Paracoccidioides brasiliensis-reactive antibodies in Brazilian blood donors


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In a survey for primary paracoccidioidomycosis (PCM) infection (and not the clinical disease), two groups of blood donors were analyzed. One study group was drawn from donors living in a rural area where PCM is endemic, and the other group from urban residents of a large city, São Paulo. Anti-Paracoccidioides brasiliensis (Pb) specific antibodies (IgG) in sera were analyzed by ELISA, using crude Pb exoantigens (exoAg) and purified specific Pb 43 kDa glycoprotein (gp43). The results showed that 21% of 700 rural samples and 0.9% of 350 urban samples were positive for exoAg and gp43. To avoid cross-reactions, the sera were adsorbed first with Histoplasma capsulatum antigens and secondly with Leishmania amazonensis antigens. In the first adsorption with H. capsulatum, reactivity to gp43 fell to 12.8% in the rural group and to 0% in the urban group. In the succeeding adsorption with L. amazonensis, this reactivity fell to 12.3% in the rural group. There was a statistically greater proportion of persons with gp43-reactive antibodies in rural group than in the urban group, indicating that rural residents had frequently become exposed to Pb and contracted primary, subclinical PCM. The present report is the first epidemiological study using ELISA to detect antibodies against gp43 in blood donors.

Keywords Paracoccidioidomycosis-infection, antibodies, blood donors, Paracoccidioides brasiliensis, gp43, exoantigens, ELISA.

Introduction

Paracoccidioides brasiliensis (Pb), a thermally dimorphic fungus, is the etiologic agent of paracoccidioidomycosis (PCM), the most prevalent systemic mycosis in South America [1].

The disease may develop in multiple forms ranging from benign and localized to severe and disseminated, depending on the extent of depression of cellular immunity [2,3,4]. Airborne fungal propagules consisting of conidia or hyphal fragments begin the infection and undergo conversion to the yeast phase, the infective stage of Pb, in the lungs, progressing secondarily to lymph nodes, skin, and mucosa, following haematogenic or lymphatic dissemination [5]. Most exposed individuals develop an asymptomatic infection (primary PCM infection) with well-organized granulomatous lesions called primary complexes in the lungs and lymph nodes. Epidemiological patterns are established using paracoccidioidin skin tests [6,7] and they reveal that exposure resulting in transient subclinical infection is high in adult populations, ranging from 11.2% to 87% [8]. PCM is more frequent in adult males and in agricultural workers; however, there is no sex difference in primary PCM infection, as established by skin tests [8].
PCM can be diagnosed by direct observation of the characteristic multiple-budding yeast cells in biological materials [9]. Nevertheless, because of its greater sensitivity, detection of serum antibodies against components of Pb has been one of the main tools used to diagnose PCM. Various serological procedures are frequently used, mainly immunodiffusion (ID) [10] and immunoenzyme assays [11–17]. Occasionally, the ID test is negative, even in previously diagnosed PCM patients, at times when the Pb cells can be observed in clinical specimens by microscopy [18]. The major and most readily detected specific antigenic component of Pb is a 43 kDa glycoprotein (gp43) [19], and, depending on the test used, it is recognized by antibodies in up to 100% of PCM patients’ sera [9], most notably in the Western blot [14,15]. Gp43 has four different isoforms [20] that are recognized differently by PCM sera [21].

Recently, Ono et al. [22] evaluated the presence of specific antibodies against gp43 in dogs, employing the ELISA test, and found 89.5%, 48.8%, and 14.8% positivity in rural, suburban, and urban dogs, respectively. This study suggested that canine behaviors of sniffing and digging in the soil may increase the chance that dogs will contact the fungus’s microhabitat and inhale Pb propagules. The transient infection thus contracted may induce specific humoral and cellular responses. Because frank PCM was not found in these dogs, it is possible that dogs may acquire primary PCM infection but not the clinical disease, perhaps because of natural resistance.

The aim of the present study was to evaluate and compare the prevalence of specific IgG anti-Pb in two groups of blood donors, in order to determine the proportion of people sensitized by Pb in these populations. One group studied was drawn from people living in a Pb-endemic area, and was composed of donors from both rural and suburban areas. The other group was composed of residents of a large city, urban São Paulo.

**Materials and methods**

This study was approved by the Internal Scientific Commission and the Bioethics in Research Committee of the Universidade Estadual de Londrina (Londrina, Paraná [PR], Brazil) and the Fundação Pró Sangue/Hemocentro de São Paulo (São Paulo, São Paulo state [SP], Brazil).

