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Rapid and Reliable Method for Production of a Specific *Paracoccidioides brasiliensis* Immunodiffusion Test Antigen

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Previously published methods to produce *Paracoccidioides brasiliensis* antigens for serological tests have yielded antigens of inconsistent quality and have involved the use of special semisynthetic media and growth periods of 1 to 3 months to yield suitable reagents. A simple procedure that uses commercially available potato glucose agar and either SABHI broth (Difco Laboratories) or Trypticase soy broth (BBL Microbiology Systems) inoculated with the mycelial form of *P. brasiliensis* consistently yielded high-titer antigens in 2 weeks or less. This new method permits the almost exclusive production of an antigen identical to the specific E antigen described by Yarzabal (Yarzabal et al., Sabouradia **14**:275–280, 1976) and the apparently equivalent specific antigen 1 described by Restrepo and Moncada (A. Restrepo and L. H. Moncada, Appl. Microbiol. **28**:138–144, 1974). In the immunodiffusion test, the rapidly produced antigen demonstrated a sensitivity of 90% by detecting antibody in sera from 103 of 114 proven cases of paracoccidioidomycosis. The specificity of this antigen was 100% because none of 139 sera from patients with heterologous mycotic diseases demonstrated diagnostic precipitins against the *P. brasiliensis* antigen. In the complement fixation tests, the rapidly produced antigen was not as suitable as the one prepared by the method of Restrepo-Moreno and Schneidau (A. Restrepo-Moreno and J. D. Schneidau, Jr., J. Bacteriol. **93**:1741–1748, 1967).

Paracoccidioidomycosis is a systemic mycosis prevalent exclusively in Latin America that is caused by the dimorphic mold *Paracoccidioides brasiliensis*. The disease is quite variable in its clinical manifestations, and consequently, diagnosis may be delayed or inaccurate. Serological procedures have proved highly useful and are frequently decisive in establishing a diagnosis. The demonstration of specific precipitins in patient sera by immunodiffusion tests is considered presumptive evidence of paracoccidioidomycosis. Restrepo and Moncada (2) and Restrepo-Moreno and Schneidau (3) described methods for the production of a paracoccidioidin containing three precipitinogens, designated 1, 2, and 3. The production of these antigens involved the use of the yeast form of the fungus, a chemically defined medium that is difficult to prepare, and an incubation period of 4 to 6 weeks. In our laboratory, *P. brasiliensis* antigens produced by the Restrepo method (2, 3) frequently varied in quality and quantity, and many were unacceptable as reference antigens for serological tests. In 1976, Yarzabal et al. (5) isolated and purified a *P. brasiliensis* antigen from culture filtrates of the mycelial form of the fungus. Although the simple and readily available Sabouraud glucose broth was used as the growth medium, 12 weeks of incubation were required for the production of an acceptable antigen. This antigen, designated E, was reported to be specific for *P. brasiliensis*. Immunoelectrophoretic studies (2) suggested that antigen E and antigen 1 of Restrepo are identical. This finding was confirmed in our laboratory during immunodiffusion (ID) studies when a sample of the specific E antigen obtained from L. A. Yarzabal was compared with paracoccidioidin containing antigen 1 obtained from A. Restrepo. This finding as well as the problems encountered in producing reproducible lots of reactive antigen by the method of Restrepo encouraged us to investigate modifications of the method of Yarzabal to simplify production of the specific *P. brasiliensis* antigen (antigen E or the equivalent antigen 1), designated in this report as E/1. The purpose of this study was to devise a procedure which would consistently yield the specific *P. brasiliensis* E/1 antigen by rapid growth of the mold form of the fungus in commercially available media.

