

Serological diagnosis of paracoccidioidomycosis in HIV-coinfected patients

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Paracoccidioidomycosis should be differentiated from other opportunistic diseases in human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) patients who live in Latin America. Laboratory investigation can begin with serological tests, which are rapid and efficient. In the present study, double immunodiffusion (DID), counterimmunoelectrophoresis (CIEP) and an enzyme linked immunosorbent assay (ELISA) tests were assessed for the detection of anti-Paracoccidioides brasiliensis antibodies in 40 patients coinfecting with HIV. The results were compared to those obtained for 75 non-HIV-infected patients with endemic paracoccidioidomycosis. Anti-P. brasiliensis antibodies were detected in 65% (DID), 79% (CIEP) and 95% (ELISA) of the patients with HIV/AIDS, significantly lower rates than those detected in cases of endemic paracoccidioidomycosis, which were 89%, 99% and 100%, respectively. The reactive sera of HIV-infected patients also showed lower anti-P. brasiliensis antibody titres than those of non-HIV-infected patients. Despite the lower intensity of the specific humoral response, serological tests are useful for the diagnosis of opportunistic paracoccidioidomycosis in the HIV/AIDS population. We suggest optimization of the laboratory diagnosis by combining the ELISA test with CIEP or DID.

Key words: paracoccidioidomycosis - HIV - AIDS - double immunodiffusion - ELISA - counterimmunoelectrophoresis

In a vast area of South America, paracoccidioidomycosis is the fourth most common systemic fungal infection among patients with human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS). Opportunistic infection with *Paracoccidioides brasiliensis* is generally disseminated among patients with a low CD4 cell count, involving the lungs, tegument, lymph nodes, monocyte-macrophage system and other tissues (Silva-Vergara et al. 2003, Morejón et al. 2009). This clinical picture must be differentiated from other opportunistic infections, particularly those involving mycobacteria and histoplasmosis.

The detection of serum anti-*P. brasiliensis* antibodies is a rapid, non-invasive and efficient method for the laboratory diagnosis of endemic paracoccidioidomycosis not associated with HIV/AIDS. The recommended serological tests are double immunodiffusion (DID), counterimmunoelectrophoresis (CIEP) and enzyme linked immunosorbent assay (ELISA) (Shikanai-Yasuda et al. 2006). With the use of appropriate antigens and procedures, these tests reach a sensitivity of 90% or more and high specificity (Mendes-Giannini et al. 1994). The titration of anti-*P. brasiliensis* antibodies is important for the laboratory diagnosis and follow up of paracoccidioidomycosis. Patients with active disease usually have high antibody levels, which decline with antifungal therapy and clinical recovery (Del Negro et al. 2000).

Immunosuppressed patients with opportunistic paracoccidioidomycosis may have a reduced specific humoral immune response. A significant proportion of false-negative results of serological tests for the detection of antibodies against *P. brasiliensis* have been observed in patients with AIDS-associated paracoccidioidomycosis (Marques et al. 2000). Few cases of this coinfection have been reported and they are distributed among various medical services with no uniformity regarding the serological tests (Goldani & Sugar 1995). Thus, the objective of the present study was to compare the performance of DID, CIEP and ELISA for the serological diagnosis of paracoccidioidomycosis in patients infected with HIV. Serum samples from patients with endemic paracoccidioidomycosis and not infected with HIV were subjected to the same tests and the results obtained were used as reference for the habitual serological response to this fungal disease.

PATIENTS, MATERIALS AND METHODS

Patients and serum samples - The serological tests were applied to serum samples from 40 patients coinfecting with *P. brasiliensis* and HIV and from 75 patients with endemic paracoccidioidomycosis not infected with HIV. The diagnosis of paracoccidioidomycosis was based on the observation of the fungus in a mycological or histopathological exam and/or on the isolation of *P. brasiliensis* in culture. Blood samples were collected before the beginning of antifungal treatment and the sera were stored at 20°C. Blood samples were collected from 1991-2008 for both patient groups.

The patients with opportunistic paracoccidioidomycosis and coinfecting with HIV were 16-57 years old (median = 32 years old) and the man:woman ratio was 4:1 and 25 out of 33 patients (75.8%) had CD4 cell counts < 200 cells/ μ L. The disease was limited to the lungs, oral mucosa and upper airways in seven patients and 33 patients had disseminated

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lesions particularly involving the lungs, skin and lymph nodes, commonly associated with hepatosplenomegaly. HIV infection was determined by laboratory tests such as ELISA HIV 1/2® (Abbott Laboratories, Abbott Park, USA), particle agglutination (Serodia HIV®, Fujirebio, Inc) and western blot for anti-HIV antibodies (Genelabs Diagnostics, Singapore).

The 75 patients with endemic paracoccidioidomycosis and not infected with HIV were 17-74 years old (median = 48 years old), with a man:woman ratio of 11.5:1.0. The acute/subacute form of paracoccidioidomycosis affected 16 out of 75 subjects (21.3%), while 59 out of 75 (78.7%) had the chronic form, according to the accepted classification of the disease (Franco et al. 1987).

