Artigo / Article

Paracoccidioidomycosis disease seven years after positive serology: serological reactivity analysis in the period before and after disease onset

Manifestação da paracoccidioidomicose sete anos após sorologia positiva: análise de reatividade sorológica no período anterior e posterior ao início da doença

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Abstract
Paracoccidioidomycosis (PCM) is one of the most important systemic mycoses in Latin America. Several cases of positive serology in healthy individuals without clinical manifestation have been detected, particularly in rural areas. However, there are no reports of serologic investigation pre- and post-manifestation of PCM. In this study, serological reactivity in the period before and after disease onset was evaluated in a patient who presented clinical manifestations of PCM disease seven years after a positive serology test. In 2003, the patient did not show any oral lesions and was apparently healthy but had a positive ELISA (enzyme-linked immunosorbent assay) result. In 2010, the patient presented disease manifestations: lesions on the labial and jugal mucosa. A biopsy was obtained from the area for histopathological exam and fungal culture analysis, and a fungus with *Paracoccidioides brasiliensis* characteristics was detected. The Serological analysis in the pre- and post-disease onset periods revealed ELISA reactivity (≥1/800) in both samples; immunodiffusion reaction was negative prior to disease onset and positive (1/16) during the PCM disease phase. Immunoblotting results showed recognition of antigens of high molecular mass and gp43 in both phases, with gp70 also observed during the disease phase. This case highlights the importance of periodic medical supervision and the need for further investigation when serology tests are positive for PCM. Additionally, the findings of serum reactivity with *P. brasiliensis* high molecular mass and gp43 antigens are noteworthy.
Key words: diagnosis; gp43; gp70; ELISA; immunoblotting.

Resumo
Paracoccidioidomicose (PCM) é uma das mais importantes micoses na América Latina. Tem sido detectado muitos casos de sorologia positiva em indivíduos saudáveis sem manifestação clínica, particularmente em áreas rurais. Entretanto, não há publicações de investigação sorológica pré e pós manifestação da PCM. Neste estudo, a reatividade sorológica no período anterior e posterior ao início da doença foi avaliada em um paciente que apresentou manifestações clínicas de PCM sete anos após a sorologia positiva. Em 2003, o paciente não apresentava nenhuma lesão oral e era aparentemente saudável, mas apresentava resultado positivo para PCM em ELISA *(enzyme-linked immunosorbet assay)*. Em 2010, o paciente apresentou manifestações da doença como: lesões na mucosa labial e jugal. O exame histopatológico da biopsia da área de lesão e por cultivo foi observada a presença/crescimento de fungo com características de *Paracoccidioides spp*. A análise sorológica no período anterior e posterior ao início da doença revelou reatividade no ELISA (≥1/800) em ambas as amostras; a reação de imunodifusão foi negativa no período anterior ao desenvolvimento da doença e positiva durante a PCM. Os resultados de *immunobloting* apresentaram reconhecimento de antígenos e alta massa molecular e gp43 em ambas as fases, e gp70 observado na fase de doença. Este caso destaca a importância da supervisão médica periódica e a necessidade de uma investigação mais detalhada quando os testes sorológicos são positivos para PCM. Além disso, destacam-se os achados de reatividade sérica com antígenos de alta massa molecular e gp43.
Palavras-chave: diagnóstico; gp43; gp70; ELISA; immunoblotting.

INTRODUCTION

Paracoccidioidomycosis (PCM) is one of the most important systemic mycoses in Latin America and is endemic to Brazil, Argentina, Venezuela, and Colombia. PCM is caused by the thermo-dimorphic fungus *Paracoccidioides brasiliensis*, and it is believed that airborne fungal propagules cause the infection [1,2,3].

Most exposed subjects develop an asymptomatic infection, though some individuals present clinical manifestations that vary from benign and localized to severe and disseminated forms [4,5]. Two forms of the disease, the more severe and rare acute or subacute and the chronic form, are distinguishable. The acute form is characterized by a rapid course and reticuloendothelial system involvement, causing lymph node enlargement and hepatosplenomegaly; the chronic form occurs more frequently, largely in adult males and agricultural workers [4,5].