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MATERIALS AND METHODS

Antigen production. *P. brasiliensis* B339, B341, and B1183 were obtained from Angela Restrepo. Additional strains L6043, L6712, and L6833 were obtained from Alberto Londero. The mold form of these six isolates of *P. brasiliensis* was inoculated onto potato glucose (Difco Laboratories) agar slants. The slants were incubated at 24 to 26°C until cultures developed growth covering an area of at least 2 cm^2 (usually 7 to 10 days). The entire growth from the potato glucose agar was used to inoculate 500-ml flasks containing 200 ml of either Trypticase soy broth (TSB) (BBL Microbiology Systems) or SABHI broth (50:50 Sabouraud broth) (Difco) and brain heart infusion broth (Difco). Cultures were incubated at 25°C while rotating at 150 rpm. Portions (10 ml) of each broth culture were aseptically withdrawn from each flask at 7, 10, 14, 18, 21, 28, and 30 days and placed in tubes. To kill the growth, Formalin was added to a final concentration of 0.5% to these tubes, and they were left at room temperature for 24 h. The soluble antigens were separated from the mycelial elements by centrifugation at 1,500 × *g* for 30 min or filtration through Millipore Millex-HA filter units. Antigens were concentrated 10 times in Amicon B 15 stationary concentrators or Amicon stir-cell concentrators by using a PM 10 membrane.

A concentrated, crude culture filtrate from yeast-form growth (paracoccidioidin) containing antigens 1, 2, and 3 was prepared by the method of Restrepo-Moreno and Schneidau (3). A reference paracoccidioidin containing these antigens was obtained from A. Restrepo. Reference monospecific anti-E antiserum and E antigen were obtained from L. A.

Yarzabal. By using these reference reagents, the E/1 antigen was identified in each lot of antigen produced in this study. The optimal E/1 antigen concentration was determined for each lot by block titration against specific anti-E rabbit reference serum in an ID test.

Antiserum production. Female albino rabbits weighing 5 to 7 lbs (2.3 to 3.2 kg) were immunized with paracoccidioidin prepared by both the method of Restrepo-Moreno and Schneidau (3) and the method described in this paper. Equal volumes of culture filtrate antigens known to contain E/1 as well as other antigens were mixed with Freund incomplete adjuvant and emulsified thoroughly. Each rabbit received 0.5 ml of the mixture subcutaneously in each of six sites in the scapular area. Rabbits were test bled at days 7 and 10. All sera from rabbits immunized with the Restrepo culture filtrate antigens as well as sera from rabbits immunized with the culture filtrates prepared by our procedure demonstrated at day 10 precipitating antibody only to antigen E/1. Intravenous injections of 0.5 ml of the respective culture filtrate antigens were given on days 17, 24, and 31 to produce antibody to antigens 2 and 3. Test bleedings were done before each of the intravenous injections, and the rabbits were bled out at day 40.

Patient sera. Sera from human cases of aspergillosis, blastomycosis, candidiasis, coccidioidomycosis, histoplasmosis, and paracoccidioidomycosis were used to evaluate the sensitivity and specificity of the paracoccidioidin antigens produced in this study. Each of the sera from these cases had been previously evaluated with standardized antigens in complement fixation (CF) and ID tests to verify the presence of specific diagnostic homologous antibodies.

CF test. The Centers for Disease Control Laboratory Branch micro-CF test was used (4). The optimal antigen dilution for each *P. brasiliensis* culture filtrate antigen was determined by block titration against sera from human cases of paracoccidioidomycosis. Five 50% units of complement were used with the optimal concentration of antigen and test sera. Sera demonstrating 30% hemolysis or less at a given dilution were considered positive. The initial dilution of patient and control sera was 1:8.

ID test. A micro-immunodiffusion (MID) test was performed in a gel composed of 1% purified agar in a medium (pH 6.4) containing 0.9% NaCl, 0.4% sodium citrate, 7.5% glycine, and 0.25% phenol. An acrylic template with 17 seven-well patterns (1) was secured to the agar medium contained in a plastic petri dish (15 by 100 mm). Antigen was placed in the central well of a pattern, reference serum was placed in wells above and below the antigen well, and patient sera were added to the four remaining lateral wells. The patient sera and reference sera were prediffused for 45 min at room temperature before the antigen was added. Plates containing the reactants were placed in a moist chamber at room temperature and incubated for 24 h. Before the precipitin reactions were observed, the plates were rinsed with distilled water, and the templates were removed. Precipitin bands were examined by obliquely transmitted light.

