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Diagnosis of Paracoccidioidomycosis by Detection of Antigen and Antibody in Bronchoalveolar Lavage Fluids▽

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Paracoccidioidomycosis (PCM) is a systemic infection caused by the fungus *Paracoccidioides brasiliensis* and is believed to be the leading cause of fungal pulmonary infection. In this study, we used an inhibition enzyme-linked immunosorbent assay to diagnose pulmonary PCM based on the detection of 43-kDa and 70-kDa molecules in bronchoalveolar lavage fluids. The results were compared with results obtained by classical methods for antibody detection.

Paracoccidioidomycosis (PCM) is a systemic endemic mycosis caused by the dimorphic fungus *Paracoccidioides brasiliensis* that affects rural workers in Latin American countries (1, 6, 9). It has a wide spectrum of clinical manifestations, ranging from mild pulmonary lesions to severe disseminated forms involving many organs, especially the mucosae, skin, lymph nodes, adrenals, and central nervous system (6, 9). The primary pulmonary infection is unapparent or oligosymptomatic in most cases, and individuals may remain infected throughout their lives without ever developing PCM. In most cases, symptomatic patients develop the disease years after acquiring the infection as a result of reactivation of the quiescent foci (chronic form) (3, 9, 10). Clinical findings in these patients generally include severe pulmonary involvement, followed by extrapulmonary dissemination. In PCM, lung destruction involves the alveoli, interstitium, and bronchial tree, resulting in fibrosis, ventilatory dysfunction, and hypoxemia (22). Tobón et al. (23) recently reported that late diagnosis and disseminated lung involvement are two conditions associated with a higher rate of pulmonary sequelae.

Definitive diagnosis of pulmonary PCM is based on the visualization of fungal elements characteristic of *P. brasiliensis* in biopsy material, respiratory secretion, or sputum culture. However, processing respiratory secretion for direct examination is time-consuming. Culture is difficult because sputum is contaminated with bacteria and other yeasts such as *Candida* sp. that inhibit the growth of *P. brasiliensis*, a fastidious organism, and bronchoscopy and lung biopsy may be difficult to perform in patients with severe respiratory dysfunction. Hence, serological methods based on antibody or antigen detection may be useful tools for diagnosis of the disease. Marques-da-Silva et al. (15–18) recently described an antigen detection assay (the inhibition enzyme-linked immunosorbent assay [inh-ELISA]) for the gp43 and gp70 molecules of *P. brasiliensis* with good potential for use in diagnosis and follow-up of patients with PCM. The detection of *P. brasiliensis* antigens in body fluids might facilitate early diagnosis of PCM even in patients with pulmonary involvement.

In the present study, gp43 and gp70 antigens of *P. brasiliensis* were detected in bronchoalveolar lavage (BAL) fluid samples from patients with pulmonary PCM using an inh-ELISA. The results were compared with those obtained for anti-*P. brasiliensis* antibodies detected by immunodiffusion (ID) tests and ELISA.

BAL fluid and serum samples were obtained from 27 patients with pulmonary PCM. Patients were selected based on clinical, serological, and chest roentgenogram findings as well as on direct examination of sputum, in which characteristic *P. brasiliensis* multibudding yeast cells were seen in all patients. The patients enrolled in this study were from Hospital São Paulo, São Paulo Federal University (UNIFESP), and Hospital das Clínicas, State University of Campinas (UNICAMP), Campinas, São Paulo, Brazil. All patients were male, with an average age of 46 years. They were subjected to bronchoalveolar lavage procedures. None were suffering from AIDS or other profoundly immunosuppressive conditions. Each lavage was performed with five 20-ml aliquots of preservative-free normal saline, and lavage fluids were then centrifuged at 2,500 × *g* for 10 min in a tabletop centrifuge to prepare sediments for direct examination and cultures. The supernatants were stored at 4°C and heated to 56°C for 30 min before being tested for antigens. The cellular sediment was separated for direct examination with 30% KOH (characteristic *P. brasiliensis* multibudding yeast cells were visualized in all sediments) and for culture, but no growth was obtained. In addition, serum samples from patients were tested for anti-*P. brasiliensis* antibodies (immunodiffusion and ELISA) and for specific *P. brasiliensis* antigens (inh-ELISA). Control groups included 10

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BAL fluid samples from patients with noninfectious diseases and 10 from patients with other infectious diseases such as tuberculosis. The study was evaluated and approved by the Ethics Committee of the Federal University of São Paulo.

