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Detection of antibodies anti-gp43 of *Paracoccidioides brasiliensis* in sera samples by double-sandwich ELISA

Luís Felipe Minozzo Figueiredo[1]; Barbara Ribeiro Fonseca-de-Souza[1]; Alessandra Becker-Finco[2]; Elizângela Almeida Rocha[1]; Luísa Lasmar Lima Oliveira[1]; Ricardo Machado-de-Ávila[4]; João Carlos Minozzo[5]; Fernanda Costal-Oliveira[1]; Larissa Magalhães Alvarenga[2]; Vanete Soccol[3]; Carlos Chávez-Olórtegui[1]*

[1]Departamento de Bioquímica e Imunologia, ICB, Universidade Federal de Minas Gerais (UFMG), Brazil

[2]Departamento de Patologia Básica, Universidade Federal do Paraná (UFPR), Brazil

[3]Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia da Universidade Federal do Paraná-Curitiba, PR, Brazil

[4]Programade Pós-Gradução em Ciência da Saúde da Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil

[5]Centro de Produção e Pesquisa de Imunobiológicos (CPPI), Piraquara, PR, Brazil.

***Corresponding Author(s): Carlos Chávez-Olórtegui**

Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais (UFMG), Antônio Carlos Avenue, Belo Horizonte, Brazil

Tel: 55 31 3409 2625; Fax: 55 31 3409 2613

Email: olortegi@icb.ufmg.br

Abstract

Monoclonal antibodies (mAbs) were raised against 43 kDa glycoprotein (gp43) of *Paracoccidioides brasiliensis*. One mAb anti-gp43 and gp43 were used in a double-antibody sandwich ELISA to detect antibodies in sera from patients with paracoccidioidomycosis. This ELISA diagnosis showed 91.18% sensibility and 100.0% specificity against sera of patients with histoplasmosis, aspergillosis or chromomycosis.

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Introduction

Paracoccidioidomycosis (PCM) is the most important systemic mycosis in Latin America, with the majority of cases in Brazil [1]. The major etiological agent is the fungus P*aracoccidioides brasiliensis* [2]. There are two forms of the disease: acute/subacute form, that represent 3 to 5% of the cases and chronic form corresponding to more than 90% of the cases. The 43 kDa glycoprotein (gp43) is the main exocellular protein of *P. brasiliensis* and is considered the major diagnostic antigen of PCM [3]. Previous findings suggest that studies of gp43 might have great potential for the development of vaccines and diagnostics for PCM patients [4,5,6].

Gp43 has homology to fungal exoglucanases but has no enzymatic activity due to a mutation (NEP -> NKP) at the catalytic site from these fungal enzymes [7], and acts as adhesin, intensifying the infection of the etiologic agent, due to its ability to bind to extracellular matrix proteins of the cells as laminin and fibronectin [8,9]. It can induce the formation of granulomas *in vitro* in a B-1 cell-dependent mechanism [10]. The process of binding and phagocytosis of the fungus by macrophages may be related to the recognition of the sugar moiety of gp43 by surface receptors on these cells [11], but gp43 can cause a down regulation in phagocytosis as well as may reduce the release of reactive oxygen and nitrogen intermediates [12].

In this work, seventy-three sera samples were tested. Thirty-four were from patients with confirmed PCM, and of these, fourteen with different titles in agar double immunodiffusion were obtained at Hospital de Clínicas do Paraná, Brazil (HCPR); and the twenty others at the Hospital das Clínicas de Minas Gerais, Brazil (HCMG), from individuals with primary infection (n=9) and from patients with recurrence of the disease or in treatment (n=11). Six samples from patients with chromomycosis, six with aspergillosis and four from patients with histoplasmosis were also obtained at the HCPR. Other fifteen samples from patients with histoplasmosis were from individuals from Porto Alegre, Brazil; totaling nineteen samples from this disease. Eight sera from individuals with no history of diseases were obtained at HCMG, and they were used as negative control. Procedures were approved by the Human Research Ethics Committee of Universidade Federal de Minas Gerais (UFMG), Brazil (CAAE 41618814.0.1001.5149); and by Animal Research Ethics Committee of the UFMG (299/2014).

