Article/Artigo

High molecular mass fraction in clinical isolates of *Paracoccidioides brasiliensis*

Fração de alta massa molecular em isolados clínicos de *Paracoccidioides brasiliensis*

Andréa Longoni Fredrich[1], Luciene Airy Nagashima[1], Wander Rogério Pavanelli[1], Audrey de Souza Marquez[2], Mari Sumigawa Kaminami[1], Nilson de Jesus Carlos[1], Ayako Sano[3], Mario Augusto Ono[1] and Eiko Nakagawa Itano[1]

ABSTRACT

Introduction: Different serum levels of the IgG/IgE for *Paracoccidioides brasiliensis* high mass molecular (hMM) fraction (~366kDa) in the acute and chronic forms of the disease have been reported. Considering the nonexistence of hMM fraction investigation involving clinical isolates of *P. brasiliensis*, the present study aimed to investigate the presence of the hMM fraction (~366kDa) in cell free antigens (CFA) from *P. brasiliensis* clinical isolates. **Methods:** CFA from 10 clinical isolates and a reference strain (Pb18) were submitted to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by gel image capturing and densitometer analysis. Additionally, CFA from 20 isolates and Pb18 were analyzed by capture ELISA (cELISA) using polyclonal (polAb) or monoclonal (mAb) antibodies to the hMM fraction. **Results:** The presence of the hMM component was observed in CFA of all samples analyzed by SDS-PAGE/densitometry and by cELISA. In addition, Pearson's correlation test demonstrated stronger coefficients between hMM fraction levels using pAb and mAb (R = 0.853) in cELISA. **Conclusions:** The soluble hMM fraction was present in all the *P. brasiliensis* clinical isolates analyzed and the reference strain Pb18, which could be used as a source of this antigen. The work also introduces for first time, the cELISA method for *P. brasiliensis* hMM fraction detection. Analysis also suggests that detection is viable using polAb or mAb and this methodology may be useful for future investigation of the soluble hMM fraction (~366kDa) in sera from PCM patients.

Key-words: Paracoccidioidomycosis. Soluble antigen. Capture ELISA. Monoclonal antibodies. IgG.

RESUMO

Introdução: Diferentes níveis sorológicos de IgG/IgE contra a fração de alta massa molecular (hMM) (~366kDa) de *Paracoccidioides brasiliensis* têm sido encontrados na PCM aguda e crônica. Considerando a inexistência de investigação sobre esta fração em isolados clínicos de *P. brasiliensis*, o objetivo deste estudo foi investigar a presença da fração hMM (~366kDa) no preparado livre de células (CFA) de *P. brasiliensis* obtidos de isolados clínicos. **Métodos:** CFA de 10 isolados e de cepa de referência (Pb18) foram submetidas à eletroforese em gel de SDS-poliacrilamida (SDS-PAGE) seguida de captura de imagem e análise por densitometria. Adicionalmente, CFA de 20 isolados e de Pb18 foram analisados por ELISA captura (cELISA) utilizando anticorpos policlonal (polAb) ou monoclonal (mAb) para fração hMM. **Resultados:** A presença do componente de hMM foi observada em todas as amostras analisadas por SDS-PAGE/densitometria e por cELISA. Adicionalmente, o teste de correlação de *Pearson* demonstrou forte relação entre os níveis de fração hMM usando pAb e mAb (R = 0.853) no cELISA. **Conclusões:** Conclui-se que a fração hMM está presente em todos os isolados clínicos de *P. brasiliensis* analisados e no isolado referencial, sugerindo a possibilidade dos mesmos serem utilizados como fonte desta fração antigênica. Este trabalho também introduz pela primeira vez o método de cELISA para detecção da fração hMM de *P. brasiliensis*, sugerindo que detecção utilizando anticorpos polAb ou mAb é viável e essa metodologia poderá ser útil para investigação futura desta fração solúvel (~366kDa) em soros de pacientes com PCM.

Palavras-chaves: Paracoccidioidomicose. Antígeno solúvel. ELISA de captura. Anticorpos monoclonais. IgG.

1. Department of Pathology Science, Biological Science Center, State University of Londrina, Londrina, PR, Brazil. 2. Health Science Research Center, University North of Paraná, Londrina, PR, Brazil. 3. Medical Mycology Research Center, Chiba University, Chiba, Japan.