RESULTS

The quantity of *P. brasiliensis* antigen produced and the time necessary for its production varied among the isolates tested and the media used (Table 1). Four of the six isolates (B339, B341, L6712, and L6833) produced the specific E/1 antigen more rapidly in the SABHI broth than in the TSB. Two isolates (B1183 and L6043) produced E/1 antigen in the same time in SABHI broth and TSB. The specific E/1 antigen produced in the SABHI broth was of equal or greater concentration than the antigen produced in the TSB. Isolate B339 exceeded the others both in the relative concentration of the E/1 antigen produced and in the shortness of the incubation period in SABHI broth. To determine the quality of the rapidly produced antigen, we performed CF and MID studies with three lots (A, B, and C) of culture filtrate antigen produced by strain B339 under various conditions. Lots A and B were filtrates of B339 grown in SABHI broth for 14 and 25 days, respectively. Lot C was from a B339 culture filtrate grown in TSB for 14 days. The optimal dilutions of lots A, B, and C in the CF test were 1:32, 1:64, and 1:32, respectively.

TABLE 1. E/1 antigen production by six *P. brasiliensis* mold-form isolates in TSB and SABHI broth

Isolate	Broth medium	E/1 antigen production	
		Minimum time (day) for detection	Concn[a]
B339	TSB	10	100
B339	SABHI	7	320
B341	TSB	28	10
B341	SABHI	21	10
B1183	TSB	14	10
B1183	SABHI	14	10
L6043	TSB	21	20
L6043	SABHI	21	50
L6712	TSB	14	10
L6712	SABHI	10	80
L6833	TSB	14	20
L6833	SABHI	10	50

[a] Obtained by multiplying the concentration factor (10) by the reciprocal of the optimal dilution for use of the antigen in the MID test.

All of the rapidly produced antigens demonstrated sensitivities equal to that of the Restrepo reference antigen in both the MID and CF tests. Of the 18 paracoccidioidomycosis case sera tested by CF, 17 were positive with the Restrepo and E/1 antigens. Of the 114 sera tested by MID, 103 were positive with the Restrepo and E/1 antigens. A positive result was indicated by titers of 1:8 or greater in the CF test and bands of identity with reference E/1 antibody in the MID test. The CF titers ranged from 1:8 to 1:1,024 with the Restrepo antigen and the rapidly produced antigen of lots A and B, but they were lower with lot C.

The specificity of the rapidly produced antigen was comparable to that of the Restrepo antigen in the MID test (Table

TABLE 2. Comparison of the specificity of the rapidly produced *P. brasiliensis* antigen lots A, B, and C with the Restrepo antigen 1 in MID tests with human sera from heterologous fungus diseases

Disease	No. of cases	No. positive by nonspecific reaction[a]	
		Restrepo antigen	Rapidly produced antigen[b]
Aspergillosis	32	2	2
Blastomycosis	10	0	0
Candidiasis	32	0	0
Coccidioidomycosis	32	2	0
Histoplasmosis	33	7	7

[a] There were no specific anti-E/1 reactions with either the Restrepo antigen or the rapidly produced antigen.

[b] Identical results were observed with three different lots produced by isolate B339 in either SABHI broth or TSB.

2) but not in the CF test (Table 3). None of the heterologous sera produced lines of identity with the reference E/1 precipitate after reaction with the rapidly produced antigens. Cross-reactions, however, were evident with other antigenic components of the preparations studied. The three lots of rapidly produced antigen that were evaluated in the CF test demonstrated degrees of nonspecificity ranging from 28% (lot A) to 52% (lot C) compared to 8% nonspecificity noted with the antigen produced by the method of Restrepo. The CF titer range of the nonspecific reactions with the Restrepo antigen was 1:8 to 1:16 and occurred with only two histoplasmosis case sera. The rapidly produced antigen showed nonspecific titers that ranged from 1:8 to 1:32 with lots A and B and 1:8 to 1:64 with lot C and occurred with almost all of the heterologous case sera tested.

