



# Distinct Pattern of *Paracoccidioides lutzii*, *P. restrepiensis* and *P. americana* Antigens Recognized by IgE in Human Paracoccidioidomycosis

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## Abstract

Paracoccidioidomycosis (PCM) is caused by the fungi *Paracoccidioides* spp. The main antigens recognized by IgE are known for *P. brasiliensis* species complex, but not for *P. lutzii*. Current research investigated the major *P. lutzii* (LDR2) antigens recognized by IgE, in comparison to *P. restrepiensis* and *P. americana* (former *P. brasiliensis* species complex), besides IgG recognition. Cell-free antigens (CFA) from *P. lutzii* (LDR2), *P. restrepiensis* (B339) and *P. americana* (LDR3) were analyzed by ELISA and immunoblotting (IB) by detecting specific IgG and IgE from sera from patients with PCM presumable by *P. brasiliensis* species complex ( $n=24$ ). Additionally, somatic antigen (SA) was analyzed by IB. *P. lutzii* (LDR2) antigens showed significantly lower reactivity than *P. restrepiensis* (B339) and *P. americana* (LDR3) in ELISA for both IgE and IgG ( $p<0.05$ ). The IgE-IB pattern was different between *P. lutzii* (LDR2) and the other species, regarding components with ~30 kDa and ~70 kDa in CFA and a ~200 kDa in SA. *P. lutzii* (LDR2) present at least three antigens recognized by IgE which mainly differ from *P. restrepiensis* (B339) and, to a lesser extent, from *P. americana* (LDR3). Current research evidenced for the first time the major *P. lutzii* (LDR2) antigens recognized by IgE.

## Introduction

Paracoccidioidomycosis (PCM) is one of the most important systemic mycoses in Latin America and is prevalent in Brazil. The disease is caused by thermally dimorphic fungal pathogens of the *Paracoccidioides* genus that grow in mycelial form at room temperature and as yeasts at 37 °C. PCM affects mainly rural workers, as the possible natural habitat of *Paracoccidioides* spp. is the soil [1].

Due to genetic variability among PCM isolates, in 2006 Matute et al. [2] used gene genealogies study to divide *P. brasiliensis* into distinct phylogenetic clades: S1, PS2, PS3, PS4. Other atypical isolates [3, 4] that did not match any of the proposed groups were described, and thereafter, based in comparative genomics data, a new species, *Paracoccidioides lutzii*, was proposed by Teixeira et al. [5]. Currently, new taxonomic species for the genus *Paracoccidioides* have been proposed, being *P. brasiliensis* S1, *P. americana* PS2, *P. restrepiensis* PS3 and *P. venezuelensis* PS4 species within the *Paracoccidioides brasiliensis* species complex [6].

Since the International Colloquium on Paracoccidioidomycosis in 1986 in Medellín, Colombia, PCM is classified as acute (AF) and chronic (CF) forms. The AF is considered to be more rare and severe, characterized by a rapid course of the disease and compromised reticuloendothelial system, causing lymph node enlargement and hepatosplenomegaly. The CF occurs more frequently and the disease may develop in multiple forms, ranging from benign and localized (unifocal) to severe and disseminated (multifocal), depending on host's cellular immunity. Those with the multifocal form may experience an evolution of the disease which can be compared in severity to the AF [1, 7].

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The cellular Th<sub>1</sub> response is considered protective in PCM caused by *P. brasiliensis* species complex, while Th<sub>2</sub> response is associated with disease progression with high levels of specific IgE [8, 9]. The main antigen from *P. brasiliensis* species complex, gp43 [10–12], shows high reactivity with serum IgE in PCM and is described as possible stimulators of the Th<sub>2</sub> response [13–15]. The gp70 is an antigen also recognized by IgE from PCM patients' sera. Rigobello et al. [15] demonstrated IgE reactivity to this antigen and its greater amount in somatic antigen than in cell-free antigens from *P. brasiliensis*, corroborating the cytoplasmic location of gp70 in *P. brasiliensis* [16].

The polydispersed high molecular mass antigens (hMM) are other important antigens produced by *P. brasiliensis* [17]. Marquez et al. [18] demonstrated higher serum levels of IgG anti-hMM from *P. brasiliensis* in CF than in AF, suggesting it as a further differential characteristic between the two PCM forms.

