JOÃO PAULO ASSOLINI

DIFERENTES PADRÕES DE RECONHECIMENTO DE ANTÍGENOS DE *Paracoccidioides brasiliensis* E *Paracoccidioides lutzii* POR IgE E IgG NA PARACOCCIDIOIDOMICOSE HUMANA

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Dissertação apresentada ao Programa de Pós Graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito para obtenção do título de Mestre.

Orientador: Profª. Drª. Eiko Nakagawa Itano.

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BANCA EXAMINADORA

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Orientador: Profª. Drª. Eiko Nakagawa Itano
Universidade Estadual de Londrina - UEL

_____

Profª. Drª. Berenice Tomoko Tatibana
Instituto Federal do Paraná - IFPR

_____

Prof. Dr. Emerson José Venancio
Universidade Estadual de Londrina - UEL

Londrina, 24 de janeiro de 2016

Este trabalho foi desenvolvido no Laboratório de Imunologia Aplicada, Departamento de Ciências Patológicas, Centro de Ciências Biológicas da Universidade Estadual de Londrina, sob orientação da Profª Drª Eiko Nakagawa Itano e com apoio da Fundação Araucária, PPSUS/Fundação Araucária CNPq, CAPES, PROPPG/UEL, PROEX/UEL.

Dedico esse trabalho:

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RESUMO

Paracoccidioidomicose (PCM) é uma das micoses sistêmicas mais importantes da América Latina, causada pelos fungos dimórficos *Paracoccidioides brasiliensis* (S1, PS2, PS3) e *Paracoccidioides lutzii*, na qual a gravidade da doença está relacionada com resposta Th2 e altos níveis de Imunoglobulina E (IgE). O presente estudo teve como objetivo avaliar o padrão de reconhecimento/reatividade de IgG e IgE séricos aos antígenos de *P. brasiliensis* S1 (B339), PS2 (LDR3) e *P. lutzii* (LDR2) na PCM humana crônica. Foi realizado *Immunoblotting* para verificar quais os componentes imunogênicos presentes no antígeno solúvel total (CFA) e antígeno somático (AS) de *P. brasiliensis* (S1 e PS2) e *P. lutzii* que são reconhecidos por IgG e IgE na PCM humana. A reatividade de IgG e IgE séricos de pacientes com PCM crônica (n = 24) foram analisados por Ensaio Imunoenzimático indireto (ELISA), utilizando antígeno solúvel total (CFA), fração de alta massa molecular (hMM) e gp70 de *P. brasiliensis* (S1 e PS2) e *P. lutzii*. Nos resultados de *Immunoblotting* foram observadas diferenças no reconhecimento de componentes imunogênicos de antígenos totais das três cepas, por IgG e IgE. As principais diferenças observadas por IgG foram: a ausência de gp43 em *P. lutzii* (LDR2), banda de gp70 intensamente corada em CFA de *P. lutzii* e presença de antígeno de alta MM e ausência de banda hMM difusa em *P. lutzii*. A IgE reconheceu gp70 e antígeno de ~30 kDa em CFA de *P. lutzii*, sugerindo que os mesmos não são exclusivamente intracelulares. Por ELISA, IgG de pacientes com PCM apresentaram maior reatividade para CFA e hMM de *P. brasiliensis* S1 em relação ao *P. lutzii* ($p < 0.05$), no entanto houve maior reatividade para gp70 de *P. lutzii* e *P. brasiliensis* PS2 do que S1 ($p < 0.05$). Anticorpos IgE apresentaram maior reatividade para CFA de *P. brasiliensis* do que *P. lutzii* ($p < 0.05$), diferente com hMM, com maior reatividade para *P. lutzii* do que *P. brasiliensis* S1 ($p < 0.05$). Em conclusão, o padrão de reconhecimento de antígeno por IgG e IgE difere de acordo com *Paracoccidioides* sp. Antígeno de hMM devido à maior reatividade para IgE, e antígenos de ~70 kDa e ~30 kDa produzidos, liberados e reconhecidos por IgE podem ser os principais fatores de virulência de *P. lutzii*, sugerindo um potencial de induzirem uma resposta Th2.

Palavras-chave: Anticorpos. Fatores de virulência. Fungo. Imunodiagnóstico. Imunomodulação. Micose.

ASSOLINI, João Paulo. **Different pattern of recognition of *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii* antigens by IgE and IgG in human Paracoccidioidomycosis**. 2016. 46 s. Dissertation (Master's Degree in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2016.

ABSTRACT

Paracoccidioidomycosis (PCM) is one of the most important systemic mycosis in Latin America, caused by fungi dimorphic *Paracoccidioides brasiliensis* (S1, PS2, PS3) and *Paracoccidioides lutzii*, in which the severity of disease is related to Th2 response and high levels of immunoglobulin E (IgE). The present study aimed to evaluate the pattern recognition/reactivity of IgG and IgE sera to *P. brasiliensis* S1 (B339), PS2 (LDR3) and *P. lutzii* (LDR2) antigens in chronic human PCM. Immunoblotting was performed to verify which the immunogenic components present in the total soluble antigen (CFA) and somatic antigen (SA) from *P. brasiliensis* (S1 and PS2) and *P. lutzii* that are recognized by IgG and IgE in the human PCM. The reactivity of IgG and IgE sera from patients with chronic PCM (n = 24) were analyzed by indirect enzyme linked immunosorbent assay (ELISA), using total soluble antigen (CFA), high molecular weight fraction (hMM) and gp70 from *P. brasiliensis* (S1 and PS2) and *P. lutzii*. In the results of immunoblotting were observed differences in the recognition of immunogenic components of total antigens of the three strains, by IgG and IgE. The main differences evidenced by IgG were: the absence of gp43 in *P. lutzii* (LDR2), gp70 band intensely stained in CFA from *P. lutzii,* and presence of hMM antigen and absence of diffuse hMM band in *P. lutzii*. IgE recognized gp70 and ~30 kDa antigen in CFA from *P. lutzii*, suggesting that they are not exclusively intracellular. By ELISA, IgG from PCM patients showed higher reactivity to CFA and hMM from *P. brasiliensis* S1 in relation to P. lutzii ($p < 0.05$), however was higher reactivity for gp70 from *P. brasiliensis* PS2 and *P. lutzii* than S1 ($p < 0.05$). IgE antibodies had higher reactivity to CFA from *P. brasiliensis* than *P. lutzii* ($p < 0.05$), different for hMM, with higher reactivity reactive to *P. lutzii* than *P. brasiliensis* S1 ($p < 0.05$). In conclusion, the antigen recognition pattern by IgG and IgE differs according *Paracoccidioides* sp. hMM antigen due to higher reactivity to IgE, and ~70 kDa and ~30 kDa antigens produced, released and recognized by IgE can be the main virulence factors of *P. lutzii*, suggesting a potential to induce a Th2 response.