MID tests with selected reference sera confirmed that the E/1 antigen, rapidly produced by the six isolates, was identical to the Yarzabal E antigen and the Restrepo antigen 1 (Fig. 1). Although nonspecific precipitins were observed in the MID test (Table 2; Fig. 1 and 2c), the use of specific antigen E/1 and anti-E/1 reference serum permitted the identification of diagnostic precipitins in sera from cases of paracoccidioidomycosis.

DISCUSSION

A reproducible method for obtaining high-titer antigen for specifically detecting paracoccidioidomycosis antibody by the MID test has been developed. This method is simple in that it utilizes commercially available media and a mold-form *P. brasiliensis* culture. It is also more rapid than the methods previously described. High-titer specific *P. brasiliensis* serodiagnostic antigens may be produced in two weeks or less by the newly described rapid method compared to 4 to 6 weeks reported by Restrepo-Moreno and Schneidau (3) and 12 weeks reported by Yarzabal et al. (5). The rapidity of antigen production achieved by our procedure might be attributed to our use of filtrates from shake cultures rather than static cultures as described by Yarzabal et al. (5). The variability in the time for antigen production and the quantity of antigen produced (Table 1) appears to result from both the isolate selected and the growth medium used. The mean time for detection of antigen produced by all isolates in TSB was 16.8 days, whereas the mean time in SABHI broth was 13.8 days. Optimal concentrations of E/1 antigen prepared by the rapid method were obtained by diluting the culture filtrates (concentrated 10 times) from 1:1 to 1:32. The optimal concentration of the Yarzabal E antigen, when used in our MID system, was found in a 1:8 dilution of a suspension (200 mg/ml) of lyophilized culture filtrate antigen. The concentration of E/1 antigen produced in a given time by the rapid method was greater with SABHI broth medium. These qualities make SABHI broth the medium of choice for *P. brasiliensis* serodiagnostic antigen production.

TABLE 3. Comparison of the specificity of the rapidly produced *P. brasiliensis* antigen lots A, B, and C with the Restrepo antigen 1 in CF tests with human sera from heterologous fungus diseases

Disease[a]	No. positive[b]			
	Restrepo antigen	Rapid antigen lot		
		A	B	C
Aspergillosis	0	2	2	3
Blastomycosis	0	0	4	5
Candidiasis	0	1	2	2
Coccidioidomycosis	0	2	1	1
Histoplasmosis	2	2	3	2

[a] There were five cases of each disease.
[b] Titers of 1:8 or greater.

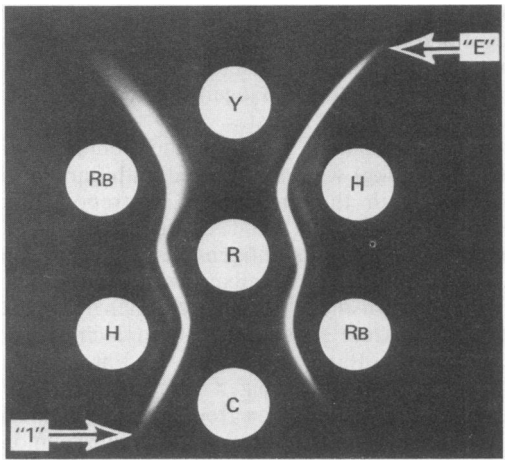

FIG. 1. MID test for paracoccidioidomycosis demonstrating the identity of the E/1 antigen to the E antigen of Yarzabal and the Restrepo antigen 1. H, Serum from a human case of paracoccidioidomycosis (contains antibody to antigens 1, 2, and 3). RB, Serum from a rabbit immunized with culture filtrate antigens of *P. brasiliensis* (contains antibody to antigen E/1). R, Culture filtrate antigen of *P. brasiliensis* prepared by the Restrepo method (contains antigens 1, 2, and 3). Y, Yarzabal culture filtrate with *P. brasiliensis* E antigen. C, Culture filtrate antigen of *P. brasiliensis* prepared by the rapid culture method described in this report (E/1).