The exoantigen, produced according Camargo et al. (1988) using *P. brasiliensis* strain B-339, was used as immunogen to produce mAbs anti-gp43. Procedures for BALB/c mice immunizations, ELISA screenings and splenocyte-Sp2/0 myeloma cell fusions were based in a previously described protocol [13]. MAbs specifity was tested by indirect ELISA and Western Blot using *Candida albicans, Aspergillus fumigatus, Aspergillus niger, Cryptococcus neoformans, Histoplasma capsulatum, Trichophyton rubrum, Leishmania braziliensis and Mycobacterium tuberculosis* antigens as previously described [14]. MAb 4E9F6 against gp43 (anti-gp43 mAb) was purified by immunoaffinity chromatography using HiTrap Protein G-Sepharose HP column coupled to a Äkta Pure system.

For the purification of gp43 using Sepharose-Protein G™ 4 Fast Flow column, anti-gp43 mAb was covalently bound to Sepharose-Protein G column in the ratio of 6 mg of IgG for 1 mL of resin. To purify gp43, protease inhibitor mix was added to the exoantigen of *P. brasiliensis* following the manufacturer instructions and then incubated with the resin for 1.5 hour at room temperature under stirring. After washing with PBS pH 7.4, purified gp43 was eluted with 0.1 M glycine pH 2.7, and fractions were neutralized using 1 M Tris-HCl pH 9.0. Fractions purity was analyzed by a 12% SDS-PAGE (data non-shown).

Double antibody sandwich ELISA format was tested for diagnosis of PCM in patient's sera using anti-gp43 mAb and purified gp43. Costar® plates were coated overnight at 4˚C with 50 µL of a 250 ng/mL solution of anti-gp43 mAb in coating buffer (0.05 M Na_2CO_3, pH 9.6). Wells were blocked using Pierce® Protein-Free T20 Blocking Buffer for one hour at 37˚C. After washing, a solution containing purified gp43 (300 ng/mL) was incubated for 1 hour at 37˚C, followed by incubation with patient's sera (1:4,000). Next, anti-human IgG Fc specific-peroxidase A0170 (Sigma-Aldrich) was added (1:10,000). Absorbance was measured at 492 nm using iMark™ Microplate Absorbance Reader. All measurements were performed in triplicate. Sera from patients without PCM were used as negative control.

Sandwich ELISA results analysis was performed using simple Student t-test for normal distribution and the Wilcoxon test for non-parametric distribution. ROC curve was constructed using the Prism 5 (GraphPad) software, and the cut-off value was chosen for giving the best values of both sensitivity and specificity.

Twelve stable hybridomas secreting anti-gp43 antibodies were selected by screening using gp43 from *P. brasiliensis* coated to ELISA plates. All mAbs cell culture supernatants were reactive with gp43 antigen. ELISA experiments with *P. brasiliensis exoantigen, C. albicans, A. fumigatus, A. niger, H. capsulatum, Trichophyton mentagrophytes, T. rubrum* and *M. tuberculosis* were also performed in order to test cross-reactivity. The 2A7C5, 3A11H7 and 4E9F6 mAbs had high reactivity with gp43 antigen and showed low cross-reactions with other antigens, when compared with the other mAbs (Figure 1A). For molecular characterization, it was examined the cross-reactivity of the three selected mAbs using Western Blot against the same antigens tested for ELISA experiments, except that *T. mentagrophytes* antigen was replaced by *L. braziliensis* (Figure 1B). MAbs 2A7C5, 3A11H7 and 4E9F6 were specifically reactive with gp43. Affinity constant of the mAbs and gp43 was determined by microscale thermophoresis method using fluorescent marker mAbs (data not shown). These experiments suggested that 4E9F6 mAb has higher affinity for gp43 antigen and therefore it was chosen to be used in immunodiagnostic assays. This mAb was also selected to construct an affinity column which was able to specifically purify gp43 from *P. brasiliensis* (detected by silver stained SDS-PAGE - data not shown).