Keywords: Antibodies. Virulence factor. Fungus. Immunodiagnosis. Immunomodulation. Mycosis.

LISTA DE FIGURAS E TABELAS

LISTA DE ABREVIATURAS E SIGLAS

°C	Graus Celsius
B339	*Paracoccidioides brasiliensis* (S1) isolado B339
CCB	Centro de Ciências Biológicas
CFA	Antígeno livre de células (*Cell Free Antigen*)
ELISA	Ensaio imunoenzimático (*enzyme linked immunosorbent assay*)
gp43	glicoproteína de 43kDa
gp70	glicoproteína de 70kDa
H_2O_2	Peróxido de Hidrogênio
H_2SO_4	Ácido Sulfúrico
HC	Hospital das Clínicas
hMM	Fração de alta massa molecular (*high Molecular Mass*)
HU	Hospital Universitário
IFN	Interferon
IgE	Imunoglobulina E
IgG	Imunoglobulina G
ID	Imunodifusão
IL	Interleucina
kDa	quilodalton
LDR2	*Paracoccidioides lutzii* isolado LDR2
LDR3	*Paracoccidioides brasiliensis* (PS2) isolado LDR3
M	Molar
mg	miligrama
mL	mililitro
MM	Massa Molecular
mM	miliMolar
N	Normal
NHS	Soro Humano Normal (*Normal Human Serum)*
nm	nanômetro
NO	Óxido Nítrico

OPD	Orto-fenilenediamina (*ortho-phenylenediamine*)
PBS	Tampão Salina Fosfato (*phosphate-buffered saline)*
PCM	Paracoccidioidomicose (*Paracoccidioidomycosis*)
PMSF	Fluoreto de phenilmetilsulfonil (*Phenylmethanesulfonyl Fluoride*)
PS2	Espécie Filogenética 2 (*Phylogenetic Species 2)*
PS3	Espécie Filogenética 3 (*Phylogenetic Species 3)*
S1	Espécie 1 (*Species 1)*
SA	Antígeno Somático (*Somatic Antigen*)
SDS-PAGE	Eletroforese em gel de poliacrilamida com Dodecil Sulfato de Sódio (*polyacrylamide gel electrophoresis with Sodium Dodecyl Sulfate*)
TGF-β	Fator de Transformação de Crescimento β (*Transforming Growth Fator*)
Th1	T *helper* 1
Th2	T *helper* 2
TMB	3,3,5,5-tetrametilbenzidina (*3,3,5,5-tetramethylbenzidine*)
UEL	Universidade Estadual de Londrina
UNIOESTE	Universidade Estadual do Oeste do Paraná
α	alfa
β	beta
γ	gama
µg	micrograma
µL	microlitro

SUMÁRIO

1 INTRODUÇÃO

A paracoccidioidomicose (PCM) é uma micose sistêmica causada pelos fungos termodimórficos *Paracoccidioides brasiliensis* (*P. brasiliensis*) e *Paracoccidioides lutzii* (*P. lutzii*). Devido a variabilidade genética dos agentes causadores da PCM, recentes estudos demonstraram a existência de três espécies filogenéticas distintas de *P. brasiliensis*: Espécie 1 (S1 com 38 isolados no Brasil, Argentina, Paraguai, Venezuela e Peru, Espécie Filogenética 2 (PS2 com 6 isolados no Brasil e Venezuela), e Espécie Filogenética 3 (PS3 com 21 isolados na Colômbia) (CARRERO et al., 2008; MATUTE et al., 2006a; MATUTE et al., 2006b; MATUTE et al., 2007; SALGADO-SALAZAR et al., 2010). Além disso, análises genômicas comparativas mostraram que isolados atípicos (Pb-01 like) devem ser agrupados em uma nova espécies *P. lutzii* (TEIXEIRA et al., 2009).

A PCM é considerada uma das micoses sistêmicas mais importante da América Latina, devido ao grande número de casos registrados, por ser considerada a oitava causa de morte entre as doenças infecciosas e parasitárias, e por ter a mais alta taxa de mortalidade entre as micoses sistêmicas (COUTINHO et al., 2002).

Os fungos das espécies *P. brasiliensis* e *P. lutzii* apresentam na forma de levedura à 35ºC caracterizando a forma parasitária no hospedeiro, e à temperatura ambiente (25ºC) na forma micelial, considerada a forma infectante e encontrada normalmente na natureza (FRANCO et al., 1994; LACAZ et al., 2002).

A PCM pode ser dividida em infecção e doença. Na PCM infecção os indivíduos são assintomáticos e não apresentam manifestações clínicas, mas com reação intradérmica positiva para a paracoccidioidina. A PCM doença pode ser uma forma aguda (tipo juvenil) que acomete principalmente crianças e jovens com a mesma proporção entre sexos, sendo a forma rara, porém mais grave da doença. Por outro lado, a forma crônica (tipo adulto) da doença acomete principalmente adultos do sexo masculino entre 30 e 60 anos. A forma crônica pode ser subdividida em unifocal onde acomete apenas um órgão ou multifocal onde vários órgãos podem ser afetados, essa última pode ser comparada à forma mais grave da doença (FRANCO et al., 1987).

A prevalência da doença na forma crônica em homens, deve ser devido a proteção conferida pelos estrógenos às mulheres. *P. brasiliensis* apresentam

proteínas que se ligam aos esteroides, possivelmente agindo como receptor para estrógenos dos mamíferos (LOOSE et al., 1983) inibindo a transição de micélio para levedura (ARISTIZABAL et al., 1998; RESTREPO et al., 1984; SALAZAR et al., 1988;). Foi descrito a influência dos hormônios, estrógeno e testosterona, na resposta imunológica em modelo de paracoccidioidomicose experimental, onde evidencias mostraram que camundongos fêmeas têm uma resposta T *helper* 1 (Th1) aumentada, com aumento de produção de citocinas pró-inflamatórias, Óxido Nítrico (NO), expressão do fator de transcrição T-bet, e diminuição na produção de IL-4 e IL-10, com menor carga fúngica. Por outro lado, em camundongos machos com altos níveis de testosterona, foi observado o predomínio de uma resposta T *helper* 2 (Th2), com baixa produção de citocinas pró-inflamatórias e Óxido Nítrico (NO), e aumento da expressão do fator de transcrição GATA-3 e citocinas como IL-4 e IL10, com maior carga fúngica (PINZAN et al., 2010; SANO et al., 1992).

A resposta imune Th1 possui um papel protetor, responsável pela imunidade celular, produção de Imunoglobulinas G (IgG) opsonizantes, citocinas pró-inflamatórias, ativação de fagócitos e formação de granulomas mais compactos, evidenciados nas formas mais brandas da PCM. A resposta do tipo Th2 é responsável pela progressão da doença, com hipergamaglobulinemia, altos níveis de IgE específicos, aumento da produção de IL-4, IL-5, IL-10 e TGF-β, onde inibem uma resposta inflamatória (Th1), e com formação de granulomas mais frouxos e fibrose, evidenciados nas formas mais graves da PCM (BERNARD et al.,1997; BORGES-WALMSLEY et al., 2002; CALICH; KASHINO, 1998).