Gel DID - The DID test was carried out on glass plates covered with agarose gel (Sigma®) punched with a metal mould to form a central well and six peripheral ones. The central well was filled with a pool of exoantigens from eight *P. brasiliensis* clinical isolates (RP 61, RP 68, RP 73, RP 74, RP 76, RP 79, RP 86 and RP 89). The exoantigens were prepared by dialysis and concentration of the liquid medium of *P. brasiliensis* mycelial cultures. The exoantigen pool had a protein content of 1.84 mg/mL. Patient serum and its dilutions in 0.9% saline were placed in the peripheral wells. The plates were incubated for 72 h and then immersed in 0.9% saline for 24 h. After drying, the gel was stained with Coomassie brilliant blue (Sigma®). The highest dilution that still formed a precipitate band was considered to be the antibody titre of the serum.

CIEP - CIEP was carried out on 8 x 10 cm glass plates covered with type II agarose gel (Sigma®) diluted in sodium barbital buffer (Merck®), pH 8.2. Two parallel rows of wells punched in the gel respectively received the antigen and serial serum dilutions in 0.9% saline. The antigen was obtained from yeasts of six *P. brasiliensis* isolates (Pb 339, Pb 18, BOAS, BAT, RP 51 and RP 54). The cells were ruptured by ultrasound sonication and the antigenic pool had a

protein concentration of 4.07 mg/mL. The plates were subjected to a 30-mAmp electric current for 60 min and then immersed in 0.9% saline for 24 h. After drying, the gel was stained with Coomassie brilliant blue (Sigma®). The serum antibody titre was considered to be the highest dilution that formed immune precipitate bands with the antigen.

ELISA - Polystyrene plates (Immulon®, Dynatech Laboratories, USA) were sensitized with 200 µL/well of a pool of exoantigens from eight *P. brasiliensis* clinical isolates (RP 65, RP 68, RP 69, RP 73, RP 75, RP 76, RP 86 and RP 91) after dilution with carbonate-bicarbonate buffer and overnight incubation at 4°C. The exoantigen pool was prepared as described for DID and had a protein concentration of 1.36 mg/mL. After the initial step and before the addition of other reagents, the plates were washed three times with phosphate buffered saline containing 0.05% Tween 20 (PBS-T). Unbound sites were blocked with 4% bovine albumin (Sigma®) and the plates received ≥ 1:200 serum dilutions in PBS-T. After incubation at 37°C for 60 min, peroxidase-conjugated goat anti-human IgG (Sigma®) was added. The immunological reaction was revealed with o-phenylenediamine dihydrochloride (Sigma®) and absorbance at 492 nm was measured with a Multiskan® plate reader (Lab Systems). The cut-off was determined as the serum absorbance of healthy individuals plus two standard deviations. The antibody titre of paracoccidioidomycosis sera corresponded to the highest dilution at which absorbance remained higher than the cut-off.

Statistical analysis - Data were analyzed statistically by the Chi-square test and Fisher test (rate of reacting sera) and also by the Mann-Whitney test with normal approximation (comparison of serological titres).

RESULTS

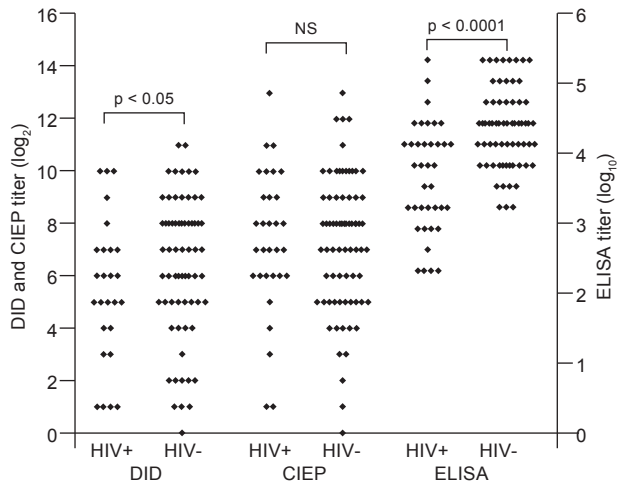
In both groups of patients, ELISA showed the highest positivity, followed by CIEP and DID. The proportion of patients with anti-*P. brasiliensis* antibodies detected

TABLE

Anti-*Paracoccidioides brasiliensis* antibodies determined by double immunodiffusion (DID), counterimmunoelectrophoresis (CIEP) and enzyme linked immunosorbent assay (ELISA) tests in patients with paracoccidioidomycosis and infected with human immunodeficiency virus (HIV+) or not infected (HIV-)

	DID		CIEP		ELISA	
	HIV+	HIV-	HIV+	HIV-	HIV+	HIV-
Patients n	40	75	40	75	40	75
Test positivity ^a %	65	89	77.5	98.7	95	100
Titer range ^b (minimum/maximum)	NR/1024	NR/2048	NR/8192	NR/62536	NR/204800	1600/204800
Titer ^b , mean ± SD	121 ± 273	272 ± 451	507 ± 1329	1439 ± 7550	15910 ± 35255	45376 ± 60138
Median titer ^b	12	64	96	256	4800	25600
p ^c	< 0.001		< 0.05		< 0.0001	

a: number of positive samples/total number of samples x 100; b: inverse of the titer expressed as dilution of the serum sample; c: comparison of the titers of each serological test between HIV-coinfected and non-coinfected patients (Mann-Whitney test); NR: non-reactive; SD: standard deviation.