For the double antibody sandwich ELISA, the plate was coated with anti-gp43 mAb. Next, gp43 was incubated to be captured, and then patient sera were added to allow the immunoglobulins to interact with gp43. Results showed no false positives or cross-reactions with patients diagnosed with aspergillosis and chromomycosis and the reaction with serum from patients with histoplasmosis was abolished, however one less serum from a PCM patient, with relapse or in treatment, reacted (9/11) (Figure 2 and Table 1). The difference between the response of the PCM groups from both Paraná and Minas Gerais to the other groups was statistically significant.

Twelve mAbs anti-gp43 were obtained, however, only three IgG1-kappa mAbs were able to specifically recognize gp43 antigen by ELISA or Western Blot, without any cross-reaction with the heterologous antigens tested in this paper.

ELISA assays could evaluate a large number of samples in a shorter time [15], but for PCM it has lower specificity when compared to DID [16]. The indirect ELISA can be used as a screening test, though. However, it requires subsequent immunological assays when it is positive [17]. The sensitivity and specificity of an ELISA depends on the quality of the antibodies used [15]. The specificity of ELISA assays for fungal diseases could be improved if a sandwich model was used instead of an indirect one [18,19]. The mAb 4E9F6 showed a high specificity for gp43 molecule, therefore it was choose because it could capture the gp43 exposing it to the antibodies from patient sera reducing other interferences. Although patient's antibodies are variable the double sandwich assay was actually more specific. Perhaps due to the greater dilution of the patients sera used, the concentration of antibodies recognizing less specific epitopes of gp43 is reduced, which may be the reason for increasing the specificity of the assay. Another possibility is that the previous interaction with an antibody may cause a steric effect that affects the interaction with these epitopes. Therefore, due to the improvement in specificity, maintaining a good sensitivity, the double sandwich ELISA could be an alternative to the diagnosis of paracoccidioidomycosis.

Conclusion

In this study, gp43 was purified by immunoaffinity chromatography and tested by ELISA for the detection of specific antibodies. Using a double antibody sandwich ELISA, the plate was coated with a monoclonal antibody to capture gp43 in order to detect specific anti-gp43 antibodies. This format exhibited high performance and was able to detect antibodies in 91.18% of PCM serum samples. No cross-reaction was observed with sera from patients with histoplasmosis, aspergillosis and chromomycosis. The sensitivity (91.18%) and specificity (100.0%) of this assay indicated that it is a successful immunodiagnostic method.

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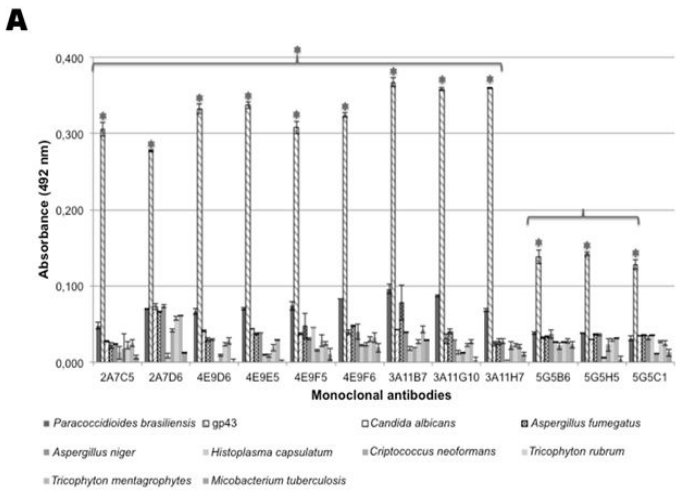
Figures