O fungo *P. brasiliensis* apresenta fatores de virulência e mecanismos de escape importantes, sendo o dimorfismo um dos mais importantes. A β-1-3-glucana, encontrada em maior quantidade na parede do fungo na forma micelial, é reconhecida por receptores dectin-1 de células do hospedeiro, ativando o sistema imune e impedindo a instalação do fungo no hospedeiro. A transição para levedura faz com que ocorra um aumento na expressão de α-1-3-glucana na parede celular fúngica possivelmente ocultando a β-1-3-glucana e impedindo a ativação das células imunes e produção de citocinas pró-inflamatórias (KLEIN; TEBBETS, 2007).

O antígeno livre de células (CFA) é composto por várias moléculas ligadas a superfície das células de leveduras, onde são facilmente retiradas e suspensas em solução aquosa, podendo mimetizar os antígenos liberados pelo fungo *in vivo* no local da infecção ou na corrente sanguínea. O antígeno somático (AS) é obtido

através da ruptura da célula do fungo e é constituído principalmente de moléculas citoplasmáticas. Várias moléculas de pesos moleculares distintos (10 kDa até de alta massa molecular) podem ser detectados nesses antígenos totais. O CFA foi descrito por ser um bom preparado antigênico para ser utilizado em testes sorológicos para diagnóstico da PCM, como em imunodifusão (ID) e Ensaio imunoenzimático (ELISA) devido a sua sensibilidade e especificidade, todavia pode apresentar reatividade cruzada com outras micoses, devido a presença de epítopos em comum com *Paracoccidioides* sp.(BLOTA; CAMARGO 1993; CAMARGO et al., 1991; FREDRICH et al., 2010; MENDES-GIANNINI et al., 1994; PUCCIA et al., 1986).

Além dos antígenos totais, diversas glicoproteínas de *P. brasiliensis* com pesos moleculares diferentes já foram descritas como sendo importantes fatores de virulência e marcadores sorológicos, sendo que a glicoproteína de 43 kDa (gp43), glicoproteína de 70 kDa (gp70) e antígeno de alta massa molecular (hMM) são os principais antígenos reconhecidos por soros de pacientes com PCM (BLOTTA; CAMARGO, 1993; CAMARGO et al., 1991; CAMARGO et al., 1989; MARQUEZ et al., 2005; PUCCIA et al., 1986).

A gp43 é a principal glicoproteína produzida e liberada por *P. brasiliensis*, é um importante antígeno utilizado para o diagnóstico, sendo reconhecido por 100% dos soros de pacientes com PCM (CAMARGO et al., 1991; CAMARGO et al., 1989; PANUNTO-CASTELO et al., 2003; TABORDA; CAMARGO, 1994). A gp43 é um fator de virulência devido a sua capacidade de se ligar a laminina (VICENTINI et al., 1994), apresentar atividade proteolítica sobre o colágeno, caseína e elastina (MENDES-GIANNINI et al., 1990), bem como induzir apoptose em células epiteliais A549 *in vitro* (SILVA et al., 2015), dessa forma desempenhando um papel importante na invasão e no desenvolvimento de lesões no hospedeiro.

A gp70 é um marcador da PCM reconhecido por mais de 90% dos soros de pacientes com PCM, bem como um fator de virulência alterando a atividade de macrófagos, inibindo a fagocitose e liberação de NO e Peróxido de Hidrogênio (H_2O_2) (MATTOS GROSSO et al., 2003). Rigobello et al. (2013) mostraram que pacientes com PCM crônica apresentam maior reconhecimento de gp70 por IgE, sugerindo que essa glicoproteína poderia estimular uma resposta Th2.

Fredrich et al. (2010) demostraram que a fração de hMM está presente em vários isolados de *Paracoccidioides* sp. e pacientes com PCM crônica apresentaram IgG anti-hMM elevados, mas não IgE, sugerindo uma resposta Th1 (MARQUEZ et

al., 2005). Pavanelli et al. (2007), evidenciaram que a imunização de camundongos com fração de hMM em PCM experimental induz a produção IFN-γ, aumento de IgG específicos, formação de granulomas compactos e organizados, levando a uma proteção do animal. A resposta Th1 foi confirmada *in vitro*, onde hMM estimulou uma linfoproliferação e produção de IFN-γ em células esplênicas (PAVANELLI et al., 2007).

A proteína de 30 kDa de *P. brasiliensis* é uma adesina capaz de interagir com a matriz extracelular (MEC) pela a ligação com a laminina, participando da invasão de células do hospedeiro, e também tem capacidade de causar apoptose em células epiteliais A549 *in vitro*, sendo um importante fator de virulência do fungo (ANDREOTTI et al., 2005; SILVA et al., 2013; SILVA et al., 2015)

A nova espécie *P. lutzii* descrita por Teixeira et al. (2009) difere de *P. brasiliensis* justamente no gene da gp43 (TAKAYAMA et al. 2010). A gp43 não é o principal antígeno produzido por *P. lutzii*, podendo estar ausente em algumas cepas (BATISTA et al., 2010).

Oliveira et al. (2015) demonstraram uma maior virulência de *P. brasiliensis* em relação ao *P. lutzii*, todavia foram descritos casos onde a PCM causada por *P. lutzii* foi mais grave (HAHN et al., 2014; MARQUES-DA-SILVA et al., 2012), e possíveis impactos em manifestações clínicas devido a diversidade de agentes causadores da PCM tem sido discutido por Teixeira et al. (2014). Esses autores observaram frequente presença de acometimento linfático-abdominal em pacientes com PCM da região do estado de Goiás, onde a prevalência é de *P. lutzii*.

Por ser uma espécie diferente, e possivelmente apresentar diferenças na produção de antígenos, *P. lutzii* pode estimular diferentes respostas imunológicas no hospedeiro em relação ao *P. brasiliensis*. Lenhard-Vidal et al. (2013) evidenciaram diferenças no reconhecimento de CFA e hMM de *P. brasiliensis* e *P. lutzii* por IgG de pacientes com PCM crônica, e soros de pacientes com PCM causada por *P. brasiliensis* podem não reconhecer antígenos de *P. lutzii*, com isso sendo necessário a utilização de antígenos das duas espécies de *Paracoccidioides* sp. para o diagnóstico da PCM (GEGEMBAUER et al., 2014).

A fim de contribuir para o entendimento sobre semelhanças e diferenças entre as espécies e a resposta imune produzida, esse trabalho avaliou a reatividade de anticorpos IgG e IgE aos antígenos de *P. brasiliensis* e *P. lutzii* na PCM humana.