Address to: Dra. Eiko Nakagawa Itano. Deptª Ciências Patológicas/CCB/UEL. Campus Universitário, 86051-970 Londrina, PR, Brasil.

Phone: 55 43 3371-4469; Fax: 55 43 3371-4207

e-mail: itano@uel.br

Received in 21/01/2010

Accepted in 27/07/2010

INTRODUCTION

Paracoccidioidomycosis (PCM), a deep mycosis endemic in Latin America, is caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis*, which develops as yeast at body temperature and as mycelium at room temperature. *P. brasiliensis* causes natural infections by inhalation of conidia or mycelial elements[1]. Most exposed subjects develop an asymptomatic infection, although some individuals present clinical manifestations that can vary from benign and localized to severe and disseminated forms[2]. Two forms of the disease are distinguished: the acute or subacute and chronic form. The acute form is more severe and rare, while the chronic form occurs more frequently and mostly affects adult males[3,4].

Usually, PCM diagnosis is inferred from indirect evidence obtained via serological tests and clinically relevant antigens have been identified and adapted for use in immunoassays for the detection of specific antibodies[5]. For this purpose, several fungal components have already been identified. The antigens most frequently identified in PCM patient sera are the glycoproteins of 43kDa (97-100%)[6-8], the main PCM diagnostic antigen[6,7,9,10], 160kDa (78%) and 70kDa (60%)[8].

The other alternative laboratory approach for diagnosis of PCM is the detection of circulating *P. brasiliensis* antigens. The inhibition ELISA methodology (inh-ELISA) is able to detect gp43 in 96.3% of PCM patients, mainly in those with the acute form of the disease (100%)[11]. Gp70 has also been detected in the urine or in cerebrospinal fluid (CSF) of PCM patients[12,13]. Gómez et al[14,15] reported the use of inh-ELISA and detected gp87 circulating antigen in sera from patients with active disease.

Puccia et al[9] demonstrated polydispersed high-molecular mass glycoprotein, with heterogeneous electrophoresis migration. From this heterogeneous electrophoresis migration Marquez et al[16] isolated fractions ranging from 278kDa to 466kDa (mean approximately 366kDa) obtained from sonicated

or cell free antigens (CFA) preparations. Moreover, different serum levels of the IgG/IgE to the hMM fraction was verified in sera of acute and chronic PCM patients and the authors suggested the analysis as a new characteristic to differentiate between these two clinical forms of the disease[16].

Taking into account the lack of data regarding the soluble hMM fraction (~366kDa) in *P. brasiliensis* clinical isolates and the immunological methodology for identifying it, in the present study, the hMMAg antigen was investigated in different clinical isolates and capture ELISA (cELISA) was introduced. In principle, observation verified that all the *P. brasiliensis* samples analyzed produced the hMM fraction (~366kDa). In addition, this hMM fraction was detected by cELISA using monoclonal or polyclonal antibodies to the hMM fraction.

METHODS

Fungal isolates

Clinical isolates of *P. brasiliensis* of the chronic form of PCM disease were obtained: 17 isolates (LDR1 to LDR17) from Londrina State University (MOOI/CCS, HC, HC, Londrina State University, Londrina, Paraná) patients (2000 to 2006); two isolates (RC-Wang and RC-Hori) from Chiba University, Chiba, Japan; one isolate (EPM-01) and reference strain (Pb18) from UNIFESP, São Paulo, Brazil. *P. brasiliensis* strains were maintained on potato dextrose agar (Difco Laboratories, MI, USA) slants at room temperature. Prior to experiments, samples of the isolates were inoculated onto a slant of brain heart infusion agar (BHI, Difco Laboratories) supplemented with 1% dextrose and cultured at 35ºC to produce the yeast form and maintained by subculturing at 35ºC at 5-day intervals on Sabouraud agar (Micromed, Rio de Janeiro, RJ, Brazil).

Cell free antigens preparation

Yeast cells were collected and the CFA samples were obtained according to Camargo et al[17], modified by the addition of PMSF protease inhibitor at 2.5mM to the supernatant. The protein concentration was determined by the Lowry method[18], adjusted to 3mg/mL and stored in -80ºC freezer until ready to use.