**Serum samples**

Serum samples were obtained from 700 blood donors at the Hemocentro Regional de Londrina (HRL), mainly external collection, 82% of them male and 27 ± 5 years of age; and from 350 blood donors at the Fundação Pró-

Sangue Hemocentro de São Paulo (FPS); 80% of them male and 29 ± 5 years of age. Positive controls consisted of 10 serum samples from male PCM patients, 29 ± 5 years of age; negative controls consisted of 10 serum samples drawn from healthy individuals from a previously selected urban area, 80% men and 27 ± 5 years of age. Informed consent was obtained from all individuals participating in this study.

**Pb exoantigens and gp43**

A lyophilized exoantigen (exoAg) was prepared from a yeast-phase culture of Pb B-339, a strain producing isofom A [20] of gp43 according to Camargo et al. [10]. Briefly, Pb was grown in neopeptone medium (Difco, Detroit, MI, USA) for 7 days at 35°C on a gyrating shaker at 50 rev min⁻¹ (Etica Equipamentos, São Paulo, SP). Cultures were killed with merthiolate (2 g l⁻¹) at 4°C overnight, and filtered through filter paper. The crude filtrates were concentrated, dialyzed, and lyophilized. Purification of gp43 from exoantigens was performed by gel filtration chromatography using Sephadex G-75 (Sigma Chemical Co., St. Louis, MO, USA). The gp43 fraction was detected by dot blot, performed according to Taborda & Camargo [16], using rabbit whole serum anti-gp43 as the indicator. The purification was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

**ELISA**

Pb exoAg (1 mg ml⁻¹ dry weight) or gp43 (250 ng per well) was diluted in 0.1 M carbonate buffer, pH 9.6, and 96-well flat polystyrene plates were sensitized and incubated for 1 hr at 37°C and overnight at 4°C. The plates were washed five times with phosphate-buffered saline (PBS)-0.05% Tween 20 (PBS-T), blocked with PBS-T-5% skim milk (PBS-T-M) for 2 h at 37°C, and then incubated for 1 h with diluted sera (1/100, 1/200, 1/400, and 1/800 dilutions). The plates were washed with PBS-T (5x) and incubated with mouse anti-human IgG peroxidase-labeled (Sigma Chemical Co., St. Louis, MO, USA) (100 μl per well, 1/7,000 dilution), washed 5 times, and a substrate solution (5 mg orthophenylenediamine—Sigma Chemical Co., St. Louis, MO, USA, 10 ml of 0.1 M citrate buffer, pH 4.5 and 10 μl H₂O₂) (100 μl well⁻¹) was added. The reaction was halted with 50 μl of 4N H₂SO₄ and absorbance was read in a Titertek Multiscan EIA reader (Labsystems, Helsinki, Finland) at 492 nm. Cutoff was established by the mean ± standard deviation of absorbance obtained previously with sera from 50 healthy individuals (1:200).
Serum adsorption

ELISA exoantigen-positive sera were adsorbed with whole yeast cells from three strains of *Histoplasma capsulatum* var. *capsulatum*. These strains, designated RP, VMF and CFLA, were maintained at the Medical Mycology Laboratory, Instituto de Medicina Tropical de São Paulo, São Paulo, Brazil. The adsorption procedure used was that of Mendes-Giannini et al. [11]. Serum samples diluted at 1/20 (v:v) in a mixture of soluble antigens (50 μg ml⁻¹) and *H. capsulatum* yeast cells (6 × 10⁵ cells ml⁻¹) were incubated overnight at 4°C and for 1 hour at 37°C. Adsorbed sera were obtained by centrifugation (10 min, 400g). An aliquot of the adsorbed serum was reabsorbed with *L. amazonensis* strains MHOM/BR/73/M2269 (6 × 10⁵ cells ml⁻¹) for 3 hours at 37°C and then centrifuged at 400g for 10 min, after which the serum was recovered.

**ID**

ID tests were performed according to Camargo et al. [10].

**Statistical studies**

Comparisons between groups were evaluated by the chi-square test and a probability value of *P* < 0.05 was considered statistically significant.

**Results**

A total of 150 (21%) of 700 serum samples collected at the HRL study centre and 3 (0.9%) of 350 serum samples collected at FPS were reactive to ELISA with Pb exoAg; the positive and negative controls reacted 100% and 0% of the time, respectively (Fig. 1). In the second step, the same sera were also found to be positive in the same percentages with gp43 antigen. To avoid possible cross-reactions, positive sera were adsorbed with *H. capsulatum* antigens, and positivity to gp43 fell to 12.8% in the HRL group and to 0% in the FPS group. After the second adsorption with *L. amazonensis* antigens, positivity to gp43 fell to 12.3% (HRL) (Table 1). ELISA-positive sera were negative by ID. In all steps, the HRL group showed a statistically significantly higher positivity (*P* < 0.05) than the FSP group or negative controls at serum dilutions of 1/400 and 1/800.

