
PROFILE OF EXOANTIGENS FROM CLINICAL ISOLATES OF *Paracoccidioides lutzii* IN MATO GROSSO, BRAZIL

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ABSTRACT

The objective of this study was to determine the immunochemical profile of three exoantigen preparations obtained from clinical samples of *Paracoccidioides lutzii* from the state of Mato Grosso, Brazil. The exoantigens were prepared according to a protocol standardized by Camargo et al. and their immunochemical profiles were analyzed using SDS-PAGE. The observed protein bands exhibited molecular weights of 20, 30, and 130 kDa. The 130 kDa glycoprotein was detected in all three preparations. The detection of this high-molecular-weight protein suggests a distinct immunochemical profile. These differences may help to explain the false negative reactions obtained when using the antigen from *P. brasiliensis* reference strain B-339. This information is essential for increasing the specificity of serological tests used to monitor the prognosis of patients with paracoccidioidomycosis.

KEY WORDS: *Paracoccidioides lutzii*; profiles; Mato Grosso.

RESUMO

Perfil dos exoantígenos de isolados clínicos de *Paracoccidioides lutzii* no Estado de Mato Grosso, Brasil

Este trabalho teve como objetivo determinar o perfil imunológico de três exoantígenos, obtidos de amostras clínicas de *Paracoccidioides lutzii* procedentes do estado de Mato Grosso. Para a obtenção dos exoantígenos, seguiu-se o protocolo padronizado por Camargo et al. e o perfil imunológico foi obtido com o emprego da técnica de SDS Page. As frações observadas demonstraram pesos moleculares de 20, 30 e 130 kDa; a glicoproteína de 130 kDa foi evidenciada tendo por base os três exoantígenos utilizados. A detecção desta fração de alto

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peso molecular sugere um perfil imunológico distinto, o que pode contribuir para elucidar as reações falso-negativas obtidas com o antígeno usualmente utilizado (obtido da cepa de referência B-339 de *P. brasiliensis*). Este achado assume grande importância no sentido de aumentar a especificidade das reações sorológicas utilizadas, para monitorar o prognóstico dos pacientes acometidos pela paracoccidiodomicose.

DESCRITORES: *Paracoccidioides lutzii*; perfil; Mato Grosso.

INTRODUCTION

Paracoccidiodomycosis (PCM) is a severe systemic mycosis caused by fungi of the genus *Paracoccidioides* (*Paracoccidioides brasiliensis* and *P. lutzii*), which exhibit thermal dimorphism. The disease occurs in Latin American countries (Brazil, Colombia, and Venezuela). In Brazil, new *P. lutzii* species (Teixeira et al., 2009; Teixeira et al., 2013) are classified based on phylogenetic differentiation and micromorphological differences of the conidia. *P. lutzii* is distributed primarily in the Midwest region of Brazil (states of Mato Grosso, Goiás, Mato Grosso do Sul). It usually affects farm labourers and its incidence is related to soil management activities. The habitat of *Paracoccidioides* spp. remains unknown (Restrepo, 1985), but other than soil, it has been isolated from animals such as armadillos (viscera), penguins (feces), and soil contaminated dog food (Bagagli et al., 1998; Ferreira et al., 1990; Gezuele, 1989; San-Blas et al., 2002; Silva-Vergara et al., 2000). Compulsory notification of PCM cases is currently not required in the state of Mato Grosso. PCM is considered a neglected disease among other diseases of national relevance in Brazil. In 2002, the annual mortality rate for the disease reached 1.45 per million inhabitants, and the State of Mato Grosso, in particular, showed a high rate of 3.22 per million inhabitants (Bocca et al., 2013; Coutinho et al., 2002; De Souza, 2010; Gegembauer et al., 2014).

PCM is contracted through the inhalation of fungal propagules or conidia, which enter the respiratory tract, reach the pulmonary alveoli, and transform into yeast. In the lungs, an infectious process begins that may either regress or progress into two clinical forms: the acute form, which primarily affects children and adolescents, and the chronic form, which primarily affects male adults. From the lungs, the infection may spread through the bloodstream to various organs, such as the ganglia, oral mucosa, adrenals, and even the central nervous system (Bocca et al., 2013; San-Blas et al., 2002).

The gold standard diagnostic test for PMC is direct microscopic examination, with visualization of the fungus showing cells with multiple buds in the clinical samples analyzed. Clinical and histological findings, along with serological techniques, aid in the diagnosis of the disease. The decline in serum antibody levels detected by double radial immunodiffusion (RID) is used to monitor and track the antifungal treatment.

