ADRIANE LENHARD VIDAL

ANÁLISE DA REATIVIDADE DE IGG/IGG2 A EPÍTOPOS DE CARBOIDRATOS DE ESPÉCIES DE *PARACOCCIDIOIDES* E DETECÇÃO DE CASOS DE PARACOCCIDIOIDOMICOSE INFECÇÃO NA POPULAÇÃO DE GUARAPUAVA, PR

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

Orientadora: Profa. Dra. Eiko Nakagawa Itano

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Ficha de identificação da obra elaborada pelo autor, através do Programa de Geração Automática do Sistema de Bibliotecas da UEL

Lenhard-Vidal, Adriane.

Análise da reatividade de IgG/IgG2 a epítopos de carboidratos de espécies de *Paracoccidioides* e detecção de casos de Paracoccidioidomicose Infecção na população de Guarapuava, PR / Adriane Lenhard-Vidal. - Londrina, 2018.

66 f. : il.

Orientador: Eiko Nakagawa Itano.

Tese (Doutorado em Patologia Experimental) - Universidade Estadual de Londrina, Centro de Ciências Biológicas, Programa de Pós-Graduação em Patologia Experimental, 2018.

Inclui bibliografia.

1. Paracoccidioidomicose - Tese. 2. Resposta imune - Tese. 3. Antígenos de fungos - Tese. 4. Imunodiagnóstico - Tese. I. Nakagawa Itano, Eiko. II. Universidade Estadual de Londrina. Centro de Ciências Biológicas. Programa de Pós-Graduação em Patologia Experimental. III. Título.

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Londrina, 09 de fevereiro de 2018.

AGRADECIMENTOS

Devo muita gratidão à Profa. Dra. Eiko Nakagawa Itano, por todos estes anos de apoio, incentivo e valorização. Aceitou-me como sua orientada no mestrado mesmo sem me conhecer e foi uma das incentivadoras para que eu continuasse em um doutorado. Sou especialmente grata pela paciência e por ter aceitado me orientar mesmo sabendo que parte seria à distância. Obrigada por este voto de confiança.

Agradeço ao Programa de Pós-graduação em Patologia Experimental e ao seu corpo docente, por proporcionarem qualidade de ensino de altíssimo nível. Obrigada ao secretário do programa, Sérgio C. Sisti, pelo excelente serviço prestado.

Obrigada à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela bolsa de estudos concedida

Agradeço aos professores doutores que integram minha banca examinadora, Audrey de Souza Marquez, Berenice Tomoko Tatibana, Emerson José Venancio e Mario Augusto Ono, pois certamente contribuíram para o aperfeiçoamento deste trabalho.

Obrigada à toda a equipe do Laboratório de Imunologia Aplicada, em especial, Nilson de Jesus Carlo, Paula Leonello. Álvares e Silva, Flávia Raquel Bender, Janeth Escobar e Franciele Ayumi Semêncio Chyoda, pela ajuda sempre disponível e pela companhia.

O trabalho desenvolvido em Guarapuava não seria possível sem o apoio de diversas pessoas no município. Agradeço a Secretaria de Saúde Municipal, pela autorização das pesquisas; ao Ambulatório de Pneumologia e Dermatologia Sanitária (AMPDS; Elisa Katayama e Marlene Borecki), pela constante colaboração; a Faculdade Campo Real, pelo apoio em infraestrutura; aos acadêmicos de Biomedicina que foram meus orientados de trabalho de conclusão de curso, Ana Elisa Mayumi Maruo, Caroline Geovana Bensberg, Ellen Cristina Pereira e Luiz Tiago Vieira; e aos laboratórios de análises clínicas que abriram suas portas para a realização de coletas, Biolab (Dra. Débora Thomson Milazzo Foroni), CELAPE (Dr. Léo Aghpito Periolo) e Góes (Dr. Celso Fernando Góes).

Além disso, preciso agradecer aos que sempre me apoiaram de outras formas, seja de longe ou de perto. Ao meu querido marido, Luiz Henrique Ilkiu Vidal,

pela compreensão, companhia e apoio financeiro. Aos meus pais, Arnildo Jacó Lenhard e Elfi Lenhard, pelo incentivo e amor. Aos meus sogros, Luiz Cláudio Swain Vidal e Deise Maria Ilkiu Vidal, pela companhia e apoio na fase final de redação desta tese. A Flávia Raquel Bender, pelas cuias de chimarrão e risadas. Aos amigos e familiares que sempre abriram as portas de suas casas para me albergar em Londrina, em especial, Paula Leonello Álvares e Silva e Ricardo Inácio Álvares e Silva, tio Carlos Alberto Swain Vidal e tia Solange Krüger Vidal, Luciana Basso Schul e Alexandre Oliveira, Raquel Bergamasco e Pedro Sant'ana Jardim.

Gratidão ao universo, por ter me enviado somente aquilo que podia suportar e que me fez crescer como pessoa, pesquisadora e professora nestes 4 anos de Doutorado.

“A educação, em seu sentido pleno, começa depois da idade escolar, após a época da universidade, quando o homem se converte no sujeito de sua própria educação e sente motivo para continuar a se instruir e formar.”

Paul Lengrand

LENHARD-VIDAL, Adriane. **Análise da reatividade de IgG/IgG2 a epítopos de carboidratos de espécies de *Paracoccidioides* e detecção de casos de Paracoccidioidomicose Infecção na população de Guarapuava, PR.** 2018. 68 f.Tese (Doutorado em Patologia Experimental)-Universidade Estadual de Londrina, Londrina, 2018.