Due to the great variability of species and types of responses induced in the host, it is necessary to understand the reactivity of IgG and IgE antibodies in patients with PCM presumably caused *P. brasiliensis* species complex to *P. americana*, *P. restrepiensis* and *P. lutzii* antigens. Therefore, the recognition patterns can be different, because some studies have already shown significant antigenic differences between species, especially between *P. lutzii* and the *P. brasiliensis* species complex, as well as IgG responses against some of these antigens [19–24].

As there is a lot to be investigated about the recently described species, *P. lutzii*, current research has evaluated IgG and IgE pattern of recognition/reactivity to somatic antigens (SA) and to the widely used cell-free antigens (CFA), from *P. lutzii*, *P. restrepiensis* and *P. americana*, in order to identify their major soluble or not soluble immunogenic antigens. Interestingly different patterns of recognition of the antigens of the two sources are demonstrated.

## Materials and Methods

### Serum Samples

PCM group consisted of 24 serum samples from chronic patients (53.95 ± 10.5 years old, 83.3% male and 16.7% female) from the northern and west regions of the Paraná State, Brazil [Clinics Hospital, University Hospital and University Dental Clinic, from State University of Londrina (HC/HURNP/COU-UEL); Municipal Laboratory from Foz do Iguaçu; Clinics Hospital from State University of Western Paraná (HC/UNIOESTE)], all from the biological samples bank (Applied Immunology Laboratory, CCB, State University of Londrina, Londrina, PR, Brazil). The present study was approved by the Institutional Human Research

Ethics Committee of Londrina State University, Paraná, Brazil and a term of free and informed consent was signed by participants.

The authors assumed that the cases were presumably caused by *P. brasiliensis* species complex, predominant species in the study area.

### Fungal Strains

Three species were used: *P. lutzii* (LDR2; IFM 54,648), *P. restrepiensis* (*P. brasiliensis* PS3) (B339, IFM 41,630) and *P. americana* (*P. brasiliensis* PS2) (LDR3, IFM 54,649) [6, 22]. *P. lutzii* strain LDR2 was isolated from patient living in north region of Paraná, Brazil [22]. The fungi were grown on Sabouraud dextrose agar slants at 35 °C, with subcultures every 5 days.

### Cell-Free Antigens (CFA) and Somatic Antigens (SA)

Cell-free antigens (CFA) and somatic antigens (SA) were produced from *P. lutzii* (LDR2; IFM 54,648), *P. restrepiensis* (*P. brasiliensis* PS3) (B339, IFM 41,630) and *P. americana* (*P. brasiliensis* PS2) (LDR3, IFM 54,649). CFA was obtained from the fungal mass according to Camargo et al. [25] modified by the addition of 2.5 mmol phenylmethylsulfonyl fluoride (PMSF). SA was obtained according to the method described by Marquez et al. [18]. The supernatants were stored at – 80 °C until use.

### Serum IgG Reactivity to CFA by iELISA

Serum samples were analyzed in duplicate by indirect enzyme-linked immunosorbent assay (iELISA) to evaluate the serum IgG reactivity to CFA. Briefly, 96-well polystyrene plates were coated with the different CFAs (25 µg/mL) and high affinity 96-well polystyrene plates (*Costar, Corning Incorporated, Corning, NY, USA*) using 100 µL/well for 1 h at 35 °C and 18 h at 4 °C. Blocking was performed for 1 h at room temperature and the plates were washed 4 times. Serum samples at 1/400 dilution were added to the plates (100 µL/well) and incubated for 2 h at 35 °C. After new washes, anti-human IgG peroxidase-labeled antibody was incubated at 1/4000 dilution (100 µL/well) for 1.5 h at 35 °C (*Sigma A6029, Sigma Chemical Co., St. Louis, MO, USA*), followed by the addition of o-phenylenediamine (OPD) substrate solution. The reaction was stopped using 4 N H<sub>2</sub>SO<sub>4</sub> and the plates were read at 492 nm in Multiskan EX Reader (*Labsystems, Helsinki, Finland*).