Epidemiological studies using a paracoccidioidin intradermal test to detect prior contact with *P. brasiliensis* have revealed that exposure resulting in transient subclinical infection is high in the healthy adult population [6,7]. An immunoenzymatic assay (enzyme-linked immunosorbent assay, ELISA) to detect specific IgG antibodies against the main gp43 antigens in humans or domestic animals has also been introduced for PCM eco-epidemiology investigation [8,9]. Thus, several cases of positive intradermal test or serology in healthy individuals without clinical manifestation have been detected, particularly in rural areas [6-9].

According to McEwen, approximately 10 million people are infected with the fungus, 2% of whom may develop the disease [10]. The most frequent form of the disease, the chronic form, presents slowly and silently progresses; it is frequently diagnosed several years after infection [4,5]. The current study is the first investigation of serology before the onset of illness and at the beginning of clinical disease manifestation in a chronic case of PCM.

MATERIAL AND METHODS

Cell-free antigen (CFA)

CFA was obtained from *P. brasiliensis* yeast cells (strain Pb B339) cultured in Sabouraud agar (Micromed, Rio de Janeiro, RJ, Brazil) and maintained by subculturing at 35ºC in 5-day intervals. CFA was obtained according to the method of Camargo et al. (11), with modification by the addition of 2.5 mM phenylmethylsulfonyl fluoride (PMSF) to the supernatant, which was subsequently frozen at -80ºC.

Clinical history and biological samples

The patient was a 55-year-old male, Caucasian rural worker and smoker who was born in Irerê and was a resident of Guaravera, municipality of Londrina, northern Paraná, Brazil. In May 2003, he participated in our extension program titled "Contribution to the promotion of health among rural workers by means of epidemiology, diagnosis and treatment of paracoccidioidomycosis and work development to raise awareness about oral health". During this visit, the patient, who was healthy and did not show any oral lesions, signs or symptoms of the disease, filled out an epidemiological form and was requested to donate blood to perform a preventive serology test for PCM. ELISA and immunodiffusion (ID) were performed using *P. brasiliensis* (Pb B339) CFA, resulting in a positive ELISA. Given the positive serology for PCM, the patient was referred to the regional basic health unit, but no clinical sign of the disease was detected. The patient was released without any treatment or periodic follow-up request for further evaluation of disease progression.

Seven years later, the patient sought treatment in the emergency room of the University Dental Clinic from Londrina State University (UDC/UEL). He reported that two months before, three lower teeth and one upper tooth from the left side had been extracted due to pain he was feeling, with swelling and redness in the upper left gum; though no improvement was achieved. He also reported that during this period, he had a dry cough sometimes with hyaline sputum, which was worse in the morning. After one month, the lesion began to spread to the bottom right side of the mouth (Figure1). A biopsy sample was collected from the oral mucosa for anatomopathological analysis and for fungal culture. As the lungs are the main target organ of *P. brasiliensis* in the chronic form of PCM, a chest radiograph was evaluated, and pulmonary changes characteristic of PCM were detected (Figure 2).

ELISA

ELISA microplates were coated (100 µl/well) with CFA (25 µg/mL) for 1 hour at 37ºC and then overnight at 4ºC. After blocking, diluted serum samples (1:100 to 1:800) were added (100 µl/well) and incubated for 2 hours at 37ºC. The plates were washed four times, and goat anti-human IgG conjugated to peroxidase (Sigma A-8775 Sigma Chemical Co., St. Louis, MO, USA) at 1:4000 dilution (100 µl/well) was added and incubated for 90 minutes at 37ºC, followed by washing and addition of ortho-phenylenediamine (OPD) substrate solution (100 µl/well). The reaction was stopped with 50 µl 4 N H_2SO_4, and absorbance was measured at 492 nm using a Multiskan EX reader (Labsystems, Helsinki, Finland).