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3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar o padrão de reconhecimento/reatividade de anticorpos IgG e IgE de pacientes com PCM crônica aos antígenos de *P. brasiliensis* (S1 e PS2) e *P. lutzii.*

3.2 OBJETIVOS ESPECÍFICOS

Avaliar o reconhecimento de componentes imunogênicos presentes em antígenos totais (CFA e AS) de *P. brasiliensis* (S1 e PS2) e *P. lutzii* por anticorpos IgG e IgE, por *Immunoblotting*.

Avaliar a reatividade de anticorpos IgG e IgE aos antígenos CFA, gp70 e hMM de *P. brasiliensis* (S1 e PS2) e *P. lutzii* por ELISA.

4 ARTIGO: Different pattern of recognition of *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii* antigens by IgG and IgE in human Paracoccidioidomycosis

Assolini J. P.[1], Lenhard-Vidal A.[1] , Bredt C. S. O[2], Tano Z.N.[1], Sano A[3]., Itano E. N.[1]
[1]*State University of Londrina, Londrina, PR,* Brazil.
[2]*State University of Western Paraná, Cascavel, PR, Brazil*
[3]*Department of Animal Sciences, University of the Ryukyus, Okinawa, Japan*

4.1 Abstract

Purpose Paracoccidioidomycosis (PCM) is caused by the dimorphic fungus *Paracoccidioides brasiliensis* (S1, PS2, PS3) and *Paracoccidioides lutzii*. The severity of PCM caused by *P. brasiliensis* is associated with Th2 profile with increased levels of IgE anti-gp43, but this response remains unknown for the new species *P. lutzii*. Current research aimed to evaluate IgE and IgG pattern of recognition/reactivity to *P. lutzii* and *P. brasiliensis* antigens in human PCM.
Methods Immunoblotting evaluated soluble (CFA) and somatic (SA) antigens from *P. lutzii* (LDR2), *P. brasiliensis* S1 (B339) and PS2 (LDR3) recognized by IgE/IgG from chronic PCM patients' sera (n=24). ELISA assessed the reactivity of IgG/IgE to CFA and purified fractions (high molecular mass antigen = hMM; 70kDa = gp70).
Results IgE/IgG pattern of recognition in immunoblotting was different among the strains, showing the absence of gp43 in *P. lutzii* and the reaction with gp70 antigens in both CFA and SA from *P. lutzii*. IgE also recognized 30 kDa antigens in CFA or SA from *P. lutzii*, absent in others. By ELISA, there was higher IgE and lower IgG reactivity to hMM fractions from *P. lutzii* than *P. brasiliensis*, the opposite with CFA and higher IgG anti-gp70 with *P. lutzii* and *P. brasiliensis* PS2 than S1 ($p<0.05$).
Conclusions The pattern of recognition of antigens by IgG and IgE differs according to strain and possibly the hMM, 70kDa and 30kDa antigens from *P. lutzii* stimulates IgE response, suggesting the potential to induce Th2 profile.

Key-Words: antibodies, antigens, mycosis, immune response, immunodiagnosis, virulence factor.

4.2 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 [1-6]. PCM affects mainly rural workers, as the possible natural habitat of *Paracoccidioides* spp. is the soil [7].

Due to genetic variability among PCM isolates, in 2006 Matute et al. [1] used gene genealogies study to divide *P. brasiliensis* into three distinct clades: S1 (*species 1*; 38 isolates from Brazil, Argentina, Paraguay, Peru e Venezuela), PS2 (*phylogenetic species 2*, with 6 isolates from Brazil and Venezuela), and PS3 (*phylogenetic species 3*, with 21 Colombian isolates). Other atypical isolates [4, 8-10] that did not fit in any of the proposed groups were described, and thereafter Teixeira et al. [6], based in comparative genomics data, proposed grouping the atypical isolates (*Pb-01 like*) as the new species *Paracoccidioides lutzii.*

Until recently, the etiological agent of PCM was only known to be *P. brasiliensis* and most researches about PCM's immunodiagnosis were performed solely with antigens from *P. brasiliensis*. In 2013, Lenhard-Vidal et al. [11] detected a larger number of PCM patients from northern and west regions of Paraná (south of Brazil) presenting higher IgG levels to antigens from *P. brasiliensis* than *P. lutzii* (strain LDR2) and suggested that antigens from *P. brasiliensis* S1 may be more appropriate for serology in this region of Brazil. In 2014, Gegembauer et al. [12] investigated sera from the central-west and southeast of Brazil, where *P. lutzii* and *P. brasiliensis* are prevalent, respectively. The authors demonstrated higher antibody titers in sera from patients with PCM caused by *P. lutzii* than *P. brasiliensis* and that the sera from these patients were able to recognize antigenic molecules from *P. lutzii*-cell free antigens, while sera from patients with PCM caused by *P. brasiliensis* could not recognize *P. lutzii* molecules.

Since the International Colloquium on Paracoccidioidomycosis held in 1986 in Medellín, Colômbia, PCM is classified into acute (AF) and chronic (CF) forms. The AF is rarer and more severe, and is characterized by a rapid course of the disease and by reticuloendothelial system involvement, causing lymph node enlargement and hepatosplenomegaly. The CF occurs more frequently and the disease may be developed in multiple forms, ranging from benign and localized (unifocal) to severe

and disseminated (multifocal), depending on the cellular immunity. Those with the multifocal form may experience an evolution of the disease that can be compared in severity to the AF [7,13].

The possible impacts in clinical manifestations due to PCM diversity of *Paracoccidioides* spp. has been discussed by Teixeira et al. [14], considering the frequent presence of the lymphatic-abdominal form of PCM in patients from the Brazilian state of Goiás (*P. lutzii* prevalence area), besides reports of serious cases of PCM caused by *P. lutzii* [15,16]. Yet, *P. lutzii*'s virulence factors remain unknown.

The cellular Th1 response is considered protective in PCM caused by *P. brasiliensis*, while Th2 response is associated with disease progression with high levels of specific IgE [17-20]. The main antigens of *P. brasiliensis* used for the diagnosis of PCM, gp43 and gp70 [21-26], are described as possible stimulators of the Th2 response due to their high reactivity with serum IgE in PCM [27-30]. But in *P. lutzii* isolates, gp43 is not the main antigen produced and can also be absent [31]. In addition, there is no data about the antigens from *P. lutzii* that are recognized by IgE.

Considering the importance of the antigens that contribute to Th2 immune response in infection caused by intracellular microorganisms such as by *Paracoccidioides* spp., current research assessed the IgE and IgG pattern of recognition/reactivity to *P. lutzii* and *P. brasiliensis* antigens, using PCM patients' sera from northern and west regions of Paraná, south of Brazil. The results point to differences between the species of *Paracoccidioides*, concerning the release of immunogenic components with distinct molecular masses (>250 kDa, 70 kDa and 30 kDa) that are recognized by IgE in human PCM.