P. brasiliensis B-339 (ATCC 200273) was obtained from the culture collection of the Cell Biology Discipline, Federal University of São Paulo, and transformed to the yeast phase; exoantigen was produced according to the method of de Camargo et al. (4, 5), and gp43 and gp70 antigens were purified from this exoantigen (20, 21). Protein content was determined by the Bradford method (2). Anti-gp43 and anti-gp70 monoclonal antibodies (MAbs) were supplied by R. Puccia (21) and D. Mattos Grosso (20), respectively.

inh-ELISA was performed as previously described (11, 15). Aliquots of BAL fluid and serum samples (200 μl) were mixed with an equal volume of 0.1 M EDTA (Sigma), pH 7.2, and boiled at 100°C for 5 min. After cooling, tubes were centrifuged at 13,000 × *g* for 30 min, and the supernatants were used for the test. First, a standard inhibition curve was prepared by adding known concentrations of gp43 or gp70 to a pool of normal human sera or BAL fluid controls in different plates (inhibition standards) (11, 15). The inhibition reaction occurred when constant aliquots of anti-gp43 or anti-gp70 MAbs were mixed with the inhibition standards, PCM patients' sera or BAL fluid samples, and normal human sera and BAL fluid control samples. Samples were then plated on a previously blocked microtiter plate (inhibition plate) and incubated overnight at 4°C. The reaction plate was coated with gp43 or gp70 and incubated overnight at 4°C. Free sites on the polystyrene were blocked with 5% skim milk in phosphate-buffered saline (PBS)–Tween 20, and samples from each well in the inhibition plate (containing a mixture of MAb [anti-gp43 or anti-gp70] bound to circulating antigen and free MAb) were transferred to the respective wells in the reaction plate. The plate was washed, probed with goat anti-mouse immunoglobulin–peroxidase conjugate, and developed with a chromogenic substrate as previously described (11, 15). Optical density (OD) readings at 492 nm were then plotted on a standard curve constructed from the data derived from MAb titration with the inhibition standards. The antigen concentrations in the patients' BAL fluid and serum samples were calculated with a regression model constructed with the reciprocal values of fixed concentrations of gp43 or gp70 and the OD values. All tests were performed in duplicate. All specimens included here had been tested previously when they were first obtained and were tested again using the same technique in this study. Inhibition standard curves were performed in duplicate for at least four independent assays. Data were statistically analyzed using the Stata 7.0 program for Windows 98/95/NT (Stata Corporation, College Station, TX), and specificity and sensitivity were analyzed using the receiver operator characteristic (ROC) curve. The sensitivity of the test ranged from 0.001 to 30 μg/ml.

ID tests were performed with BAL fluid samples and serum samples at the time of diagnosis as previously described (4). For the ELISAs, microtiter plates (Costar) were coated with 100 μl of purified gp43 or gp70 (500 ng/well), diluted in 0.06 M carbonate-bicarbonate buffer, pH 9.6, and incubated for 2 h at 37°C and overnight at 4°C. The plates were washed three times with PBS-Tween 20 (0.05%), and free sites were blocked with 5% skim milk in PBS-Tween (200 μl/well) for 2 h at 37°C. After three washes, 100 μl of BAL fluid samples (1:50 to 1:51,200 dilution) from each patient was added to each well, and the plates were incubated at 37°C for 1 h. Plates were then washed again, 100 μl of peroxidase-labeled goat anti-human immunoglobulin (1:1,000 dilution; Sigma) was added to each well, and the plates were incubated for 1 h at 37°C. After 3 washes, the reaction was developed by adding 100 μl of a mixture of *O*-phenylenediamine (0.2 mg/ml; Sigma) and 0.05% (vol/vol) H_2O_2 in 0.1 M citrate buffer, pH 4.5. After 8 min of incubation in the dark, the reaction was stopped with 50 μl of 4 N H_2SO_4. OD was measured at 492 nm using an ELISA microplate reader (Titertek Multiskan MCC/340).