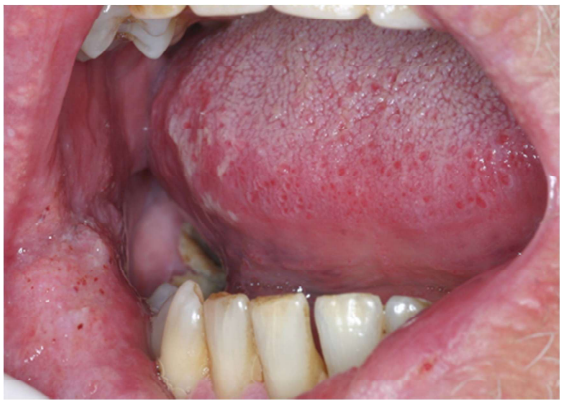

Figure 1. Oral lesion with a moriform surface and small whitish points, presenting seven years after positive serology for PCM.

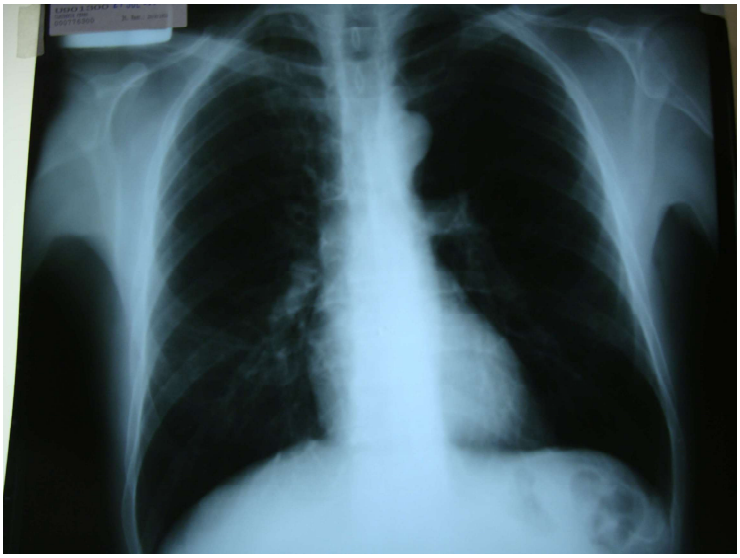

Figure 2. Chest radiograph showing changes characteristic of pulmonary paracoccidioidomycosis.

Immunodiffusion (ID)

Immundiffusion tests were performed according to Camargo et al. [12].

Immunoblotting

Five microliters CFA and molecular weight protein standards were separated by 5-20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Tris-glycine buffer, pH 8.2, at 125 V. The proteins were transferred to a nitrocellulose membrane in Tris-HCl-methanol buffer at 23 V overnight and at 60 V for 1 hour. The membrane was incubated with blocking buffer (skim milk 5%-Tween 0.5% in phosphate-buffered saline (PBS) for 1 hour at room temperature, washed four times with washing buffer (skim milk 0.5%-Tween 0.05%-PBS) and cut into strips. The strips were incubated individually with serum samples diluted 1:40 for 2 hours at 37ºC followed by washing and the addition of goat anti-human peroxidase-conjugated IgG diluted 1:2000 (Sigma A-8775 Sigma Chemical Co., St. Louis, MO, USA) for 2 hours

at 37ºC. After washing, the proteins were detected using diaminobenzidine (DAB), and the reaction was stopped with distilled water.

RESULTS

Culture of the biopsy sample revealed cerebriform fungal growth characteristics of *P. brasiliensis* (Figure 3), with microscopy showing the characteristic multiple-budding yeast form (Figure 4).