4.3 Materials and methods

4.3.1 Serum samples

The 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 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]). Ten samples of normal human serum (NHS) were used as

negative controls, all from the biological samples bank (Applied Immunology Laboratory, CCB, State University of Londrina, Londrina, PR, Brazil). Current study was approved by the Internal Scientific Commission and the Research Bioethics Committee of the State University of Londrina.

4.3.2 Fungal Strains

Three strains were used: *P. brasiliensis* species 1 (S1) (B339, IFM 41630) [1,10], *P. brasiliensis* phylogenetic species 2 (PS2) (LDR3, IFM 54649) [10] and *P. lutzii* (LDR2; IFM 54648) [10]. The fungi were grown on Sabouraud dextrose agar slants at 35 °C, with subcultures every 5 days.

4.3.3 Cell Free Antigen (CFA) and Somatic Antigen (SA)

Cell-free antigen (CFA) was obtained from the fungal mass according to Camargo et al. [32] modified by the addition of 2,5 mM PMSF. Somatic antigens (SA) was obtained according to the method described by Marquez et al. [33]. The supernatants were stored at -80 °C until use.

4.3.4 Immunoblotting

CFA and SA samples (5 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 ProteinTM Kaleidoscope cat. #161-0375, Bio-Rad Laboratories, Hercules, CA, USA*) and transferred to a nitrocellulose membrane (18 h at 4 ºC, 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, which is the most abundant immunoglobulin in the serum. The process reduces epitopes blocking, keeping accessible for the IgE that is present in lower concentration. Each serum sample was individually incubated with Sepharose G protein (*Sigma-P3296*; v/v, 2 h at 35 ºC 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 ºC. 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 ºC) and then with peroxidase-labeled antibody (*Sigma-A3415, Sigma Chemical Co., St. Louis, MO, USA*; 1/1000, 1.5 h, 35 ºC). 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 Biotecnologia, São Paulo, SP, Brazil*) and were expressed as percentages.

4.3.5 High molecular mass (hMM) and 70 kDa antigens fractions obtainment

The hMM fraction was obtained from the CFAs subjected to exclusion chromatography on a Sephacryl S200-HR column (*GE Healthcare Bio-Sciences AB, Uppsala, Sweden*) followed by SDS-PAGE elution, according to Lenhard-Vidal et al. [11], while the 70 kDa fraction (gp70) was obtained from SA, according to Rigobello et al. [30].

4.3.6 Serum IgG reactivity to CFA, hMM and gp70 by ELISA

Serum samples were analyzed in duplicate by indirect enzyme linked immunosorbent assay (iELISA) to evaluate the serum IgG reactivity to CFA, hMM and gp70. 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*) with hMM or gp70 (1 µg/mL), 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 ºC (*Sigma A6029, Sigma Chemical Co., St. Louis, MO, USA*), followed by the addition of o-phenylenediamine (OPD) substrate solution. The reaction was stopped

using 4N H_2SO_4 and the plates were read at 492 nm in Multiskan EX Reader (*Labsystems, Helsinki, Finland*).

4.3.7 Serum IgE reactivity to CFA, hMM and gp70 by ELISA

The following procedures were performed according to Rigobello et al. [30]. Each serum sample was previously adsorbed with Sepharose G protein as described in immunoblotting section. 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, hMM and gp70, 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 ºC), followed by anti-sheep IgG peroxidase-labeled antibody (*Sigma-A3415, Sigma Chemical Co., St. Louis, MO, USA*; 1/2000, 1.5 h, 35 ºC). OPD was added, the reaction was stopped with 4N H_2SO_4 and the plates were read at 492 nm.

4.3.8 Statistical analysis

T test, one-way ANOVA with Tukey's post-hoc test for multiple comparisons were calculated using the 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 Biotecnologia, São Paulo, SP, Brazil*) was employed for the analysis of the area percentage of the antigens recognized by IgE or IgG in immunoblotting.

4.4 Results

4.4.1 Immunoblotting

In immunoblotting, IgG and IgE showed different patterns of recognition of the immunogenic components from the different strains, either in CFA or SA (Fig. 1). The main difference was the absence of gp43 in *P. lutzii* and the detection of 70 kDa antigens in both CFA and SA from *P. lutzii*. IgE also recognized 30 kDa antigens in CFA or SA from *P. lutzii*, which were not recognized by other strains. The presence of the 70 and 30 kDa components in the CFA and SA recognized by IgE suggests

that both are not restricted as intracellular components and therefore they are also released in the soluble form. The components of hMM with heterogeneous electrophoresis migration were detected in CFA from *P. brasiliensis*, while in *P. lutzii* a compact band with >250 kDa was detected. Computer analysis of the corresponding areas of each band resulted in a greater area corresponding to the hMM antigens from *P. brasiliensis* S1 and PS2, but not from *P. lutzii* (Table 1). Our previous knowledge about high molecular mass antigens were only about *P. brasiliensis*, which presents diffused electrophoretic migration, therefore we consider as a high molecular mass fraction all the components with more thant 150 kDa, according to the LabImage analysis.

4.4.2 IgG and IgE anti-total soluble antigens

The serum reactivity of IgG and IgE to CFA are shown in Fig. 2. Comparing the strains, there was a significantly higher reactivity of IgG anti-CFA from *P. brasiliensis* S1 than PS2 ($p < 0.05$) and *P. lutzii* ($p < 0.01$) (Fig. 2a). There was a greater reactivity of IgE anti-CFA from *P. brasiliensis* S1 than PS2 ($p < 0.01$) and *P. lutzii* ($p < 0.001$), and there were also differences between *P. brasiliensis* PS2 and *P. lutzii* ($p < 0.05$) (Fig. 2b).

4.4.3 IgG and IgE to hMM fraction

The serum reactivity of IgG and IgE to hMM are shown in Fig. 3. Comparing the strains of *Paracoccidioides* spp., there was a significant increase in reactivity of IgG to hMM from *P. brasiliensis* S1 compared to *P. lutzii* ($p < 0.01$), but not with *P. brasiliensis* PS2 (Fig. 3a). For IgE, there was higher reactivity to hMM from *P. lutzii* than *P. brasiliensis* S1 ($p < 0.01$), but not with PS2 (Fig. 3b).

4.4.4 IgG and IgE anti-gp70

The serum reactivity of IgG and IgE to gp70 are shown in Fig. 4. Comparing the strains, there was a significantly higher reactivity of IgG anti-gp70 from *P. brasiliensis* PS2 and *P. lutzii* than S1 ($p < 0.05$) (Fig. 4a). Differently, there was no

significant difference between the serum reactivity of IgE anti-gp70 using all strains under study (Fig. 4b).