Anti-*Paracoccidioides brasiliensis* antibody titers in reactive sera of patients with paracoccidioidomycosis and infected with human immunodeficiency virus (HIV+) or not infected (HIV-) in three tests: double immunodiffusion (DID), counterimmunoelectrophoresis (CIEP) and enzyme linked immunosorbent assay (ELISA). Non-reactive sera were excluded. NS: non-significant ($p > 0.05$).

in serum was lower among HIV-infected subjects than among those with endemic paracoccidioidomycosis. The difference was more marked in the DID (65% vs. 89%, $p < 0.01$) and CIEP (77.5% vs. 98.7%, $p < 0.05$) tests than in ELISA (95% vs. 100%, $p > 0.05$) (Table). In all three serological tests, the antibody titres were significantly lower among HIV/AIDS patients (Table). Even when nonreactive sera were excluded, HIV/AIDS patients had lower anti-*P. brasiliensis* antibody titres in the DID and ELISA tests than patients with endemic paracoccidioidomycosis (Figure).

DISCUSSION

The present study shows that patients with HIV/AIDS coinfecting with *P. brasiliensis* have a weaker humoral response against the fungus than patients with endemic paracoccidioidomycosis. It was also demonstrated that the detection of anti-*P. brasiliensis* antibodies can be enhanced with the use of more sensitive tests than DID.

The rate of positivity for serum anti-*P. brasiliensis* antibodies detected in patients with endemic paracoccidioidomycosis was comparable to the sensitivity observed in other studies, i.e., 65-100% for DID, 77-97% for CIEP and 66-100% for ELISA (Mendes-Giannini et al. 1994, Bueno et al. 1997, Do Valle et al. 2001). False-negative results for specific antibodies may occur in patients not infected with HIV, especially those with the chronic form of the disease and an unifocal, small lesion (Del Negro et al. 2000). Patients with this form of paracoccidioidomycosis have a less intense humoral immunological response than patients with acute/subacute form of the disease (Panunto-Castelo et al. 2003).

In patients with paracoccidioidomycosis associated with HIV/AIDS, false-negative serological tests for anti-*P. brasiliensis* antibodies were more common, cor-

responding to 1/3 of the samples in DID. In a series of coinfecting patients in which a small number of patients were assessed with serological tests for anti-*P. brasiliensis* antibodies, false-negative results were obtained in 33% (Paniago et al. 2005) and 66% (Marques et al. 1995) of the serum samples. The use of CIEP or ELISA increased the rate of reactive sera; however, in both tests the low levels of anti-*P. brasiliensis* in some HIV-infected patients were insufficient for a safe serological diagnosis of paracoccidioidomycosis because of cross-reactions and specificity lower than 100% (de Camargo 2008). In this investigation, the joint use of two serological tests increased to 100% the positivity of detection of *P. brasiliensis* antibodies (data not shown). Thus, the serological diagnosis of paracoccidioidomycosis in patients with HIV/AIDS is improved by using ELISA in combination with DID or CIEP. Alternatively, the detection of circulating fungal antigens can lead to a diagnosis of paracoccidioidomycosis in AIDS patients (Gómez et al. 1998).

The partial failure of the humoral immune response against *P. brasiliensis* in HIV-infected patients was also demonstrated by the lower antibody titres compared to patients with endemic paracoccidioidomycosis. This failure has been attributed to B cell dysfunction due to HIV infection (Benard & Duarte 2000), which also impairs the humoral response in opportunistic histoplasmosis (Kauffman 2008), coccidioidomycosis (Singh et al. 1996) and aspergillosis (Martinez et al. 2009). Surprisingly, some patients with severe cell immunodeficiency respond with high antibody titres, as observed in the present study and in previous reports (Tobon et al. 1998). There may be other causes contributing to a lower level of anti-*P. brasiliensis* antibodies, such as the more rapid progression of paracoccidioidomycosis in patients infected with HIV (Morejón et al. 2009). Moreover, in these patients, fungal disease may be the consequence of reactivation of residual foci of the primary infection that arose in the remote past, a condition in which there may be a reduced stimulus for antibody production.

Despite their low sensitivity in detecting anti-*P. brasiliensis* antibodies, serological tests have been useful in endemic areas for the differentiation between paracoccidioidomycosis and other opportunistic infections in patients with HIV/AIDS. In these cases, it is recommendable to use at least two serological tests and to determine the titre of anti-*P. brasiliensis* antibodies to improve the performance of this method of laboratory diagnosis.

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