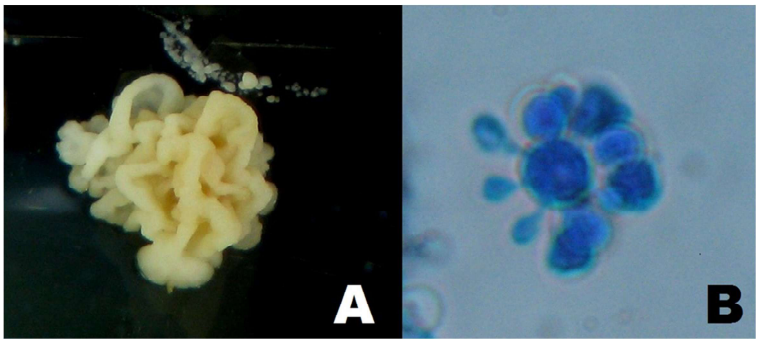

Figure 3. Photo of subculture of the fungus isolated from the biopsy sample of the oral mucosal lesion, showing characteristics of *P. brasiliensis*, **(a)** macroscopically and **(b)** microscopically at 40x magnification.

The 2003 serum sample was positive by ELISA (≥1/800) and negative by ID, confirming the previous serological diagnosis. Serological analysis of the 2010 serum sample was also positive by ELISA (≥1/800), but unlike the previous sample, was positive by ID (1/16) (Figure 4).

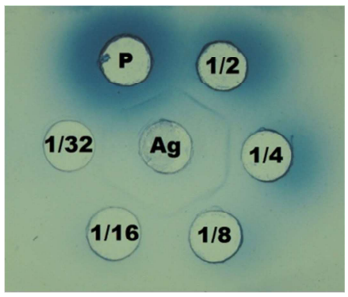

Figure 4. Double immunodiffusion (ID) using *P. brasiliensis* CFA as the antigen (Ag) with serum from the patient after disease onset, pure (P) and at dilutions of 1/2, 1/4, 1/8, 1/16 and 1/32.

Furthermore, immunoblotting showed recognition of high molecular mass (MM) and gp43 bands in both samples (2003 and 2010). In addition, a gp70 band was detected in the 2010 sample (Figure 5).

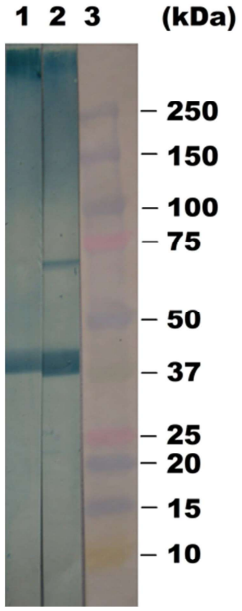

Figure 5. Immunoblotting results: 5-20% SDS-PAGE of CFA from *P. brasiliensis* strain Pb B339 using the patient's serum obtained (1) before disease onset in 2003 and (2) at seven years, after progression to PCM disease, both diluted 1/10. Molecular weight standard (3).

DISCUSSION

The current study reports for the first time a case of positive serology in an apparently healthy individual (PCM infection) who developed PCM disease and whose antibodies were reactive toward *P. brasiliensis* antigens.

The major risk factor for acquiring infection is a profession or activity related to the handling of soil contaminated with the fungus. Case-by-case analysis reveals that the vast majority of patients had performed agricultural activity in the first two decades of life, likely acquiring the infection during that period, even though clinical manifestations appear much later. Contact with the fungus, failure of host defense mechanisms and certain lifestyle habits, such as smoking, alcoholism, nutritional factors and socioeconomic conditions, may contribute to the development of PCM [13]. The patient reported herein falls within this population: male, 55 years old, smoker, farmer and rural resident.

Epidemiological studies using paracoccidioidin intradermal test for detection of prior contact with *P. brasiliensis* [3,7] or the use of immunoenzymatic tests to detect specific IgG antibodies have revealed that exposure resulting in transient subclinical infection is high in healthy adult populations or even in domestic animals, especially in rural areas [13,14]. This primary PCM infection may persist for decades in quiescent PCM foci, as observed in imported cases [15,16]. The onset of clinical symptoms or progression to disease occurs more often in adults between 30 and 60 years due to reactivation of a latent endogenous focus [13].