gp43 and gp70 antigens in BAL fluid were detected by preparing a standard inhibition curve and determining the cutoff values (0.23 μg/ml for gp43 and 0.21 μg/ml for gp70) using an ROC curve. BAL samples with concentrations above these values were considered positive. gp43 and gp70 were detected in all BAL fluid samples (100%). The mean antigen concentration found for gp43 (9.38 μg/ml) was significantly higher than that for gp70 (4.37 μg/ml, $P = 0.0001$). BAL fluid samples from individuals with diseases other than PCM gave negative results (Table 1).

gp43 and gp70 were detected in sera by preparing a standard inhibition curve and determining the cutoff values (0.23 μg/ml for gp43 and 0.21 μg/ml for gp70) using an ROC curve. Samples with concentrations above these values were considered positive. gp43 (mean, 15.53 μg/ml) and gp70 (mean, 7.86 μg/ml) antigens were detected in all of the patients' sera ($P = 0.0001$).

None of the 27 BAL fluid samples were positive for *P. brasiliensis* crude exoantigen in immunodiffusion tests. However, ID tests showed that 85.18% of the sera were positive, with antibody titers ranging from 1:8 to 1:64. When tested by ELISA, all BAL fluid samples (100%) had anti-gp43 and anti-gp70 antibodies, with titers ranging from 1:50 to 1:400 (Table 1).

Detection of circulating antigen is a useful approach for serodiagnosis in some invasive fungal diseases (7, 8, 14, 19) and may also be an alternative tool for diagnosing PCM patients. Goméz et al. (11, 12) were the first researchers to use monoclonal antibodies to detect an 87-kDa circulating antigen in PCM and achieved a sensitivity of 80.4%. Marques-da-Silva et al. (15–18) recently described an antigen detection assay (inh-ELISA) for *P. brasiliensis* gp43 and gp70 molecules with good potential for diagnosis and follow-up of patients with PCM.

PCM is acquired either by inhalation of mycelial propagules of the fungus in nature or by reactivation of latent foci of infection. Pulmonary involvement is characteristic of PCM, and BAL fluid would accordingly be expected to contain antigens. In other pulmonary diseases, such as histoplasmosis, Wheat et al. (24) found elevated levels of *Histoplasma* antigens in BAL fluid in 19 (70.3%) of 27 cases. Graybill et al. (13) also reported the presence of *Histoplasma* antigens in BAL fluid of mice with experimentally induced histoplasmosis.

In the present study, *P. brasiliensis* gp43 and gp70 were detected in all BAL fluid samples from patients with pulmonary PCM, with mean antigen concentrations of 9.38 μg/ml and 4.37 μg/ml, respectively. Our results suggest that monitoring specific antigens of *P. brasiliensis* in BAL samples may be

TABLE 1. Serological results for 27 patients with pulmonary PCM and control groups evaluated by inh-ELISA, immunodiffusion, and ELISA