Cell free antigens analysis by SDS-PAGE

Cell free antigens (3mg/mL) samples, obtained as described above, were mixed with the reducing sample buffer (62.5mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 10% β-mercaptoethanol and 0.05% bromophenol blue) and boiled for 3 min. The antigens were separated by SDS-polyacrylamide gel electrophoresis (7.5%), in tris-glycine buffer, pH 8.2, at 125v. Protein standards with the following molecular masses were used: myosin (201.1kDa); β-galactosidase (115.7kDa); bovine serum albumin (93.6kDa); ovalbumin (50.3kDa); carbonic anhydrase (37.3kDa). Using specific densitometer software, Glob-Al Scan (Cellogel Electrophoresis Co, Milan, Italy), silver stained dehydrated SDS-electrophoresis gel image was captured, the specific cutoff point of that defines the area of the hMM fraction band (~366kDa) was indicated and the result automatically calculated and expressed in percentages.

Capture ELISA for IgG-hMM fraction

ELISA immunoplates sensitized with rabbit IgG anti-hMM fraction (25µg/ml) were incubated with CFA samples at 30µg/ml at 37ºC for 1h and then with polyclonal mouse IgG anti-hMM (IgG pAb) (30µg/ml), anti-mouse IgG peroxidase conjugate and OPD (100µL well). The absorbance was read at 492nm. Additionally, cELISA for hMM fraction level determination was performed as described, substituting polyclonal mouse IgG anti-hMM fraction for monoclonal IgG anti-hMM fraction (IgG mAb) as secondary antibodies. Polyclonal rabbit and mouse antibodies were produced by using the hMM fractions obtained, according to Pavanelli et al[19] for immunizations. A monoclonal antibody was obtained by spleen cells (from immunized BALB/c mice with the hMM fraction) fused with P3U1 cell line by using PEG. Hybridomas were screened by ELISA with hMM fraction (~366kDa) and cloned by limiting dilutions. Pristane-primed BALB/c mice were injected i.p. with hybridoma and IgG purified (Sepharose-G protein column) from ascite fluid. The cutoff was determined as the mean plus 2 standard deviations of the absorbance obtained with control: primary rabbit IgG anti-hMM, secondary mouse pAb or mAb IgG and peroxidase conjugate without the CFA sample.

Statistical analysis

Statistical comparisons were performed by analysis of variance (ANOVA) and by the Tukey test. All values are reported as the mean ± SD of the mean, with significance assumed in the range of $p<0.05$. Pearson's correlation was applied between hMM fraction levels using IgG pAb and mAb anti-hMM fraction and a significant correlation was considered when $r \geq 0.50$.

Ethical

This study was approved by the Internal Scientific Commission and the Bioethics in Research Committee of the State University of Londrina (Londrina, PR, Brazil).

RESULTS

CFA analysis by SDS-PAGE

The presence of the hMM fraction was observed in CFA from all the clinical isolates and reference Pb18 by SDS-PAGE. The SDS-PAGE was submitted to densitometry in an attempt to quantify the hMM fraction. The results show that 20.5 ± 5.7% (clinical isolates) and 16.7% (Pb18) from CFA correspond to the hMM fraction (**Figure 1**). This study indicates that this antigen is present in different clinical isolates from chronic patients and could be used as source of the hMM fraction.

Capture ELISA for hMM fraction levels in CFA samples

Various researchers have attempted to detect circulating antigens in PCM patients using polyvalent antigens or antibodies in different assays. For this purpose, the presence of the hMM fraction in CFA samples was determined using cELISA expressed as optical density units (OD). Analysis of the result verified the presence of the hMM fraction in Pb18, a strain characterised as highly virulent, and in all the clinical isolates of *P. brasiliensis*, with the use of polAb or mAb to the hMM fraction (**Table 1**). All values greater than the values obtained in the control systems of polyclonal/polyclonal or monoclonal/monoclonal with free samples of CFA plus 2 x standard (0.118 ± 0.008 and 0.086 ± 0.005, respectively) were considered as positive.

Correlation analysis

Analysis of the results confirmed a strong correlation between hMM fraction levels with IgG pAb or mAb anti-hMM as the secondary antibody ($r = 0.853$) (**Figure 2**). This result demonstrates that the hMM fraction can be detected by cELISA using monoclonal or polyclonal antibodies to hMM, as a secondary antibody.