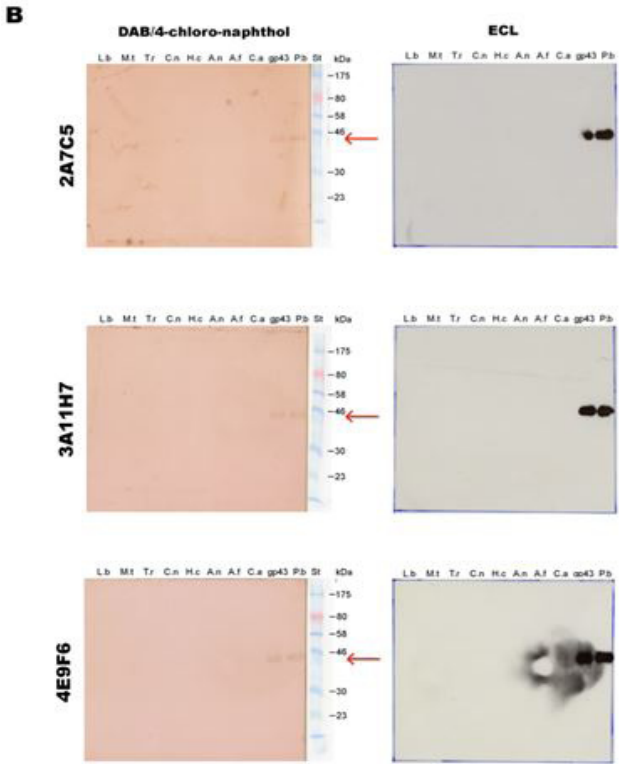


Figure 1: ELISA and immunoblot immunoreactivity of mAbs against gp43-antigen, exoantigen of *P. brasiliensis* and another pathogen antigens. (A) ELISA microtiter plates coated with gp43 or *P. brasiliensis, C. albicans, A. fumigatus, A. niger, H. capsulatum, C. neoformans, T. rubrum, T. mentagrophytes* and *M. tuberculosis* and tested against supernatants from 2A7C5, 2A7D6, 4E9D6, 4E9F5, 4E9F6, 3A11H7, 5G5B6, 5G5H5 and 5G5C1 mAbs. (B) For samples Western Blotting analysis, *P. brasiliensis* (P.b), *C. albicans* (C.a), *A. fumegatus* (A.f), *A. niger* (A.n), *H. capsulatum* (H.c), *C. neoformans* (C.n), *T. rubrum* (T.r), M. *tuberculosis* (M.t) and *L. braziliensis* (L.b) or gp43 antigens were analyzed in 12% poliacrylamide gel, transferred to nitrocellulose membranes, probed against 2A7C5, 3A11H7 and 4E9F6 supernatants and incubated with anti-mouse IgG conjugated to peroxidase.

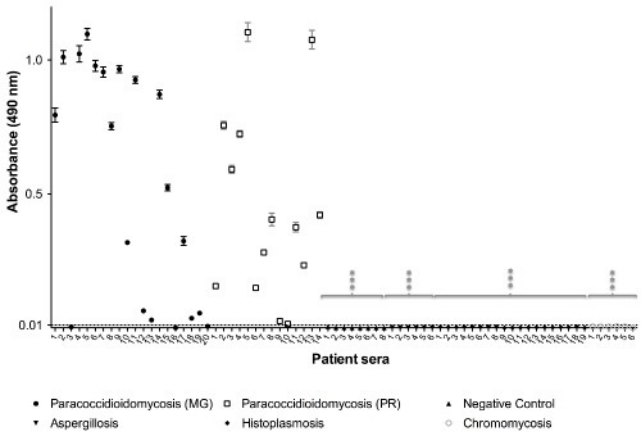


Figure 2: Immunoassays using patient's sera for paracoccidioidomycosis immunodiagnosis. Microplates were coated with mAb antigp43, subsequently gp43 was added, followed by incubation with sera from patients. Finally, anti-human IgG Fc specific-peroxidase A0170 (Sigma-Aldrich) was added (1:10,000). Patient sera were divided into the following groups: paracoccidioidomycosis (MG), Paracoccidioidomycosis (PR), histoplasmosis, aspergillosis, chromomycosis and negative control. Statistical signs and meanings: * - p-value < 0.05; *** - p-value < 0.01; *** - p-value <0.001.

Tables

Table 1: Double antibody sandwich ELISA format for immunodiagnosis of paracoccidioidomycosis using mAb anti-gp43.

Groups	Double sandwich ELISA cut-off 0.01			
	No. of serum samples tested	No. of reactive samples	No. of nonreactive samples	% reactive
PCM*	34	31	3	91.18
MG (first infection)	9	8	1	88.89
MG (relapsed / treatment)	11	9	2	81.82
PR	14	14	0	100
Histoplasmosis	19	0	19	0
Aspergillosis	6	0	6	0
Chromomycosis	6	0	6	0
Normal controls from a region of endemicity	8	0	8	0

*Total number of samples.

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