The sensitivity of the *P. brasiliensis* mold-form antigen produced by the rapid method was equivalent to that of the yeast-form or conventional antigen produced by the Restrepo method. Both antigens detected antibody to E/1 antigen or antigen 1 in the MID test with sera from 103 of 114 (90%) human cases of paracoccidioidomycosis. Restrepo and Moncada (2) have previously reported that 8 of 54 (13%) patients tested for antibody against antigens 1, 2, and 3 had precipitin bands other than the specific band 1. However, none of 114 sera examined in this study demonstrated precipitins to antigen 2 or 3. Most of the precipitin-negative sera in our study were obtained in follow-up studies many months to years after diagnosis and treatment. Restrepo and Moncada (2) noted that precipitin band 1 (E/1) disappeared in ca. 22% of cases after 1 or more years postdiagnosis and treatment. In this regard, the disappearance of band 1 (E/1) might be considered a good prognostic sign.

Although an occasional heterologous serum (Fig. 2c) demonstrated precipitins against either the Restrepo antigen or the rapidly produced antigen, the use of specific reference sera in each MID test established the identification of diagnostic precipitins only in sera from cases of paracoccidioidomycosis. The rapidly produced antigen does not appear to be suitable for use in the CF test. This antigen demonstrated a high degree of nonspecificity (Table 3) that could not be attributed to any single factor. This is indicative of the presence of multiple antigens in addition to E/1. The lower degree of nonspecific CF reactions with the Restrepo antigen may be due to differences in the quantity and types of antigens produced by the yeast-form (Restrepo) antigen (3) in contrast to the mold-form rapid antigen, or the yeast antigen may be purer since it is produced in a dialysate medium and then extensively dialyzed before use. Attempts to improve the CF specificity of the rapidly produced antigen by dialysis against distilled water, however, were not suc-