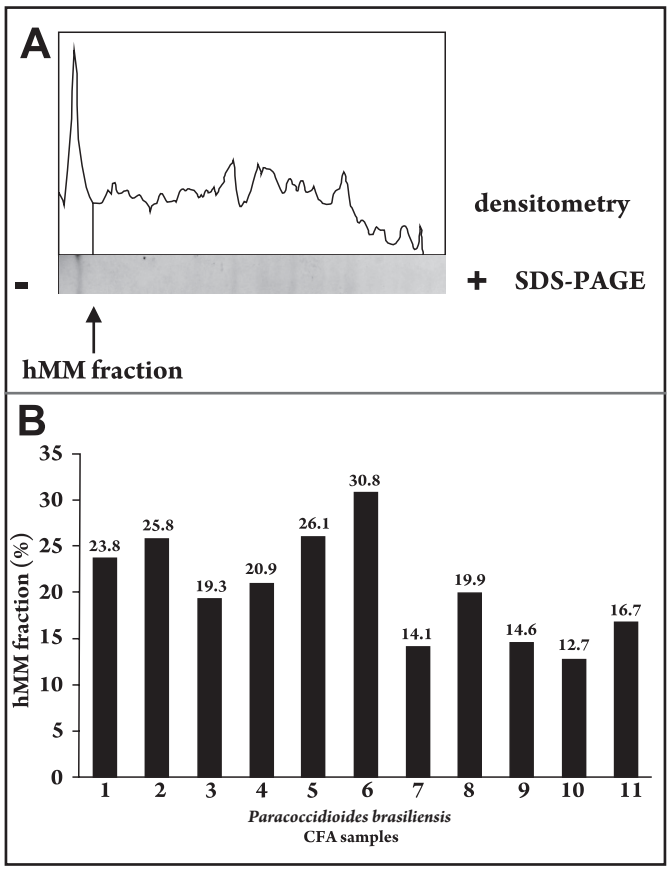

FIGURE 1 - The hMM fraction in CFA from *P. brasiliensis* by SDS-PAGE and densitometry. A) *P. brasiliensis* CFA sample was separated using 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), silver stained and dehydrated. The gel image was captured and the specific cutoff point of hMM fraction (~366kDa) was marked and equivalent area was automatically calculated and expressed in percentages of hMM fraction. B) Percentage of hMM fraction in relation to all other fractions in CFA from clinical isolates of *P. brasiliensis*: (1) LDR13, (2) LDR11, (3) EPM-01, (4) LDR16, (5) LDR17, (6) RC-Hori, (7) RC-Wang, (8) LDR12, (9) LDR14, (10) LDR10 and reference strain (11) Pb18.

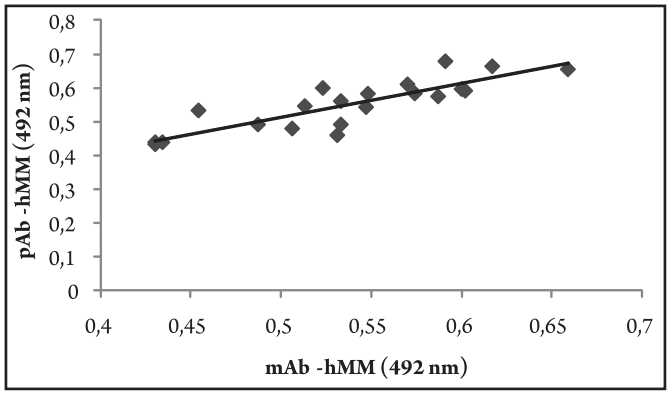

FIGURE 2 - Pearson's correlation tests between hMM fraction levels by capture ELISA (cELISA) using monoclonal (mAb) or polyclonal (polAb). The cELISA results showed a stronger correlation between hMM fraction levels with IgG pAb or mAb anti-hMM as a secondary antibody (r= 0.85).

DISCUSSION

In this work, the hMM fraction was detected in all the *P. brasiliensis* clinical isolates and the reference strain Pb18 by SDS-PAGE, suggesting that all these *P. brasiliensis* could be used as a source of this fraction.