### Serum IgE Reactivity to CFA by ELISA

The following procedures were performed according to Rigobello et al. [15]. Each serum sample was previously adsorbed

with Sepharose G protein in order to reduce or remove IgG, as it is the most abundant immunoglobulin in the serum. This process reduces epitopes blocking, keeping them accessible for the IgE, which is present in lower concentration in the sera. Each serum sample was individually incubated with Sepharose G protein (*Sigma-P3296*; v/v, 2 h at 35 °C under agitation), centrifuged, the supernatants were used. The individual samples were incubated (100 µL/well) in duplicate at 1/20 dilution for 1.5 h at 35 °C, in plates coated with CFA, as described in the previous item. Anti-human IgE (*Sigma-I6284*, *Sigma Chemical Co., St. Louis, MO, USA*) was added (100 µL/well, 30 µg/mL, 1.5 h, 35 °C), followed by anti-sheep IgG peroxidase-labeled antibody (*Sigma-A3415*, *Sigma Chemical Co., St. Louis, MO, USA*; 1/2000, 1.5 h, 35 °C). OPD was added, the reaction was stopped with 4N H<sub>2</sub>SO<sub>4</sub> and the plates were read at 492 nm.

### Immunoblotting

CFA and SA samples (1 mg/mL) were treated with mercaptoethanol sample buffer (*Bio-Rad Laboratories, Hercules, CA, USA*) and applied to individual lanes of a 10% polyacrylamide gel electrophoresis with Sodium Dodecyl Sulfate (10% SDS-PAGE) in tris–glycine buffer, pH 8.2, at 100 V, along with a pre-stained molecular weight protein standard (*Bio-Rad Precision Plus Protein™ Kaleidoscope cat. #161–0375*, *Bio-Rad Laboratories, Hercules, CA, USA*) and transferred to a nitrocellulose membrane (18 h at 4 °C, 23 V). A pool of the 24 PCM patients' sera was employed, but first, the individual samples were subjected to affinity chromatography in order to reduce or remove IgG, as it is the most abundant immunoglobulin in the serum. This process reduces epitopes blocking, keeping them accessible for the IgE, which is present in lower concentration in the sera. Each serum sample was individually incubated with Sepharose G protein (*Sigma-P3296*; v/v, 2 h at 35 °C under agitation), centrifuged, the supernatants were pooled and used to detect IgE recognition. The bounded proteins (IgG) were eluted from the resin and were pooled. For IgG detection, the membrane was incubated with 1 mg/mL of pooled IgG, followed by anti-human IgG peroxidase-labeled antibody at 1/2000 dilution (*Sigma A6029*, *Sigma Chemical Co., St. Louis, MO, USA*) for 1.5 h at 35 °C. For IgE, the membrane was incubated with 1 mg/mL of pooled IgE, followed by anti-human IgE (*Sigma-I6284*, *Sigma Chemical Co., St. Louis, MO, USA*; 30 µg/mL, 1.5 h, 35 °C) and then with peroxidase-labeled antibody (*Sigma-A3415*, *Sigma Chemical Co., St. Louis, MO, USA*; 1/1000, 1.5 h, 35 °C). The visual detection was performed using 3,3',5,5'-tetramethylbenzidine (TMB) (*Zymed cat. #00–2019*, *Zymed, San Francisco, CA, USA*). The analysis of the areas of each component from the CFA or SA that were recognized by IgG or IgE was performed using LabImage 1D 2006 Professional software (*Loccus*

*Biotechnologia, São Paulo, SP, Brazil*) and were expressed as percentages.

### Statistical Analysis

T test and one-way ANOVA with Tukey's post-hoc test for multiple comparisons were calculated in Graphpad Prism 6.01 software for Windows (*GraphPad Software, San Diego California, USA*), considering significant *P*-value ≤ 0.05. LabImage 1D 2006 Professional software (*Loccus Biotechnologia, São Paulo, SP, Brazil*) was employed for the analysis of the area percentage of the antigens recognized by IgE or IgG in immunoblotting.