As expected, the NHS had lower levels of reactivity than PCM patients' sera in all iELISA ($p < 0.05$; data not shown).

4.5 Discussion

The new species *P. lutzii* is still a mystery, because its main antigens and virulence factors, important to understand its pathogenicity, remain unknown. However, some reports have shown the species' potential severity [15,16]. The severity of PCM caused by *P. brasiliensis* is associated with the development of Th2 response [21-26], and high levels of IgE to gp43 or gp70 has been described as stimulators of this profile [27-30]. In this research, the main *P. lutzii* soluble antigens (CFA) recognized by IgE in immunoblotting were hMM (>250kDa), 70 kDa and 30 kDa components. Also, the polydispersed high-MM components with heterogeneous electrophoresis migration, as evidenced by Puccia et al. [34] and Marquez et al. [33] were recognized by both IgG and IgE in CFA from *P. brasiliensis* (S1 and PS2). This diffuse band could be distinct from hMM antigens from *P. lutzii* (LDR2) due to its higher MM and distinct pattern of migration (not diffused). However, this pattern cannot be characteristic of all isolates of *P. lutzii*, as can be observed in western blotting results from Gegembauer et al. [12].

The gp43 is the major antigen and virulence factor from *P. brasiliensis*, responsible for the evasion from the host's immune response [35]. As expected, this antigen was recognized in immunoblotting by both IgG and IgE in CFA from *P. brasiliesis* S1 and PS2, mainly S1. However, it was not possible to evidence this antigen in CFA from *P. lutzii*. Currently, it is known that the expression of gp43 may vary according to the strain [36] and *P. lutzii* differs from *P. brasiliensis* precisely in the sequence of the gene responsible for gp43 [10]. In *P. lutzii* isolates, gp43 is not the main antigen produced or may even be absent in isolates from the Brazilian central-west region, as it was not identified in *P. lutzii* by nested PCR for gp43 [31,37]. Additionally, gp43 can be discarded as the main antigen in isolate of *P. lutzii* (LDR2).

The gp70 is one of *P. brasiliensis* antigens recognized by more than 90% of sera from patients with PCM [21,22,24,25], and is considered a virulence factor

capable of inhibiting phagocytosis and protecting the fungus against the action of NO and H_2O_2 [38]. Rigobello et al. [30] showed no recognition of gp70 by IgE in CFA and was almost not visible in SA from *P. brasiliensis* in immunoblotting using sera from chronic PCM patients, while in ELISA the gp70 from *P. brasiliensis* presents high reactivity with IgE, suggesting a Th2 response. The authors detected gp70 in greater amounts in SA than in CFA from *P. brasiliensis* and discussed about the possible low concentration of gp70 in CFA preparation, which is in accordance to Benard et al. [17] who evidenced the cytoplasmic localization of gp70 with specific antibodies. Interestingly, the current research demonstrates recognition of the component of 70 kDa from *P. lutzii* in SA or CFA, or even more strongly in CFA. Therefore, the 70 kDa components could be more released as a soluble form by *P. lutzii*. Additionaly, the 70 kDa bands recognized by IgE in both CFA and SA from *P. lutzii* were thinner than the ones generated by IgG, suggesting the possibility of more than one component with approximately 70 kDa released by *P. lutzii*, and that not all of them are recognized by IgE, which requires further studies.

Although a ~ 11 kDa band was detected only in CFA from PS2, the general immunoblotting profile was more similar between S1 and PS2 than from *P. lutzii*, corroborating the phylogenetic distances investigations [6]. As for SA, IgG recognized in common a band of ~30 kDa in both S1 and *P. lutzii*, which was even more evident by IgE in *P. lutzii*. This component could be the 30 kDa adhesin protein, a laminin ligand, more highly expressed in more virulent *P. brasiliensis* isolates [39] with subcellular localization [40]. However, it was recognized by IgE in both *P. lutzii*'s SA and CFA, suggesting that it could be more released in the soluble form by *P. lutzii* (as the 70 kDa antigens) or the ~30 kDa could be different from the *P. brasiliensis* antigen, which is less recognized by IgE.

In the current research, the patients were from the southern region of Brazil and therefore most likely had PCM caused by *P. brasiliensis*. According to Gegembauer et al. [12], a high number of components were recognized by PCM patients' sera with the CFA from *P. brasiliensis* than from *P. lutzii.* We cannot rule out the possible existence of PCM due to *P. lutzii* in the southern region, because the strain used in these assays (*P. lutzii* LDR2) was isolated from a man living in the southern region of Brazil [10]. Thus, it could be a mixture of both, but with prevalence of *P. brasiliensis,* that could explain the recognition of antigens from *P. lutzii*.

The reactivity of IgG and IgE to hMM and gp70 fractions has also been investigated by ELISA. The gp43 fractions were not investigated by this method because it was not possible to obtain gp43 from *P. lutzii*, as described above.

Lenhard-Vidal et al. [11] and Gegembauer et al. [12] showed differences in the recognition of sera from patients with PCM to *P. brasiliensis* and *P. lutzii* antigens. In accordance with these studies, current research observed higher reactivity of IgG and IgE to total soluble antigens (CFA) from *P. brasiliensis* than *P. lutzii* by ELISA, which may be due to a strong participation of gp43 in the *P. brasiliensis*' CFA, an antigen not detected in *P. lutzii* on this immunoblotting. Lenhard-Vidal et al. [11] also demonstrated that hMM from *P. brasiliensis* was more recognized than *P. lutzii* by IgG from chronic PCM patients' sera. Current research had similar results concerning IgG, and adds the demonstration of the reactivity of IgE in the opposite way, with higher reactivity to hMM from *P. lutzii* than *P. brasiliensis*. The apparent discrepancy between the results of immunoblotting and ELISA using hMM may be due to the adjusted concentration of hMM protein used in ELISA. In immunoblotting, the same concentration of CFA was employed, but their hMM antigens could be present in a higher proportion in the CFA from *P. brasiliensis* than from *P. lutzii,* as shown by the percentages from the computer analyses. The IgG reactivity anti-gp70 was higher for *P. lutzii* than *P. brasiliensis,* with no differences for IgE. The antigens could be present in greater proportion in CFA from *P. lutzii* than *P. brasiliensis* S1 and therefore more evidenced by IgG or IgE in immunoblotting. Additional possible differences between ELISA and immunoblotting can be due to denaturation of proteins in SDS-PAGE, changing the exposure of epitopes in immunoblotting.