TABLE 1 - High molecular mass fraction (~ 366 KDa) levels in CFA from clinical isolates *Paracoccidioides brasiliensis* (Pb) by capture ELISA, expressed in optical density units.

Reference strain	Pb	pAb-hMM	mAb-hMM
	Pb18	0.600	0.596
	LDR1	0.659	0.655
	LDR2	0.617	0.664
	LDR3	0.587	0.574
	LDR4	0.531	0.458
	LDR5	0.434	0.437
	LDR6	0.574	0.583
	LDR7	0.548	0.582
	LDR8	0.591	0.679
Clinical isolates	LDR9	0.570	0.610
	RC-WANG	0.547	0.542
	LDR10	0.513	0.545
	EPM-01	0.533	0.490
	LDR11	0.454	0.532
	RC-HORI	0.430	0.431
	LDR12	0.506	0.478
	LDR13	0.533	0.560
	LDR14	0.487	0.490
	LDR15	0.430	0.437
	LDR16	0.523	0.599
	LDR17	0.602	0.591
Mean±SEM		**0.537±0.064**	**0.549±0.076**

ELISA immunoplates sensitized with rabbit IgG anti-hMM were incubated with CFA samples and then with polyclonal mouse IgG anti-hMM (pAb-hMM) or with monoclonal mouse IgG anti-hMM (mAb-hMM). After incubation with anti-mouse IgG peroxidase conjugate, absorbance was read at 492nm. All values higher than the controls: polyclonal/polyclonal (0.118 ± 0.008) or polyclonal/monoclonal (0.086 ± 0.005), with free CFA sample, plus 2 x standard were considered positive.

For diagnostic purposes, the presence of the target antigen in all isolates of the *P. brasiliensis* is important. The present study indicates that this antigen is present in different clinical isolates from chronic patients as the common *P. brasiliensis* antigen. However, further studies involving larger sample of isolates are required to confirm these findings.

Panunto-Castelo et al[8] identified hMM antigens with 172 or 160kDa in exoAg from three *P. brasiliensis* isolates (DGO, C-9 and BAT). In this study, the considered hMM band identified in CFA from the *P. brasiliensis* isolates presented ~366kDa and is, therefore, a different hMM antigen.

The detection of circulating antigens is a useful approach for serodiagnosis for monitoring PCM treatment. The gp43 glycoprotein, one of the most important immunodominant antigens of *P. brasiliensis*, has been extensively investigated, particularly in relation to diagnosis[20-22]. Gp43 has been detected at higher levels in PCM patient sera as circulating antigens[22] and in CSF and bronchoalveolar lavage (BAL) fluid samples from PCM patients[11]. However, recently the existence of a *P. brasiliensis* isolate presenting differences in this major antigen coding gene gp43[23,24] has been demonstrated and speculation of the possibility of a new species in the genus Paracoccidioides has been noted. Considering genetic differences in gp43 according to *P. brasiliensis* isolate, it has become important to investigate other *P. brasiliensis* antigens for diagnosis.

Besides gp43[11], the 87-kDa molecule[15], gp70[13] and the high molecular mass antigen with 160kDa[25] were also introduced as potential candidates for diagnosis and/or for follow-up of patients with PCM.

Panuto-Castelo et al[8] demonstrated that the hMM antigens (172 or 160kDa) are highly reactive with serum IgG of patients with acute or chronic PCM, indicating their potential application in the diagnosis and follow-up of the disease. In addition, Coltri et al[25] characterized this antigen as a protein of 160kDa, designated paracoccin, with selective binding to immobilized GlcNAc and able to interact with laminin.

In this study, the association between SDS-PAGE and densitometry analysis shows that approximately 16-20% of *P. brasiliensis* CFA antigens correspond to the hMM fraction. Considering the proportion and as a soluble antigen present in CFA, we speculate that its presence may also be observed as a soluble antigen during infection, similar to other antigens, such as gp43 or gp70, present in CFA and in the serum[11,13,22,26], with the potential for diagnosis and follow-up of the PCM patients. Thus, the hMM fraction is also important when considering the distinct isotypic humoral immune response to hMM antigens with ~336kDa observed in the acute and chronic forms of the PCM disease, which suggest that it has potential as a new biomarker for differentiating these two clinical forms[16].