## Results

### Serum IgG and IgE Reactivity to CFA by ELISA

Figure 1 shows serum reactivity of IgG and IgE to CFA antigens in ELISA. Comparing the species, there was a significantly higher reactivity of IgG anti-CFA from *P. restrepiensis* B339 than *P. americana* LDR3 (*P* < 0.05) and *P. lutzii* LDR2 (*p* < 0.01) (Fig. 1a). There was a greater reactivity of IgE anti-CFA from *P. restrepiensis* B339 than *P. americana* LDR3 (*P* < 0.01) and *P. lutzii* LDR2 (*P* < 0.001), and there were also differences between *P. americana* LDR3 and *P. lutzii* LDR2 (*P* < 0.05) (Fig. 1b).

### Serum IgG and IgE Reactivity to CFA and SA by Immunoblotting

In immunoblotting, IgG and IgE showed different patterns of recognition of the immunogenic components from the different species, either in CFA or SA (Fig. 2). The main difference was the absence of gp43 detection in *P. lutzii* LDR2 and the detection of ~70 kDa antigens in both CFA and SA from *P. lutzii* LDR2. IgE also recognized ~30 kDa antigens in CFA and SA from *P. lutzii* LDR2, which were not recognized in CFA from other species used. The components of hMM with heterogeneous electrophoresis migration were detected in CFA and SA from *P. restrepiensis* B339 and *P. americana* LDR3, while in *P. lutzii* LDR2 a compact band with ~200 kDa in SA was detected. Analysis by LabImage 1D 2006 Professional software of the corresponding areas of each band resulted in a larger area corresponding to the hMM antigens from *P. restrepiensis* B339 and *P. Americana* LDR3, but not from *P. lutzii* LDR2 (Table 1). Our previous knowledge about high molecular mass antigens was only about *P. brasiliensis* species complex, which presents diffused electrophoretic migration, therefore we consider as a high molecular mass fraction all the components with more than 120 kDa.

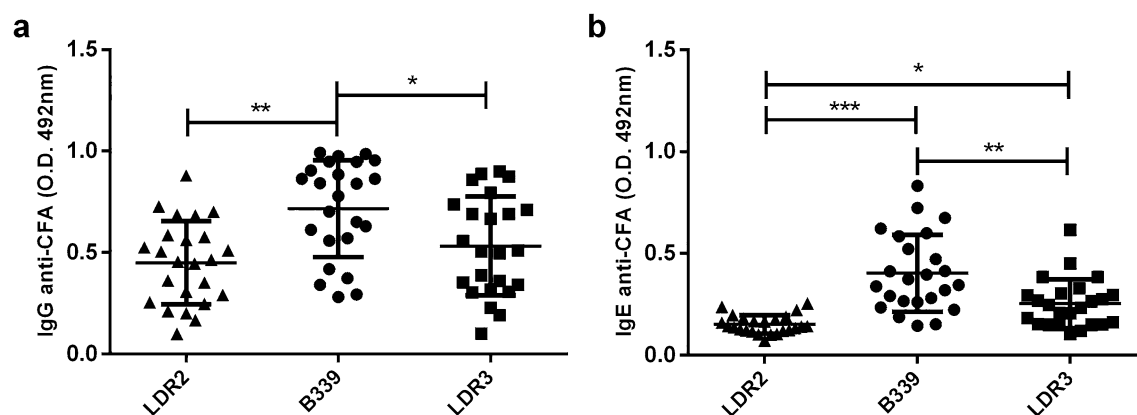


**Fig. 1** Reactivity of serum IgG (a) and IgE (b) from PCM patients' sera to CFA from *P. lutzii* (LDR2), *P. restrepiensis* (B339) and *P. americana* (LDR3), by ELISA. Immunoplates coated with CFA (25 µg/mL) from *P. lutzii* (LDR2), *P. restrepiensis* (B339) or *P. americana* (LDR3) were incubated with chronic

PCM patients' serum samples ( $n=24$ ) diluted 1/400 (for IgG) or serum samples previously adsorbed with Sepharose G protein at 1/20 final dilution (for IgE). The results are expressed as means of optical density (O.D.) at 492 nm. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$