**Discussion**

Epidemiological studies of PCM are generally carried out by skin-testing of normal people using paracoccidioides-reactive antibodies in Brazilian blood donors

<table>
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<tr>
<th>Table 1 Percentage of rural and urban blood donor samples initially reactive to purified <em>Paracoccidioides brasiliensis</em> gp43 antigen in ELISA remaining positive after adsorption of the serum with <em>Histoplasma capsulatum</em> (Hc) alone and with Hc plus <em>Leishmania amazonensis</em> (La).</th>
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<td><strong>Patient group</strong></td>
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<td><strong>Serum Dilutions</strong></td>
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Group I: 150 exoantigen-reactive sera from a total of 700 blood samples from the rural HemoCentro Regional de Londrina tested against gp43 and adsorbed with Hc and Hc + La antigens. Group II: 3 reactive sera from a total of 350 blood samples from the Fundação Pró-Sangue/HemoCentro de São Paulo treated as for group I. Group III: 10 serum samples from clinical paracoccidioidomycosis patients adsorbed with Hc and Hc + La. Group IV: 10 serum samples from healthy negative controls adsorbed with Hc and Hc + La. He⁺ = sera adsorbed with *H. capsulatum* antigens. Hc + La = sera adsorbed with *H. capsulatum* plus *L. amazonensis* antigens.

He group I × He group II *P* < 0.05 (1/400, 1/800 dilutions).

Hc + La group I × Hc + La group II *P* < 0.05 (1/400, 1/800 dilutions).
diodin. Using this test it is possible to determine the locations of PCM-endemic regions in Latin America and the level of sensitization to Pb of people living in those regions. Fava Netto et al. [6], Fava Netto & Raphael [23] and Saraiva et al. [7] have used polysaccharide or purified gp43, as well as paracoccidioidin, in delayed-type hypersensitivity (DTH) tests in epidemiological studies or in patients with PCM. The positivity of the intradermal reaction for PCM depends on the presence of the gp43 molecule [24]. Preliminary studies in the city of Londrina in southern Brazil showed that 50% of the population was reactive to gp43 in DTH tests [25]. The DTH test detects the presence of sensitized lymphocytes, indicating that the individual had previous contact with Pb, most likely starting with the inhalation of propagules in the environment and progressing to a transient or, rarely, a more serious or persistent primary infection. Because of the difficulty of doing skin tests on blood donors, the ELISA test was chosen to detect antibodies against Pb exoAg and gp43 in those individuals as an epidemiological parameter.

Cross-reaction between the carbohydrate present in the gp43 molecule with Leishmania and Trypanosoma cruzi was recently reported [26]. Therefore positive sera were adsorbed with antigens of L. amazonensis. The maintenance or slight decline in positivity following adsorption with Leishmania was expected since all sera reactive to T. cruzi had previously been eliminated during triaging of blood donors.

The difference in results between the serum samples from blood donors at the HRL and those from FPS possibly occurred because of the higher incidence of Pb in the rural surroundings of Londrina. Blood donors from the HRL are more commonly involved in agriculture, the predominant activity in the region, while those from the FPS are mainly industrial workers. PCM is known to be most common in rural areas [4]. As rural people are in constant contact with soil and other elements of the natural environment in the course of their work, they may have an increased chance of contact with the fungus' microhabitat and of inhaling Pb propagules. In this study, only two points after the cutoff (1/400, 1/800) were analyzed, but the mean ELISA titer of patients with active PCM has been shown to be still higher [27].

The detection of antibodies only by ELISA and not by ID in the blood donors could have been a consequence of the relatively high sensitivity of the former test. No blood donor showed clinical indications or symptoms, and none was considered to have active PCM. We cannot completely rule out the possibility of cross-reaction with other infectious agents, a problem also affects interpretation of paracoccidioidin skin test results [6]. The present report is the first epidemiological study to use ELISA to detect antibodies against gp43 in blood donors. This method has the advantage of being minimally traumatizing for the individual, and it facilitates epidemiological study by permitting serum samples to be stored frozen for long periods.

Acknowledgements

The authors thank Mari S. Kaminami and Nilson de Jesus Carlos for their excellent technical assistance, Dr. Janet W. Reid for her careful correction of the English text and Dr. Tiemi Matsuo for her help in statistical analysis.

References