Subject no.	BAL fluid antigen concn (μg/ml)[a]		BAL fluid ELISA result (dilution)[b]		Serum antigen concn (μg/ml)[a]		ID antibody titer in:	
	gp43	gp70	anti-gp43	anti-gp70	gp43	gp70	BAL fluid	Serum
1	11.25	5.27	+ (1:200)	+ (1:100)	15.0	6.38	0	1:16
2	12.75	4.16	+ (1:400)	+ (1:50)	15.0	9.12	0	1:32
3	3.0	0.93	+ (1:200)	+ (1:50)	21.0	11.21	0	1:16
4	16.5	5.64	+ (1:50)	+ (1:50)	19.5	7.5	0	1:8
5	4.16	2.67	+ (1:200)	+ (1:100)	13.5	7.12	0	0
6	6.38	3.39	+ (1:200)	+ (1:50)	14.25	30.0	0	1:16
7	9.0	4.53	+ (1:50)	+ (1:50)	27.0	10.2	0	1:32
8	19.5	4.90	+ (1:50)	+ (1:50)	16.5	13.87	0	1:64
9	6.75	4.22	+ (1:50)	+ (1:50)	14.25	1.87	0	1:2
10	22.5	12.75	+ (1:200)	+ (1:50)	15.0	8.83	0	1:8
11	9.65	5.02	+ (1:200)	+ (1:50)	18.0	4.53	0	1:4
12	8.71	4.87	+ (1:50)	+ (1:50)	7.12	2.85	0	1:16
13	5.92	3.01	+ (1:50)	+ (1:50)	3.29	2.76	0	1:8
14	15.05	7.15	+ (1:200)	+ (1:50)	17.57	5.27	0	1:16
15	16.0	4.89	+ (1:400)	+ (1:100)	9.45	4.64	0	1:64
16	11.75	6.0	+ (1:400)	+ (1:50)	12.76	7.23	0	1:64
17	8.06	3.79	+ (1:200)	+ (1:100)	10.67	6.63	0	0
18	3.25	1.20	+ (1:100)	+ (1:50)	12.66	8.68	0	1:16
19	9.65	4.17	+ (1:200)	+ (1:100)	19.34	6.69	0	1:16
20	11.87	7.79	+ (1:400)	+ (1:100)	29.76	11.12	0	0
21	10.21	5.71	+ (1:50)	+ (1:50)	13.46	6.93	0	1:16
22	2.71	1.00	+ (1:50)	+ (1:50)	9.55	3.86	0	1:32
23	3.09	2.01	+ (1:50)	+ (1:50)	13.89	7.78	0	1:64
24	6.58	3.79	+ (1:200)	+ (1:100)	19.33	7.44	0	1:8
25	7.21	4.29	+ (1:200)	+ (1:50)	22.37	7.93	0	1:16
26	8.87	3.0	+ (1:100)	+ (1:50)	10.52	3.03	0	1:8
27	3.0	1.89	+ (1:100)	+ (1:50)	18.65	8.98	0	0
Mean	9.38	4.37			15.53	7.86		
Control A[c]	<0.23	<0.21	0	0				
Control B[d]					<0.23	<0.21	0	0

[a] Antigen detection by inh-ELISA.
[b] Antibody detection by ELISA. +, positive. 0, negative.
[c] Ten BAL fluid samples from patients with noninfectious diseases were negative for antigens and antibodies (control patients).
[d] Ten BAL fluid samples from patients with other infectious diseases were negative for antigens and antibodies (control patients).

helpful in determining a diagnosis of pulmonary PCM, particularly when the infection is in its initial stage. Since antigen values for gp43 were always higher than those found for gp70, assaying only for gp43 may prove sufficient for that purpose. No antibodies were detected in BAL fluids when tested by ID, whereas antibodies against both antigens were detected by ELISA, although the titers were low. However, when sera from these patients were tested by ID, 85.18% of them reacted positively, with titers ranging from 1:8 to 1:64. Despite the limited number of patients in this study, the data suggest that antigen detection in BAL by inh-ELISA or the detection of specific antibodies by ELISA may be equally sensitive in identifying pulmonary PCM. The fact that gp43 and gp70 antigens were detected in the sera of 100% of these patients indicates that this test, as well as the detection of antigens in BAL fluid, has a role to play in the diagnosis of patients with suspected pulmonary PCM, diffuse pulmonary infiltrates, or unexplained febrile illnesses. However, antibody detection by ELISA as a routine procedure is less cumbersome and time-consuming than antigen detection. Hence, testing of BAL samples for antigens would be recommended only when a suspected patient has either negative or inconsistent results for antibody detection or negative results in the direct examination of sputum or when sera of suspected patients are negative by ID.