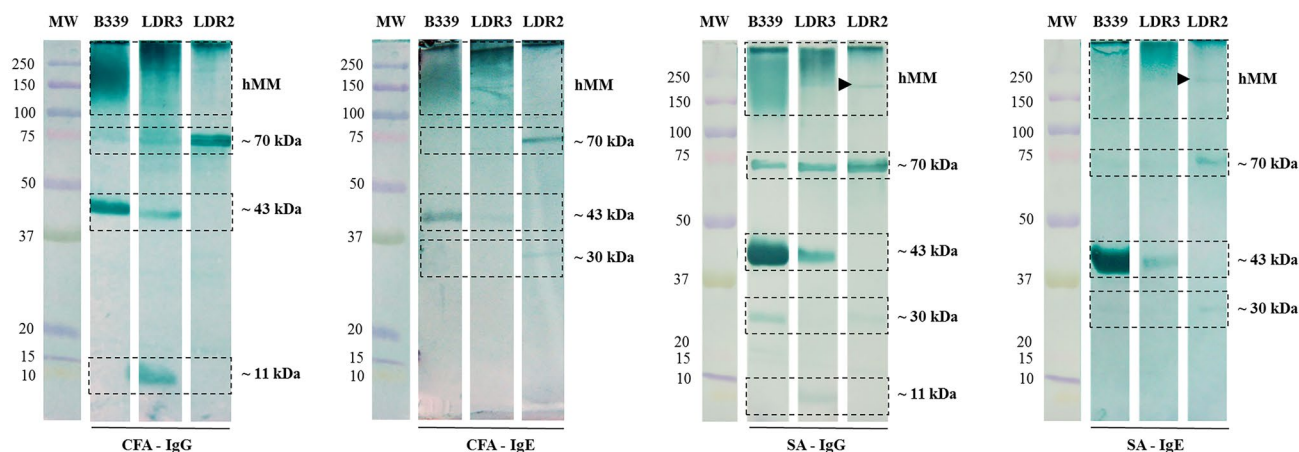


**Fig. 2** Immunoblotting of total soluble antigens (CFA) and somatic antigen (SA) from *P. restrepiensis* (B339), *P. americana* (LDR3) or *P. lutzii* (LDR2), using IgG or IgE polyclonal antibodies. CFA and SA samples (1 mg/mL) treated with mercaptoethanol sample buffer were applied to individual lanes of a 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were incubated with

purified IgG or adsorbed IgE (Sepharose G protein) from a pool of 24 PCM patients' sera. Dashed lines are indicating bands referring to antigens recognized by IgG/IgE. MW molecular weight protein standard; hMM Fração de alta massa molecular arrowhead hMM compact band ~200 kDa

**Table 1** Computer analysis of the areas of the immunoblotting bands from *P. restrepiensis* (B339), *P. americana* (LDR3) and *P. lutzii* (LDR2) CFA and SA recognized by IgG or IgE, expressed in percentages

| % Antigens | CFA-IgG |      |      | CFA-IgE |      |      | SA-IgG |      |      | SA-IgE |      |      |
|------------|---------|------|------|---------|------|------|--------|------|------|--------|------|------|
|            | B339    | LDR3 | LDR2 | B339    | LDR3 | LDR2 | B339   | LDR3 | LDR2 | B339   | LDR3 | LDR2 |
| hMM        | 80.3    | 61.2 | 48   | 90.2    | 87.3 | 33.7 | 56.9   | 57.7 | 66.4 | 53.7   | 75.8 | 66   |
| ~70 kDa    | 5.9     | 13.2 | 52   | —       | —    | 39.3 | 8.5    | 13   | 26.6 | 5      | 8.9  | 21.6 |
| ~43 kDa    | 13.8    | 11.4 | —    | 9.8     | 12.7 | —    | 26.5   | 20.2 | —    | 36.2   | 15.3 | —    |
| ~30 kDa    | —       | —    | —    | —       | —    | 27   | 8.1    | —    | 7    | 5.1    | —    | 12.4 |
| ~11 kDa    | —       | 14.2 | —    | —       | —    | —    | —      | 9.1  | —    | —      | —    | —    |

## Discussion

The progress of PCM depends on the virulence of the infecting agent [26] and on the host's immune response [27], as demonstrated in experimental infections in animals. The host's main defense response in PCM is the development of the Th<sub>1</sub> profile and, on the other hand, development of Th<sub>2</sub> profile associated with the production of IgE antibodies has been correlated with more severe disease [13, 28–30]. The main antigen from *P. brasiliensis* species complex recognized by IgE is gp43, considered a marker of the deviation to Th<sub>2</sub> immune response, and was associated with disseminated or severe PCM [13, 28–30]. Another *P. brasiliensis* species complex antigen recognized by IgE from patients with PCM presumably caused by *P. brasiliensis* species complex is gp70 [15].