Despite the high sensitivity of the RID exam (80-95%) observed in

patient samples from the South and Southeast regions of Brazil (Camargo & Franco, 2000), the same level of sensitivity is not observed in samples from Mato Grosso. Prior studies conducted with sera from Mato Grosso patients with PCM showed that when these samples were tested using the standard exoantigen B-339 (ExoAg B-339), they exhibited lower reactivity or no reactivity to exoantigen-MT. However, when an exoantigen obtained from a strain isolated from a patient in Mato Grosso (ExoAg MT) was used, the tests showed higher positive rates (Batista, 2006; Batista et al., 2009). The objective of this report was to produce the immunochemical profile of three exoantigens obtained from *Paracoccidioides* spp. clinical isolates derived from patients residing in the state of Mato Grosso.

MATERIALS AND METHODS

Clinical isolates

Three isolates of *Paracoccidioides lutzii* (31AMS, 11MFC, and 20EE) were obtained from patients diagnosed with the chronic form of PCM, who resided in Mato Grosso, and who received care at the Júlio Muller University Hospital (HUJM). The study was approved by the Research Ethics Committee at HUJM on August 8th, 2007 under protocol n° 390/CEP/HUJM/07.

Preparation of the exoantigen

Paracoccidioides lutzii were isolated in culture media containing Fava-Netto agar. After the fungal colonies developed in tubes containing Fava-Netto agar, they were then incubated at 35°C in a biological oxygen demand incubator to convert the fungi from the mycelial phase into the yeast phase. Subcultures were grown in YPD broth medium, in intervals ranging from 7 to 15 days. Once deactivated with thimerosal 0.2 g/L, the suspension cultures were filtered using filter paper and then concentrated using a vacuum rotatory evaporator. The concentrate was transferred to a dialyzing membrane, submerged in a beaker containing 1 L of bidistilled water and stored at 4-8°C.

After 24 h of dialysis, the water in the beaker was removed and 1 L of fresh bidistilled water was added once for a total of two dialyses. After obtaining the exoantigen, a Bradford assay (Bradford, 1976) was performed to quantify the amount of total protein in the preparation. In order to assess the quality of the exoantigen, immunodiffusion in agarose gel (Camargo et al., 1988; Camargo & Franco, 2000; Camargo et al., 2003) and SDS-PAGE were performed (Laemmli, 1970). The antigen profiles of the three exoantigen preparations obtained from the Mato Grosso strains were characterized using SDS-PAGE followed by silver staining. *P. brasiliensis* isolate B-339 (used in the state of São Paulo) was used as a control (Blumer et al., 1984; Camargo et al., 1988).

SDS-PAGE

Was performed using a 1.0 mm thick, 10 % separating gel with a 3% stacking gel and a Mini-PROTEAN II electrophoresis cell (Bio-Rad Laboratories, Inc., Hercules, California, USA). Two µg of protein from each exoantigen were aliquoted and diluted separately in sample buffer. β-Mercaptoethanol (5%) (Sigma-Aldrich Co., St. Louis, Missouri, USA) was added to the samples. The samples were then heated for 3 minutes at 100°C to denature the proteins. For each electrophoretic run, a low molecular weight (MW) standard was used, composed of: MBP-β-galactosidase (175 kDa), MBP paramyosin (83 kDa), glutamic dehydrogenase (62 kDa), aldolase (47.5 kDa), triose phosphate isomerase (32.5 kDa), β-lactoglobulin A 88 (25 kDa), lysozyme (16.5 kDa), and aprotinin (6.5 kDa) (New England Biolabs Inc., Woburn, Massachusetts, USA).

RESULTS

The analysis by SDS-PAGE presents the antigen profiles of the three exoantigens (MT) (Table) (Camargo et al., 1988; 2003).

Table. Immunochemical profile of exoantigens of *Paracoccidioides lutzii*.

Isolates of	Protein	SDS-PAGE
<i>Paracoccidioides lutzii</i>	Concentration	Protein band sizes (kDa)
31MAS	---	20, 30, 50, and 130
11MFC	180 µg/µL	50
20EE	520 µg/µL	50 and 130

--- No protein concentration

The electrophoretic profiles for the exoantigens from *Paracoccidioides lutzii* isolates 31AMS, 11MFC, and 20EE were obtained by SDS-PAGE. The profiles showed gp50 as the major protein in the preparations. Exoantigen preparations from isolate 20EE also revealed a protein with high MW (130 kDa), while exoantigen preparations from isolate 31AMS showed 18 different protein bands with molecular masses ranging from 20 to 130 kDa (Figure).

