.

preparative amounts of the E_2 fraction could be obtained by affinity fractionation of culture filtrates. The ELISA with E_2 fraction as antigen was as sensitive as that with culture filtrates, but cross-reactions were still present with a 0.050 OD cutoff. When the cutoff point was raised to 0.100 OD, however, only one histoplasmosis serum gave a positive reaction, and test sensitivity was not decreased.

A more practical solution could be developed by sorbing sera with *H. capsulatum* components and by using total culture filtrates to sensitize plates. A satisfactory sorbent included both yeast and mycelial antigens and was obtained by suspending 10^6 yeast cells per ml of a mycelial culture filtrate dilution with 50 μg of protein.

This ABS-ELISA showed a high sensitivity, since it was positive with all 69 sera from cases of paracoccidioidomycosis studied here, with titers between 80 and 5,120 (geometric mean titer, 140). The test was also very specific, with negative results for all non-paracoccidioidomycosis sera studied, including those from patients with other mycoses. Comparatively, the CF test with *P. brasiliensis* culture filtrates was positive for 67 of the 69 paracoccidioidomycosis sera, with titers ranging from 8 to 2,048 (geometric mean titer, 20).

A simple quantitative ABS-ELISA could be standardized for routine purposes by assaying sera diluted at 1:100 in the sorbent, as described above. The close relation observed between the OD values and the endpoint titers suggests that this simple quantitative technique is satisfactory for diagnosis of paracoccidioidomycosis and treatment follow-up.

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LITERATURE CITED

1. **Avrameas, S., and T. Ternynck.** 1969. The cross-linking of protein with glutaraldehyde and its use in the preparation of immunosorbent. Immunochemistry **6**:53–56.
2. **Casals, J. B.** 1979. Tablet sensitivity testing of pathogenic fungi. J. Clin. Pathol. **32**:719–722.
3. **Fava Netto, C.** 1961. Contribuição para o estudo imunológico da blastomicose de Lutz. Rev. Inst. Adolfo Lutz **21**:99–194.
4. **Kaufman, L., M. Huppert, C. Fava-Netto, L. Pollak, and A. Restrepo.** 1974. Manual of standardized serodiagnostic procedures for systemic mycosis. II. Complement fixation tests. Pan-American Health Organization, Washington, D.C.
5. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem **193**:265–275.
6. **Pons, L., Gimenez, C. Guilleron, and A. Szarfman.** 1976. La técnica de la inmunoperoxidase en la detection de anticuerpos especificos en la infeccion humana por *Paracoccidioides brasiliensis*. Medicina **36**:510–512.
7. **Reca, M. E., and C. C. Campbell.** 1967. Growth curves with yeast phase *Histoplasma capsulatum*. Sabouraudia **5**:267–273.
8. **Restrepo, M. A.** 1966. La prueba de immunodiffusion en el diagnostico de la paracoccidioidomicosis. Sabouraudia **4**:223–230.
9. **Restrepo, M. A., and F. L. H. Moncada.** 1972. Indirect fluorescent-antibody and quantitative agar-gel immunodiffusion tests for the serological diagnosis of paracoccidioidomycosis. Appl. Microbiol. **24**:132–137.
10. **Restrepo, M. A., and L. H. Moncada.** 1974. Characterization of the precipitin bands detected in the immunodiffusion test for paracoccidioidomycosis. Appl. Microbiol. **28**:138–144.
11. **Restrepo, M. A., and L. H. Moncada.** 1978. Una prueba de latex en lamina para el diagnostico de la paracoccidioidomicosis. Bol. Of. Sanit. Panam. **84**:520–531.
12. **Smith, C. E., E. C. Whiting, E. C. Baker, H. G. Rosenberger, R. R. Beard, and M. T. Saito.** 1948. The use of coccidioidin. Am. Rev. Tuberc. Pulm. Dis. **57**:330–360.

13. **Wilson, M. B., and P. K. Nakane.** 1978. Recent developments in the periodate method of conjugating horseradish peroxidase (HRPO) to antibodies, p. 215–223. *In* W. Knapp, K. Holuber, and G. Wick (ed.), Immunofluorescence and related techniques. North-Holland Biomedical Press, Amsterdam.
14. **Yarzabal, L. A., J. Biguet, T. Vaucelle, A. Adrieu, J. M. Torres, and S. Luz.** 1973. Anàlisis inmunoqumico de extractos solubles de *Paracoccidioides brasiliensis*. Sabouraudia **11**:80–88.
15. **Yarzabal, L. A., D. Bout, F. Naquira, J. Fruit and S. Andrieu.** 1977. Identification and purification of the specific antigen of *Paracoccidioides brasiliensis* responsible for immunoelectrophoretic band E. Sabouraudia **15**:79–85.