Negative ID results for sera positive for PCM by ELISA, as observed in this case seven years before clinical manifestation of the disease, is possibly a characteristic of PCM infection, as observed by Botteon et al. in an epidemiological investigation [8]. In

animals, negative ID results in samples positive by ELISA have also been observed, which are considered to be PCM infection [9, 18-20].

In addition, negative ID results have been observed in some PCM disease cases, even in the presence of anti-gp43 antibodies [21]. The lack of reactivity in ID tests of samples from patients with PCM disease may be related to minute titers of low-avidity IgG2 antibodies directed against carbohydrate epitopes [22]. Blotta and Camargo observed 100% gp43-specific reactivity by immunoblotting of sera from PCM patients [23]. In another study, sera from patients with clinical and mycological confirmation of PCM but lacking anti-*P. brasiliensis* antibodies based on ID were evaluated by immunoblotting against two different antigen preparations. The authors observed that 95.4% of the sera specifically recognized gp43 and 100% recognized gp70, with both antigens being described as serological markers of the disease [24]. A high MM component in some serum samples considered to be normal [25] can indicate a fungal infection. In the present case, both serum samples (from 2003 and 2010) showed a high MM fraction by immunoblotting.

Because they are high MM antigens, they may have a more immunogenic potential and are therefore able to induce an earlier immune response with a higher titer, though this needs to be examined in further studies.

This case highlights the importance of periodic medical monitoring and the need for more research when serology is positive for PCM, mainly when the patient falls within a risk group. PCM is responsible for approximately half of all deaths due to systemic mycoses in Brazil, and Paraná is one of the states with the highest mortality rates [26].

Moreover, it is important to further research biological markers that can detect PCM infection in an early stage. In our case, in addition to recognizing gp43, a high MM component was identified in the serum sample taken prior to PCM disease onset.

Acknowledgments

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REFERENCES

1.Restrepo A. The ecology of *Paracoccidioides brasiliensis*: a puzzle still unsolved. Sabouraudia. 1985;23(5):323-34.

2.McEwen JG, Bedoya V, Patiño MM, Salazar ME, Restrepo A. Experimental murine paracoccidiodomycosis induced by the inhalation of conidia. J Med Vet Mycol. 1987;25(3):165-75.

3.Brummer E, Castaneda E, Restrepo A. Paracoccidioidomycosis: an update. Clin Microbiol Rev. 1993;6(2):89-117.

4.Franco M, Montenegro MR, Mendes RP, Marques SA, Dillon NL, Mota NG. Paracoccidioidomycosis: a recently proposed classification of its clinical forms. Rev Soc Bras Med Trop. 1987;20(2):129-32.

5.Franco M, Peracoli MT, Soares A, Montenegro R, Mendes RP, Meira DA. Host-parasite relationship in paracoccidioidomycosis. Curr Top Med Mycol. 1993;5:115-49.
6.Costa EO, Diniz LS, Netto CF, Arruda C, Dagli ML. Delayed hypersensitivity test with paracoccidioidin in captive Latin American wild mammals. J Med Vet Mycol. 1995;33(1):39-42.

7.Wanke B, Londero AT. Epidemiology and paracoccidioidomycosis infection 1994:109-117.

8.Botteon FAG, Camargo ZP, Bernard G, Coelho RF, Chamone DAF, Itano EN. *Paracoccidioides brasiliensis*-reactive antibodies in Brazilian blood donors. Med Mycol. 2002;40(4):387-91.

9.Ono MA, Bracarence APFRL, Morais HS, Trappo SM, Belitardo DR, Camargo ZP. Canine paracoccidioidomycosis: a seroepidemiologic study. Med Mycol. 2001;39(3):277-82.

10.McEwen JG, Garcia AM, Ortiz BL, Botero S, Restrepo A. In search of the natural habitat of *Paracoccidioides brasiliensis*. Arch Med Res. 1995;26(3):305-6.
11. Camargo, ZP; Taborda, CP; Rodrigues, EG; Travassos, LR. The use of cell-free antigens of *Paracoccidioides brasiliensis* in serological tests. J Med Vet Mycol. 1991; 29(1): 31-8.