Our results show that *P. brasiliensis* antigen detection in BAL fluid is a valuable tool for diagnosis of pulmonary PCM. Nevertheless, the limited clinical and radiological information available for the small number of patients enrolled in our study proscribed the possibility of establishing a correlation between antigen levels and the severity of the disease.

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REFERENCES

1. **Blotta, M. H. S. L., R. L. Mamoni, S. J. Oliveira, A. S. Nouér, P. M. O. Papaiordanou, A. Gouveia, and Z. P. de Camargo.** 1999. Endemic regions of paracoccidioidomycosis in Brazil: a clinical and epidemiologic study of 584 cases in southeast region. Am. J. Trop. Med. Hyg. **61:**390–394.
2. **Bradford, M.** 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
3. **Brummer, E., E. Castañeda, and A. Restrepo.** 1993. Paracoccidioidomycosis: an update. Clin. Microbiol. Rev. **2:**89–117.
4. **de Camargo, Z. P., C. Unterkircher, S. P. Campoy, and L. R. Travassos.** 1988. Production of *Paracoccidioides brasiliensis* exoantigens for immunodiffusion tests. J. Clin. Microbiol. **26:**2147–2151.
5. **de Camargo, Z. P., R. Berzaghi, C. C. Amaral, and S. H. Marques-da-Silva.**