In this study cELISA was introduced to detect the hMM fraction in clinical isolates and the reference Pb18 strain was introduced and the presence of the hMM fraction was detected in all the samples analyzed, in agreement with SDS-PAGE. This study is qualitative and the results are expressed as optical density. cELISA has the advantage of being able to process large numbers of samples at the same time and presents high sensitivity and a high specificity. The analysis was performed using polAb or mAb to the hMM fraction as a secondary antibody and the results showed a stronger correlation between hMM fraction levels obtained using pAb or mAb anti-hMM. cELISA with specific polAb and mAb was used in previous studies by our group to determine circulating soluble gp43 levels in PCM[22] and determining plasmatic hMM fraction levels in PCM patients by cELISA will be the object of future investigations.

Unexpectedly, the results of ELISA showed homogeneity. We believe that regardless of differences in isolates or strains, these components are better preserved and produced more homogeneously, as observed with other antigens reported in the literature[8]. The same isolates showed less homogeneity in relation to gp43 determined by capture ELISA (data not shown) and the heterogeneity observed in the percentage of the hMM fraction in the electrophoresis was due to variations in the other components present in CFA (data not shown).

In conclusion, the soluble hMM fraction was present in all the *P. brasiliensis* clinical isolates analyzed and the reference strain Pb18, which could be used as source of this fraction. The work also introduced for first time the capture ELISA method for *P. brasiliensis* hMM fraction (~366kDa) detection.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FINANCIAL SUPPORT

This work was supported financial by the Financiadora de Estudos e Projetos (FINEP), the Secretaria de Estado da Ciência, Tecnologia e Ensino Superior (SETI/PR), the Fundação Araucaria/PR, Pró-reitoria de Extensão (PROEX/UEL), the Pró-reitoria de Pesquisa (PROPPG/UEL) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