12.Camargo ZP, Unterkircher C, Campoy SP. et al. Production of Paracoccidioides brasiliensis exoantigens for immunodiffusion test. *J Clin Microbiol* 1988; 26:2147-2I5I.

13.Shikanai-Yasuda MA, Telles Filho FeQ, Mendes RP, Colombo AL, Moretti ML. [Guidelines in paracoccidioidomycosis]. Rev Soc Bras Med Trop. 2006;39(3):297-310.

14. Silveira LH, Paes RCS, Medeiros EV, Itano EN, Camargo ZP, Ono MA. Occurrence of antibodies to *Paracoccidioides brasiliensis* in dairy cattle from Mato Grosso do Sul, Brazil. Mycopathologia. 2008;165(6):367-71.

15.Oliveira GG, Silveira LH, Itano EN, Soares RM, Freire RL, Watanabe MAE, Camargo ZP, Ono MA. Serological evidence of *Paracoccidioides brasiliensis* infection in chickens from Paraná and Mato Grosso do Sul States, Brazil. Mycopathologia. 2011;171(3):197-202.

16.Botas-Velasco M, Jover-Díaz F, Ortiz de la Tabla-Duccase V, Martínez-García C. Imported paracoccidioidomycosis in Spain. Enferm Infecc Microbiol Clin. 2010;28(4):259-60.

17.Onda H, Komine M, Murata S, Ohtsuki M. Letter: Imported paracoccidioidomycosis in Japan. Dermatol Online J. 2011;17(12):11.

18.Ono MA, Kishima MO, Itano EN, Bracarense AP, Camargo ZP. Experimental paracoccidioidomycosis in dogs. Med Mycol. 2003;41(3):265-8.

19.Eisele RC, Juliani LC, Belitardo DR, Itano EN, Estevão D, Bracarence APFRL, Camargo ZP, Ono MA. Immune response in dogs experimentally infected with *Paracoccidioides brasiliensis*. Med Mycol. 2004;42(6):549-53.

20.Corte AC, Svoboda WK, Navarro IT, Freire RL, Malanski LR, Shiozawa MM, Aguiar LM, Passos FC, Maron A, Camargo ZP, Itano EN, Ono MA. Paracoccidioidomycosis in wild monkeys from Paraná State, Brazil. Mycopathologia. 2007;164(5):225-8.

21.Del Negro GMB, Benard G, Assis CM, Vidal MSM, Garcia NM, Otani C, Shikanai-Yasuda MA, da S Lacaz C. Lack of reactivity of paracoccidioidomycosis sera in the double immunodiffusion test with the gp43 antigen: report of two cases. J Med Vet Mycol 1995;33(2):113-6.

22.Neves AR, Mamoni RL, Rossi CL, de Camargo ZP, Blotta MH. Negative immunodiffusion test results obtained with sera of paracoccidioidomycosis patients may be related to low-avidity immunoglobulin G2 antibodies directed against carbohydrate epitopes. Clin Diagn Lab Immunol. 2003;10(5):802-7.

23.Blotta MH, Camargo ZP. Immunological response to cell-free antigens of Paracoccidioides brasiliensis: relationship with clinical forms of paracoccidioidomycosis. J Clin Microbiol. 1993;31(3):671-6.

24.Silva DF, Assis CM, Zamboni IM, Barreto LC, Kohara VS, Vicentini-Moreira AP. Use of immunoblotting assay improves the sensitivity of paracoccidioidomycosis diagnosis. Journal of Venomous Animals and Toxins including Tropical Diseases. 2008;14(2):313-21.

25.Puccia R, Schenkman S, Gorin PA, Travassos LR. Exocellular components of *Paracoccidioides brasiliensis*: identification of a specific antigen. Infect Immun. 1986;53(1):199-206.

26.Coutinho ZF, Silva D, Lazera M, Petri V, Oliveira RM, Sabroza PC, Wanke B. Paracoccidioidomycosis mortality in Brazil (1980-1995). Cad Saude Publica. 2002;18(5):1441-54.