Current research demonstrates components from *P. lutzii* (LDR2) which are recognized by IgE. In the first step, analysis performed by ELISA showed weaker IgE reactivity to CFA from *P. lutzii* (LDR2) than from other species. IB shows two antigens recognized by IgE in CFA from *P. lutzii* (LDR2), with approximately 70 and 30 kDa. Concerning the other species' CFAs, IgE recognized mainly antigens with high MM with heterogeneous migration (hMM) and, to a lesser extent, the antigen of 43 kDa. This large proportion of hMM (Fig. 2, Table 1) may have contributed to the higher reactivity observed in ELISA in CFA from *P. restrepiensis* B339 and *P. americana* LDR3 than from *P. lutzii* (LDR2). These hMM antigens from *P. restrepiensis* B339 and *P. americana* LDR3 that had heterogeneous (diffused) electrophoretic migration, recognized by both IgE and IgG, may be the same components described by Puccia et al. [17] and Marquez et al. [18]. Although Marquez et al. [18] did not detect hMM by immunoblotting, the IgE reactivity with hMM antigens from *P. restrepiensis* B339 (former *P. brasiliensis* species complex) with either acute or chronic form of PCM, can be observed by capture ELISA.

Here it was not possible to evidence gp43 in *P. lutzii*, which was expected and in accordance to Lenhard-Vidal et al. [21], who also did not detect gp43 in antigens from *P. lutzii* LDR2 by silver/PAS staining of SDS-PAGE or by immunoblot (rabbit immune serum against *P. brasiliensis* S1 or by PCM patients' sera). The non-production of this antigen may be due to the time of cultivation of the fungus, as discussed by Lenhard-Vidal et al. [21] and further studies should be carried out for complete clarification. The gp43 is considered the major antigen and virulence factor from *P. brasiliensis* species complex, responsible for the evasion from the host's immune response in PCM [31]. Currently, it is known that the expression of gp43 may vary according to the strain [19] and *P. lutzii* differs from *P. brasiliensis*

precisely in the sequence of the gene responsible for gp43 [22]. In *P. lutzii* isolates, gp43 is not the main antigen produced or may even be absent in isolates from the Brazilian central-west region, since it was not identified in *P. lutzii* by nested PCR for gp43 [21, 32].

Regarding gp70 (~70 kDa), it was the main *P. lutzii*'s CFA component recognized by both IgE and IgG. A prominent band of ~70 kDa in *P. lutzii*'s CFA, but negligible in *P. restrepiensis* B339 and *P. americana* LDR3, indicates this antigen is more easily released in the soluble form by *P. lutzii* LDR2, bearing in mind that CFA preparations contain components that are easily released by stirring. Also, in IB with CFA from *P. lutzii* LDR2, it was evidenced a component with ~30 kDa recognized by IgE. Further investigation is needed to see if this component is an adhesion protein, a laminin ligand, highly expressed in more virulent *P. brasiliensis* isolates [33] with subcellular localization [34]. Maybe this component is produced in higher concentrations by *P. lutzii* LDR2 than the other investigated species, as it was more evident in *P. lutzii* LDR2 (Fig. 2, Table 1).

As CFA represents only part of surface antigens that are released, current research also analyzed SA, obtained by cell lysis, therefore containing the internal components of the fungi. As for CFA, profiles of SA recognized by IgG and IgE was more similar between *P. restrepiensis* B339 and *P. americana* LDR3 than *P. lutzii* LDR2. Interestingly, a change in the intensity or percentage of antigens in SA compared to CFA was detected (Fig. 2 and Table 1). The main changes were an increase in the gp43 band and a decrease in hMM from *P. restrepiensis* B339 and *P. americana* LDR3. On the other hand, a compact band of high MM (~200 kDa) became evident in *P. lutzii* LDR2 (Fig. 2).