The differences between the species may have impact on the diagnosis and also in efficacy of a vaccine against PCM. Gp43 is considered the main antigen for PCM diagnosis [26] and also a vaccine based on gp43 or gp43-derived P10 sequence has been shown to be effective in experimental models of PCM [41,42], but it may not be effective in cases of infection by *P. lutzii* which don't produce gp43. Pavanelli et al. [43] evidenced that immunization of mice with hMM fraction induces increased production of INF-γ and is protective in murine PCM, suggesting a Th1 response. The results of the current research are in agreement with Pavanelli et al. [43] concerning the hMM produced by *P. brasiliensis*, but the response induced by hMM from *P. lutzii* is possibly Th2. In the face of the diversity of PCM agents with

distinct characteristics, it becomes important to investigate a larger number of strains, mainly variety of *P. lutzii* isolates.

Taken together, the resulting data points to differences between the species of *Paracoccidioides* spp. concerning the proportion of production and the release of the immunogenic components with distinct molecular masses recognized by PCM sera. The major virulence factor of *P. lutzii* could be the hMM antigens (>250 kDa) due to its higher reactivity to IgE, and 70 or 30 kDa antigens produced and released in higher proportion and recognized by IgE, which indicates the induction of Th2 profile. Still, more researches are needed about *P. lutzii* antigens and their immune response, in order to understand the pathogenesis of PCM due to this fungus. In conclusion, the antigen recognition pattern by IgG and IgE differs according to the specie of *Paracoccidioides*.

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4.7 Figures and Tables

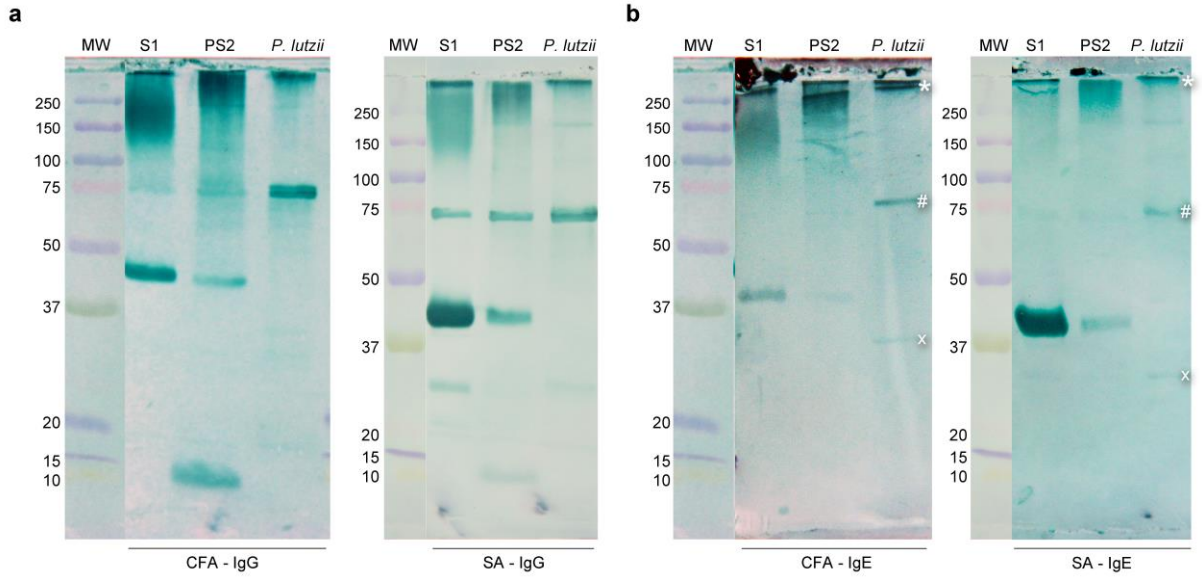

Fig.1 Immunoblotting of total soluble antigen (CFA) and somatic antigen (SA) from *P. brasiliensis* S1 (B339), PS2 (LDR3) or *P. lutzii* (LDR2), using IgG (a) and IgE (b) from a pool of PCM patients sera. CFA and SA samples (5 mg/mL) treated with mercaptoethanol sample buffer was applied to individual lanes of a 10% SDS-PAGE, along with a pre-stained molecular weight protein standard and transferred to a nitrocellulose membrane. For IgG, the membrane was incubated with purified IgG and for IgE, incubated with pool of serum samples previously adsorbed with Sepharose G protein from a pool of 24 PCM patients' sera. The reaction detection was performed by using anti-IgG or anti-IgE peroxidase and TMB. MW: molecular weight protein standard; *-hMM fraction; #-70 kDa; *x*-30 kDa.

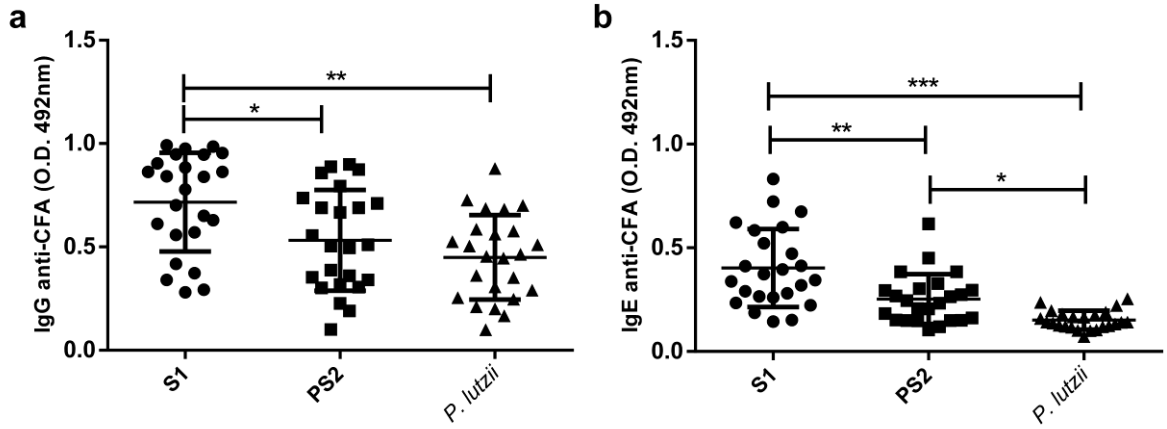

Fig.2 Reactivity of seric IgG (a) and IgE (b) from PCM patients to CFA from *P. brasiliensis* S1, PS2 and *P. lutzii*, by ELISA. Immunoplates coated with CFA (25µg/mL) from *P. brasiliensis* S1 (B339), *P. brasiliensis* PS2 (LDR3) and *P. lutzii* (LDR2), incubated with chronic PCM patients serum samples (n=24) diluted 1/400 (for IgG) and serum samples previously adsorbed with sepharose protein G at 1/20 final dilution (for IgE) were incubated, in duplicate. The reaction was evidenced with anti-human IgG or IgE peroxidase and OPD. The results are expressed as means in optical density (O.D.) at 492 nm. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p. \leq 0.001$.