2003. Simplified method for producing *Paracoccidioides brasiliensis* exoantigens for use in immunodiffusion tests. Med. Mycol. **41:**539–542.
6. **Del Negro, G., C. S. Lacaz, V. A. Zamith, and A. M. Siqueira.** 1994. General clinical aspects: polar forms of paracoccidioidomycosis, the disease in childhood, p. 225–232. *In* M. Franco, C. S. Lacaz, A. Restrepo-Moreno, and G. Del Negro (ed.), Paracoccidioidomycosis. CRC Press, Boca Raton, Fla.
7. **Dupont, B., M. Huber, S. J. Kim, and J. E. Bennet.** 1987. Galactomannan antigenemia and antigenuria in aspergillosis studies in patients and experimental infected rabbits. J. Infect. Dis. **155:**1–11.
8. **Ferreira, R. P., B. Yu, Y. Niki, and D. Armstrong.** 1990. Detection of *Candida* antigenuria in disseminated candidiasis by immunoblotting. J. Clin. Microbiol. **28:**1075–1078.
9. **Franco, M., M. R. Montenegro, R. P. Mendes, S. A. Marques, N. L. Dillon, and N. G. S. Mota.** 1987. Paracoccidioidomycosis: a recently proposed classification on its clinical forms. Rev. Soc. Bras. Med. Trop. **20:**129–132.
10. **Franco, M., M. T. S. Peraçoli, A. M. V. C. Soares, M. R. Montenegro, R. P. Mendes, and D. A. Meira.** 1993. Host-parasite relationships in paracoccidioidomycosis. Curr. Trop. Med. Mycol. **5:**115–149.
11. **Gómez, B. L., J. I. Figueroa, A. J. Hamilton, B. Ortiz, M. A. Robledo, R. J. Hay, and A. Restrepo.** 1997. Use of monoclonal antibodies in diagnosis of paracoccidioidomycosis: new strategies for detection of circulating antigens. J. Clin. Microbiol. **35:**3278–3283.
12. **Gómez. B. L., J. I. Figueroa, A. J. Hamilton, B. I. Ortiz, M. A. Tobón, R. J. Hay, and A. Restrepo.** 1998. Antigenemia in patients with paracoccidioidomycosis: detection of the 87-kilodalton determinant during and after antifungal therapy. J. Clin. Microbiol. **36:**3309–3316.
13. **Graybill, J. R., E. Palou, and J. Ahrens.** 1986. Treatment of murine histoplasmosis with UK 49,858 (fluconazole). Am. Rev. Respir. Dis. **134:** 768–770.
14. **Haynes, K. A., J. P. Latgé, and T. R. Rogers.** 1990. Detection of *Aspergillus* antigens associated with invasive infection. J. Clin. Microbiol. **28:**2040–2044.
15. **Marques-da-Silva, S. H., A. L. Colombo, M. H. S. L. Blotta, J. D. Lopes, F. Queiroz-Telles, and Z. P. de Camargo.** 2003. Detection of circulating gp43 antigen in serum, cerebrospinal fluid, and bronchoalveolar lavage fluid of patients with paracoccidioidomycosis. J. Clin. Microbiol. **41:**3675–3680.
16. **Marques-da-Silva, S. H., F. Queiroz-Telles, A. L. Colombo, M. H. S. L. Blotta, J. D. Lopes, and Z. P. de Camargo.** 2004. Monitoring gp43 antigenemia in paracoccidioidomycosis patients during therapy. J. Clin. Microbiol. **42:**2419–2424.
17. **Marques-da-Silva, S. H., D. Mattos Grosso, J. D. Lopes, A. L. Colombo, M. H. S. L. Blotta, F. Queiroz-Telles, and Z. P. de Camargo.** 2004. Detection of *Paracoccidioides brasiliensis* gp70 circulating antigen and follow-up of patients undergoing antimycotic therapy. J. Clin. Microbiol. **42:**4480–4486.
18. **Marques-da-Silva, S. H., A. L. Colombo, M. H. S. L. Blotta, F. Queiroz-Telles, J. D. Lopes, and Z. P. de Camargo.** 2005. Diagnosis of neuroparacoccidioidomycosis by detection of circulating antigen and antibody in cerebrospinal fluid. J. Clin. Microbiol. **43:**4680–4683.
19. **Matthews, R. C.** 1996. Comparative assessment of the detection of candidal antigens as a diagnostic tool. J. Med. Vet. Mycol. **34:**1–10.
20. **Mattos Grosso, D., S. R. Almeida, M. Mariano, and J. D. Lopes.** 2003. Characterization of gp70 and anti-gp70 monoclonal antibodies in *Paracoccidioides brasiliensis* pathogenesis. Infect. Immun. **71:**6534–6542.
21. **Puccia, R., S. Schenkman, P. A. Gorin, and L. R. Travassos.** 1986. Exocellular components of *Paracoccidioides brasiliensis*: identification of a specific antigen. Infect. Immun. **53:**199–206.
22. **Ratto, O. R., and J. E. Afonso.** 1994. Pulmonary lesions: clinical and functional aspects, p. 271–279. *In* M. Franco, C. S. Lacaz, A. Restrepo-Moreno, and G. Del Negro (ed.), Paracoccidioidomycosis. CRC Press, Boca Raton, Fla.
23. **Tobón, A. N., C. A. Agudelo, M. L. Osorio, D. L. Alvarez, M. Arango, L. E. Cano, and A. Restrepo.** 2003. Residual pulmonary abnormalities in adult patients with chronic paracoccidioidomycosis: prolonged follow-up after itraconazole therapy. Clin. Infect. Dis. **37:**898–904.
24. **Wheat, L. J., P. Connoly-Stringfield, B. Williams, K. Connoly, R. Blair, M. Bartlett, and M. Durkin.** 1992. Diagnosis of histoplasmosis in patients with the acquired immunodeficiency syndrome by detection of *Histoplasma capsulatum* polysaccharide antigen in bronchoalveolar lavage fluid. Am. Rev. Resp. Dis. **145:**1421–1424.