REFERENCES

1. Restrepo A, Tobón AM. *Paracoccidioides brasiliensis. In:* Mandell GL, Bennett JE, Dollin R, editors. Principles and Practice of Infectious Diseases. 6th ed. Philadelphia: Elsevier; 2005. p. 3062-3068.
2. Borges-Walmsley MI, Chen D, Shu X, Walmsley AR. The pathobiology of *Paracoccidioides brasiliensis*. Trends Microbiol 2002;10:80-87.
3. Shikanai-Yasuda MA, Telles Filho FQ, Mendes RP, Colombo AL, Moretti ML, Grupo de Consultores do Consenso em Paracoccidioidomicose. Consenso em Paracoccidioidomicose. Rev Bras Med Tropical 2006; 39:297-310.
4. Franco M, Montenegro MR, Mendes RP, Marques SA, Dillon NL, Mota NGS. Paracoccidioiodomycosis: a recently proposed classification of its clinical forms. Rev Soc Brasil Med Trop 1987; 20:129-132.
5. Ferreira-da-Cruz MF, Galvão-Castro B, Daniel-Ribeiro CT. Isolation and antigenicity of a 45-kilodalton *Paracoccidioides brasiliensis* immunodominant antigen. Infect Immun 1992; 60:2667-2671.
6. De-Camargo Z, Unterkircher C, Campoy SP, Travassos LR. Production of *Paracoccidioides brasiliensis* exoantigens for immunodiffusion tests. J Clin Microbiol 1988; 26:2147-2151.
7. Taborda CP, Camargo ZP. Diagnosis of paracoccidioidomycosis by passive haemagglutination assay of antibody using a purified and specific antigen-gp43. J Med Vet Mycol 1993; 31:155-160.
8. Panunto-Castelo A, Freitas-da-Silva G, Bragheto IC, Martinez R, Roque-Barreira MC. *Paracoccidioides brasiliensis* exoantigens: recognition by IgG from patients with different clinical forms of paracoccidioidomycosis. Microbes Infect 2003; 5:1205-1211.
9. Puccia R, Schenkman S, Gorin PA, Travassos LR. Exocellular components of *Paracoccidioides brasiliensis*: identification of a specific antigen. Infect Immun 1986; 53:199-206.
10. Puccia R, Travassos LR. 43-kilodalton glycoprotein from *Paracoccidioides brasiliensis:* immunochemical reactions with sera from patients with paracoccidioidomycosis, histoplasmosis, or Jorge Lobo's disease. J Clin Microbiol 1991; 29:1610-1615.
11. Marques-da-Silva SH, Colombo AL, Blotta MHSL, Lopes LD, Queiroz-Telles F, Camargo ZP. Detection of circulating gp43 antigen in serum, cerebrospinal fluid and bronchoalveolar lavage of patients with paracoccidioidomycosis. J Clin Microbiol 2003; 41:3675-3680.
12. Salina MA, Shikanai-Yasuda MA, Mendes RP, Barravieira B, Mendes-Giannini MJS. Detection of circulating *Paracoccidioides brasiliensis* antigen in urine of paracoccidioidomycosis patients before and during treatment. J Clin Microbiol 1998; 36:1723-1728.
13. Marques-da-Silva SH, Mattos Grosso D, Lopes JD, Colombo AL, Blotta MHSL, Queiroz-Telles F, et al. Detection of *Paracoccidioides brasiliensis* gp70 circulating antigen and follow-up of patients undergoing antimycotic therapy. J Clin Microbiol 2004; 42:4480-4486.
14. Gómez BL, Figueroa JL, Hamilton AJ, Ortiz B, Robledo MA, Hay RJ, et al. Use of monoclonal antibodies in diagnosis of paracoccidioidomycosis: new strategies for detection of circulating antigens. J Clin Microbiol 1997; 35:3278-3283.
15. Gómez BL, Figueroa JI, Hamilton AJ, Diez S, Rojas M, Tobón AM, et al. Restrepo A. Antigenemia in patients with paracoccidioidomycosis: detection of the 87-kilodalton determinant during and after antifungal therapy. J Clin Microbiol 1998; 36:3309-3316.
16. Márquez AS, Vicentini P, Ono MA, Watanabe MAE, Camargo ZP, Itano EN. Reactivity of antibodies from patients with acute and chronic paracoccidioidomycosis to a high-mass molecular antigen from *Paracoccidioides brasiliensis*. J Clin Labor Anal 2005; 19:199-204.
17. 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; 29:31-38.
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Colorimetric assays: Lowry´s method for protein determination. J Biol Chem 1951; 193:265-275.
19. Pavanelli WR, Kaminami MS, Gerez JR, Sano A, Ono MA, Camargo ICC, et al. Protection induced in BALB/c mice by the high-molecular-mass (hMM) fraction of *Paracoccidioides brasiliensis*. Mycopathologia 2007; 163:117-128.

20. Camargo ZP, Unterkircher C, Travassos L. Identification of antigenic polypeptides of *Paracoccidioides brasiliensis* by immunoblotting. J Med Vet Mycol 1989; 27:407-412.

21. Brummer E, Castañeda E, Restrepo A. Paracoccidioidomycosis: an update. Clin Microbiol Rev 1993; **6**:89-117.

22. Miura CSN, Estevão D, Lopes JD, Itano EN. Levels of specific antigen (gp43), specific antibodies, and antigen-antibody complexes in saliva and serum of paracoccidioidomycosis patients. Med Mycol 2001; 37:423-428.

23. Carrerro L, Nino-Vega G, Teixeira MM, Carvalho MJA, Soares CM, Pereira M, et al. New *Paracoccidioides brasiliensis* isolate reveals unexpected genomic variability in this human pathogen. Fungal Genet Biol 2008; 45:605-612.

24. Takayama A, Itano EN, Sano A, Ono MA, Kamei K. An atypical *Paracoccidioides brasiliensis* clinical isolate. Med Mycol 2009; 47:01-09.

25. Coltri KC, Casabona-Fortunato AS, Gennari-Cardoso ML, Pinzan CF, Ruas LP, Mariano VS, et al. Paracoccin, a GlcNAc-binding lectin from *Paracoccidioides brasiliensis*, binds to laminin and induces TNF-alpha production by macrophages. Microbes Infect 2006; 8:704-713.

26. Stambuk BU, Puccia R, De-Almeida ML, Travassos LR, Schenkman S. Secretion of the 43kDa glycoprotein antigen by *Paracoccidioides brasiliensis*. J Med Vet Mycol 1988; 26:367-373.