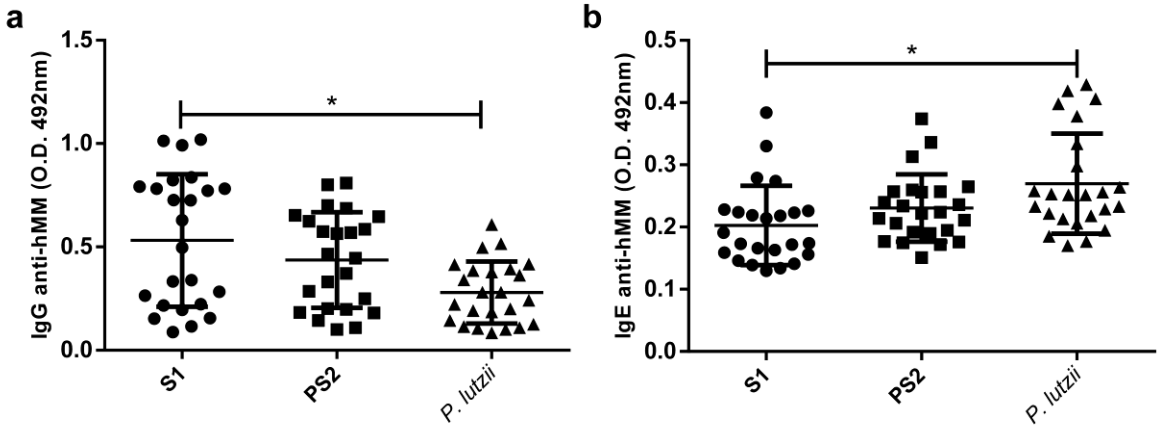

Fig.3 Reactivity of seric IgG (a) and IgE (b) from PCM patients to hMM from *P. brasiliensis* S1, PS2 and *P. lutzii*, by ELISA. Immunoplates coated with purified hMM (1µg/mL) from *P. brasiliensis* S1 (B339), *P. brasiliensis* PS2 (LDR3) and *P. lutzii* (LDR2), incubated with chronic PCM patients serum samples (n=24) diluted 1/400 (for IgG) and serum samples previously adsorbed with sepharose protein G at 1/20 final dilution (for IgE) were incubated, in duplicate. The reaction was evidenced with anti-human IgG or IgE peroxidase and OPD. The results are expressed as means in optical density (O.D.) at 492 nm. * $p \leq 0.05$.

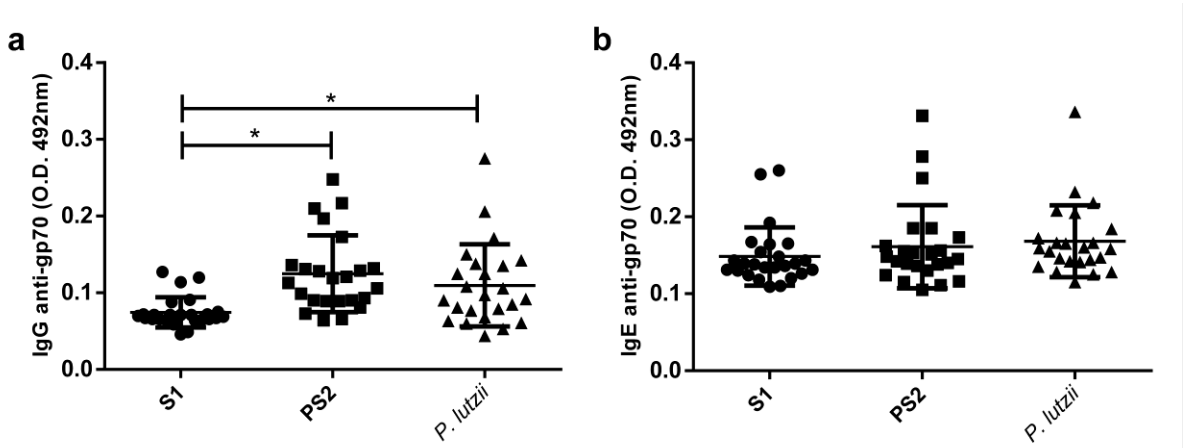

Fig.4 Reactivity of seric IgG (a) and IgE (b) from PCM patients to gp70 from *P. brasiliensis* S1, PS2 and *P. lutzii*, by ELISA. Immunoplates coated with purified 70kDa antigen (1µg/mL) from *P. brasiliensis* S1 (B339), *P. brasiliensis* PS2 (LDR3) and *P. lutzii* (LDR2), incubated with chronic PCM patients serum samples (n=24) diluted 1/400 (for IgG) and serum samples previously adsorbed with sepharose protein G at 1/20 final dilution (for IgE) were incubated, in duplicate. The reaction was evidenced with anti-human IgG or IgE peroxidase and OPD. The results are expressed as means in optical density (O.D.) at 492 nm. * $p \leq 0.05$.

Table 1 Computer analysis of the area percentages of the immunoblotting bands from CFA from *P. brasiliensis* S1, PS2 and *P. lutzii* recognized by a pool of IgG or IgE from patients with chronic PCM.

%	CFA – IgG			CFA – IgE		
Antigens	S1	PS2	*P. lutzii*	S1	PS2	*P. lutzii*
hMM	80.3	61.2	48	90.2	87.3	33.7
~70 kDa	5.9	13.2	52	-	-	39.3
~43 kDa	13.8	11.4	-	9.8	12.7	-
~30 kDa	-	-	-	-	-	27
~11 kDa	-	14.2	-	-	-	-

5 CONCLUSÃO

Com base nos resultados obtidos, conclui-se que o padrão de reconhecimento de antígenos por IgG e IgE difere de acordo com *Paracoccidioides* sp., onde observou diferenças na proporção de produção e liberação de componentes imunogênicos de massas moleculares (MM) distintas reconhecidos por soros de pacientes com PCM crônica.

Gp43 pode ser descartada como o principal antígeno de *P. lutzii* (LDR2), já que nesse isolado, ela não foi reconhecida por anticorpos IgG e IgE de pacientes com PCM.

Anticorpos IgG de pacientes com PCM crônica das regiões norte e oeste do Paraná, apresentam maior reatividade para CFA e hMM de *P. brasiliensis* do que *P. lutzii*, e para gp70 ocorre o contrário.

Anticorpos IgE de pacientes com PCM crônica das regiões norte e oeste do Paraná, apresentam maior reatividade para CFA de *P. brasiliensis* do que *P. lutzii*, e ocorre o contrário para o antígeno de hMM.

O antígeno de hMM (>250 kDa), devido à maior reatividade para IgE e antígenos de 70 ou 30 kDa produzidos e liberados em maior proporção e reconhecidos por IgE poderiam ser os principais fatores de virulência, como possíveis indutores de resposta Th2 em *P. lutzii* (LDR2).

No entanto, ainda são necessários mais estudos sobre os antígenos de *P. lutzii* e sua resposta imunológica, a fim de compreender a patogênese da PCM causado por este fungo.