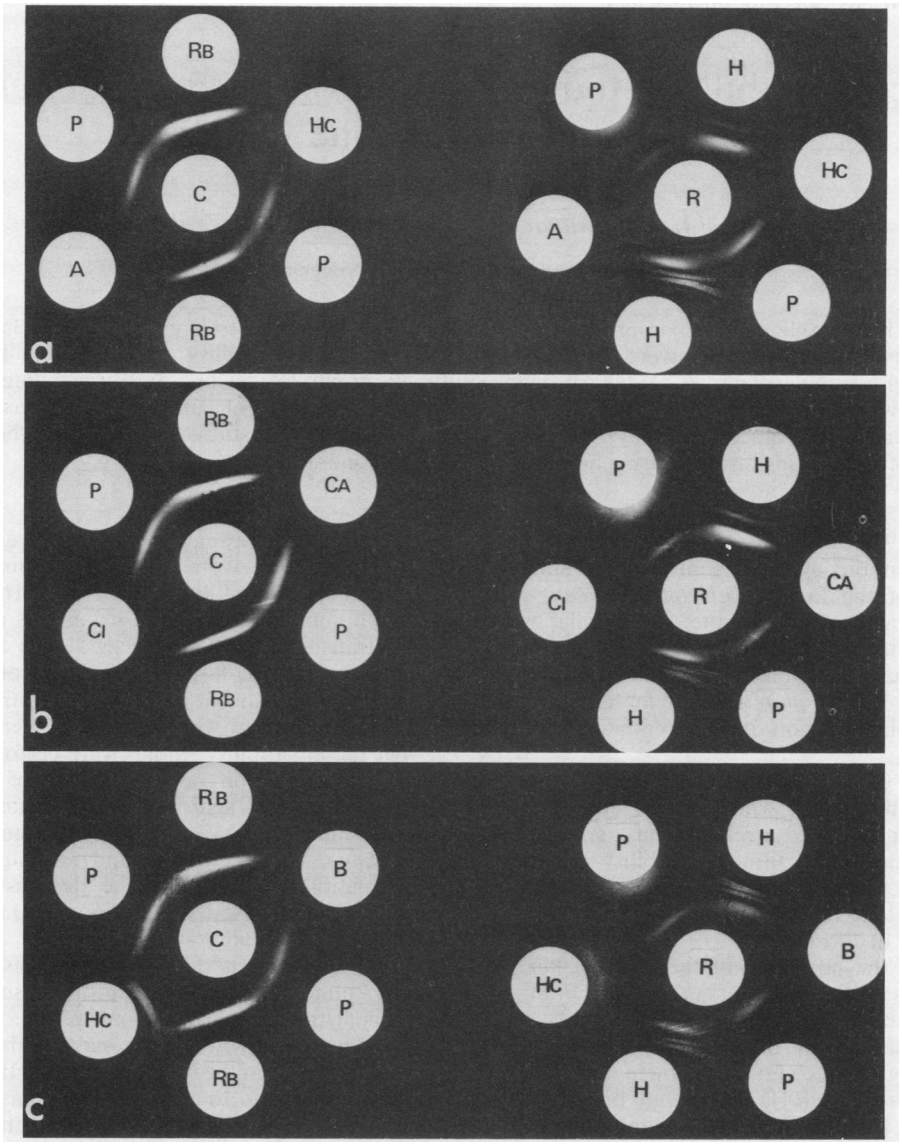

FIG. 2. Comparison of the reactivity of the Restrepo antigen 1 with the E/1 antigen. Definitions for H, RB, R, and C are the same as in the legend to Fig. 1. A, Serum from a human case of aspergillosis. B, Serum from a human case of blastomycosis. CA, Serum from a human case of candidiasis. CI, Serum from a human case of coccidioidomycosis. HC, Sera from two human cases of histoplasmosis. P, Sera from two human cases of paracoccidioidomycosis. Identical results were observed with lots A, B, and C.

cessful. Studies with paracoccidioidomycosis case sera indicated that the positive CF tests were invariably accompanied by positive ID tests and that, in many cases, the ID test was positive before the CF test (3). Therefore, the availability of a rapidly and readily produced specific *P. brasiliensis* antigen useful only in ID tests is not a handicap. The ease with which this antigen can be produced and reproduced should encourage laboratory workers to adopt the rapid method for producing the specific E/1 antigen for the diagnosis of paracoccidioidomycosis by ID.

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LITERATURE CITED

1. **Busey, J. F., and P. F. Hinton.** 1965. Precipitins in histoplasmosis. Am. Rev. Respir. Dis. **92**:637–639.
2. **Restrepo, A., and L. H. Moncada.** 1974. Characterization of the precipitin bands detected in the immunodiffusion test for paracoccidioidomycosis. Appl. Microbiol. **28**:138–144.
3. **Restrepo-Moreno, A., and J. D. Schneidau, Jr.** 1967. Nature of the skin-reactive principle in culture filtrates prepared from *Paracoccidioides brasiliensis*. J. Bacteriol. **93**:1741–1748.
4. **U.S. Public Health Service.** 1965. Standardized diagnostic complement fixation method and adaption to microtest. U.S. Public Health Service publication no. 1228. Department of Health, Education, and Welfare, Washington, D.C.
5. **Yarzabal, L. A., S. Andrieu, D. Bout, and F. Naquira.** 1976. Isolation of a specific antigen with alkaline phosphatase activity from soluble extracts of *Paracoccidioides brasiliensis*. Sabouraudia **14**:275–280.