The diffuse hMM band, described previously [17, 18] could be distinct from hMM antigens present in SA from *P. lutzii* (LDR2) due to the distinct pattern of migration and also by glycosylation as described by Lenhard-Vidal et al. [21], although it also present high MM. However, this diffuse hMM band pattern may not be characteristic of all isolates of *P. lutzii*, as observed in IB results from Gegembauer et al. [23], which makes it important to investigate IgE reactivity with others *P. lutzii* isolates.

The comparison between CFA and SA components allows the identification of whether antigens are present in greater concentrations inside the fungus or if they are released to the environment, which possibly has a major impact on host-fungus interaction. In accordance to what was already described by Rigobello et al. [15], gp70 was detected in greater concentration in SA than CFA from *P. brasiliensis* species complex. On the other hand, indirectly, our findings indicate that *P. lutzii* (LDR2) has a greater proportion of ~70 kDa antigen in CFA than in SA (Fig. 2, Table 1). If these components were more easily released during CFA

preparation, possibly they may also be released during host's infection, contributing to the pathogenesis of PCM. Gp70 is considered a virulence factor of *P. brasiliensis* species complex, capable of inhibiting phagocytosis and protecting the fungus against the action of NO and H<sub>2</sub>O<sub>2</sub> [16], so theoretically, *P. lutzii*'s gp70 present in soluble form could spread and act at distant sites, inhibiting macrophage function. Furthermore, the antigens potentially released in vivo in the soluble form, as the ~30 and ~70 kDa antigens, also have the potential to form immunocomplexes and activate the complement system, which would be another important aspect to be investigated. Additionally, knowledge about the major immunogenic antigens, released or not by *Paracoccidioides* sp., has practical relevance in choosing the source of antigen.

The limitation of this study is the non-confirmation the infectious species of the patients included in the research. Thus, the difference in the IgE pattern found in this study may be due to the differences between the species of *Paracoccidioides* or may also be the product of an infection caused by a specific species. Until very recently, *P. restrepiensis* and *P. americana* were classified as *P. brasiliensis* PS3 and PS2, respectively [2, 22]. Antigens of *P. brasiliensis* sensu stricto e *P. venezuelensis* were not used in this study, because, *P. restrepiensis* strain B339 is a known isolate that has an antigenic profile very similar to that of *P. brasiliensis* sensu stricto [20, 35–38], and, *P. venezuelensis* (PS4) is a species found exclusively in Venezuela [6].

Because patients are from a region where the predominant species are *P. brasiliensis* species complex, such as *P. brasiliensis* sensu strict and *P. americana* [22], we assumed that they had PCM by these species, although the infection with other species cannot be ruled out. In addition, *P. lutzii* is an endemic species in the central-western and northern regions of Brazil, however it has also been found in the southern region, in which *P. lutzii* used in the present study was isolated from a patient living in northern Paraná, Brazil [22]. Interestingly, Lenhard-Vidal et al. [24] showed that IgG from patients with PCM who live in an endemic region for *P. brasiliensis* recognize CFA and hMM from different species of *Paracoccidioides* with different intensities.

This research reveals that IgE and IgG from patients with PCM presumably due *P. brasiliensis* species complex recognized antigens from two *P. brasiliensis* complex species: *P. americana* and *P. restrepiensis*; and from *P. lutzii*. However, different patterns of the reactivity were observed. Taken all together, major *P. lutzii*'s immunogenic compounds recognized by IgE were antigens of ~30 kDa, ~70 kDa and a compact band of hMM with ~200 kDa. The different pattern of antigen recognition by IgG/IgE in *P. lutzii*, *P. restrepiensis* B339 and *P. americana* LDR3 CFA/SA suggests that some antigens from these species are more easily released and/or

recognized by PCM patients' sera. Future studies may be addressed with the objective of complementing the results of the present research, verifying if different species can cause different patterns of IgE reactivity or if individuals infected with a predominant species may have different IgE responses to *P. americana*, *P. restrepiensis* and *P. lutzii*.

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