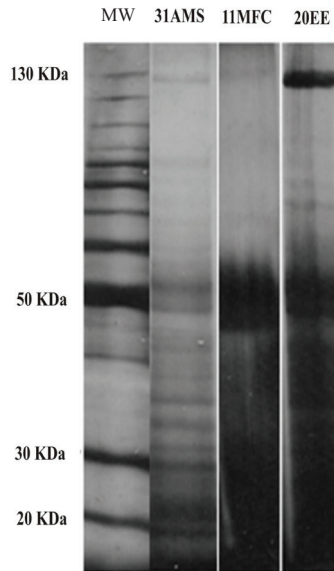


Figure. Immunochemical profile of three exoantigens determined by SDS-PAGE. MW: molecular weight

DISCUSSION

Fifteen years ago, our group reported important differences among the exoantigens obtained from clinical isolates of *Paracoccidioides lutzii* originating from patients in Mato Grosso and the exoantigen from the reference strain (B-339) (Restrepo, 1985), which is commonly used in immunodiffusion assays during serological testing for PCM. Results found by Batista Jr. et al. (2009) suggested that genetic differences must exist between the isolates originating from distinct geographic regions of Brazil, thus enabling a better understanding of the discrepancies observed, and providing a potential reason for the false negative results obtained when sera from patients in Mato Grosso were tested for reactivity with the B-339 exoantigen. Conversely, reactivity decreased when exoantigens derived from Mato Grosso clinical isolates were tested against patient sera originating from the state of São Paulo (Batista et al., 2006). Later, our research group also identified genetic differences between two clinical isolates derived from distinct anatomical sites (arm and face) in the same patient infected with PCM using random amplified polymorphic DNA (RAPD) (Batista et al., 2010; Hahn et al., 2003).

These previous findings suggested that further molecular investigations are required in order to gain a better understanding of the antigenic differences observed when performing serological reactions with exoantigens from distinct geographic regions in Brazil (Midwest and Southeast). Thus, initiatives were undertaken and, in 2009, Teixeira et al. published new findings showing a phylogenetic classification known as S1, PS2, and PS3. In this classification, all isolates originating from Mato Grosso were included in the Pb 01-like cluster, with a new species proposed (*P. lutzii*) based on contrasting genetic differences with the older species (*P. brasiliensis*) which is assigned to PS2 and S1 clusters. In this same study, morphological differences in the conidia present in the mycelia phase were also described. From this time on, studies were intensified, focusing on isolates originating from the state of Mato Grosso, in particular from patients with a clinical and epidemiological history revealing autochthonous cases, who were born in the state, farm workers for the most part, and who had never resided in other Brazilian states. As a result of these investigations, a new species was named in 2013. The name given to this new species was *P. lutzii*, as a tribute to Adolfo Lutz, a physician and researcher who described the first case of PCM in Brazil, in the State of São Paulo. In these studies, a series of isolates originating from the State of Mato Grosso were included in the phylogenetic analyses and, once again, classified as Pb 01-like, thus corroborating earlier findings from 2009 (Teixeira et al., 2009). The Pb 01 isolate originated from a patient diagnosed with chronic PCM from the state of Goiás and has been widely characterized using diverse genetic and biochemical analyses. The research group led by Soares, from the University of Goiás has been studying this isolate for over two decades (Tomazett et al., 2011). Based on these findings, the results obtained in this study, which evaluated the exoantigens from 31AMS, 11MFC, and 20EE isolates of *Paracoccidioides lutzii*, revealed the presence of a 130 kDa glycoprotein that is strongly detected and that distinguishes these isolates from others, again suggesting important antigenic differences between Mato Grosso isolates and the reference strain used for the monitoring of PCM patients, considering the serological reactions used to monitor the prognosis of patients diagnosed with PCM. The 43 kDa (gp43) glycoprotein was weakly expressed in the exoantigen preparations of the three isolates evaluated in this study. Recently, Leitão Jr. et al. (2014) proposed the presence of an active glucanase (Plp43) expressed from *P. lutzii* isolates precisely from the state of Mato Grosso. In addition, these isolates did not express high levels of gp43 suggesting that glucanase Plp43 would be evident in *P. lutzii* isolates, while gp43 would be more evident in *P. brasiliensis* isolates. On the other hand, in the last decade, Marques da Silva et al. (2004) noted the presence of gp43 and gp70 in 100% and 98.76%, respectively, of sera from patients infected with PCM. These authors also showed that reduction in the circulating levels of gp70 during the treatment was associated with reduction in anti-*P. brasiliensis*

antibodies. Given that a low expression or the absence of gp43 was observed in the three exoantigen preparations evaluated in this study, the presence of the 130 kDa glycoprotein may, in future investigations, serve as a potential marker for patients diagnosed with PCM caused by the species *P. lutzii*.

The concurrent investigation of Plp43 and the expression of the 130 kDa protein may provide new markers for tracing both the species *P. lutzii* as well as potential therapeutic follow-up based on the monitoring of circulating anti-*P. lutzii* antibody levels and the decline in the 130 kDa glycoprotein levels.

Thus, investment in obtaining and characterizing exoantigens from the Midwest region of Brazil is essential in order to select or identify an appropriate antigen candidate as the gold standard reactant to be used for analyzing sera from patients infected with *P. lutzii*. It is therefore necessary to identify specific *P. lutzii* proteins different from gp43, which is either expressed at low levels or absent in isolates of this new species.

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