RESUMO

Paracoccidioidomicose (PCM) é uma micose sistêmica causada pelos fungos termodimórficos *Paracoccidioides brasiliensis* e *Paracoccidiodies lutzii*, sendo restrita a América Latina e endêmica no Brasil. Acredita-se que a infecção ocorra pela inalação de propágulos fúngicos quando o solo é revolvido. A PCM pode ser dividida em: (1) PCM infecção, sem sintomas, mas com reação positiva aos antígenos de *Paracoccidioides* spp., com prevalência muito variável entre regiões; e (2) PCM doença, classificada em (2a) PCM aguda, condição mais grave que ocorre em crianças e jovens ou (2b) PCM crônica, que ocorre em adultos e soma aproximadamente 90% dos casos. A presente pesquisa objetivou: (a) avaliar a reatividade de IgG/IgG2 de pacientes crônicos aos epítopos de carboidrato de *Paracoccidioides* spp.; (b) avaliar epidemiologicamente os casos de PCM infecção em Guarapuava, PR, Brasil. A investigação dos epítopos de carboidrato foi feita por immunoblot (IB) de *cell free antigens* (CFA) de *P. brasiliensis* B339 (anteriormente espécie S1), *Paracoccidioides americana* LDR3 (anteriormente *P. brasiliensis* PS2) e *P. lutzii* LDR2, tratados ou não com metaperiodato de sódio (SMP). IgG de coelho anti-*P. brasiliensis* Pb18 e IgG/IgG2 de um pool de soros de pacientes com PCM doença reconheceram principalmente antígenos de alta massa molecular (hMM, >150 kDa), gp70 e gp43, sendo esta última não detectada em *P. lutzii* LDR2. A reatividade aos antígenos diminuiu com o tratamento com SMP. Conclui-se que não foi possível detectar gp43 em *P. lutzii* LDR2 e, como *P. brasiliensis*, seus principais antígenos apresentam epítopos de carboidrato reconhecidos pelos soros de pacientes com PCM. No levantamento epidemiológico sobre PCM infecção, 359 soros foram analisados por ELISA para detecção de IgG contra CFA de *P. brasiliensis* B339, *P. americana* LDR3 e *P. lutzii* LDR2. Gp43 de B339 serviu de teste confirmatório. Estes antígenos foram também testados após tratamento com SMP para redução de reações cruzadas. Reatividade em ELISA: CFA/SMP-CFA em geral 37,3/17,8%, B339 25,3/14,5%, LDR3 24,5/1,4%, LDR2 8,3/5,8%; gp43/SMP-gp43 7,2/4,7%. Houve soros reagentes para mais de uma espécie. IB de 37 soros selecionados demonstrou diferentes padrões de reconhecimento de gp43, gp70 e hMM. Residência em zona rural e profissões relacionadas ao manejo do solo foram identificados como fatores de risco. *P. brasiliensis* parece ser a espécie prevalente na área, mas 21 soros de pessoas que declararam ter vivido apenas na região foram reagentes com antígenos de *P. lutzii* LDR2. IgG anti-gp70 e -hMM foram avaliados em amostras reagentes, mas com baixa reatividade: gp70/hMM em geral 6,4/6,7%. O uso de SMP ou antígenos purificados podem não ser adequados para levantamentos sobre PCM infecção na região avaliada, portanto, recomenda-se o uso de CFA-ELISA. Apesar de Guarapuava ser uma cidade de economia agrícola e estar localizada em região endêmica de PCM, a baixa prevalência para PCM infecção era esperada, considerando-se que a região teve relatos anteriores de menor endemicidade de PCM doença do que outras áreas do Paraná, mas as características gerais encontradas assemelham-se à outros trabalhos publicados.

Palavras-chave: Ensaio imunoenzimático. Epidemiologia. Immunoblot. *Paracoccidioides brasiliensis. Paracoccidioides lutzii.*

LENHARD-VIDAL, Adriane. **Analysis of IgG/IgG2 reactivity with carbohydrate epitopes from species of *Paracoccidioides* and detection of Paracoccidiodomycosis Infection in the population of Guarapuava, PR.** 2018. 68 p. Thesis (Doctorate in Experimental Pathology). Universidade Estadual de Londrina, Londrina, 2018.

ABSTRACT

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by the thermodimorphic fungi *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii*, restricted to Latin America and is endemic in Brazil. It is believed that the infection occurs by inhalation of fungal propagules when the soil is plowed. PCM can be divided into: (1) PCM infection, with no symptoms but with positive reaction to *Paracoccidioides* spp. antigens, of variable prevalence, according to the evaluated region; (2) PCM disease, which is classified as (2a) acute PCM, a more serious condition that occurs in children and young people or (2b) chronic PCM, which occurs in adults and sums almost 90% of cases. Current research aimed to: (a) evaluate the reactivity IgG/IgG2 of chronic PCM patients to carbohydrate epitopes of *Paracoccidioides* spp.; (b) epidemiologically evaluate the cases of PCM infection in Guarapuava, Paraná, Brazil. The investigation of carbohydrate epitopes was performed using immunoblot (IB) of cell free antigens (CFA) from *P. brasiliensis* B339 (former species S1), *Paracoccidioides americana* LDR3 (former *P. brasiliensis* PS2) and *P. lutzii* LDR2, treated or not with sodium metaperiodate (SMP). Rabbit IgG anti-*P. brasiliensis* Pb18 and IgG/IgG2 from a pool of PCM patients' sera recognized mainly high molecular mass antigens (hMM, >150 kDa), gp70 and gp43, but the last was not detected in *P. lutzii*. Antigens reactivities decreased after SMP treatment. In conclusion, gp43 was not detected in *P. lutzii* LDR2 and, as *P. brasiliensis,* its major antigens present carbohydrate epitopes recognized by PCM patients' sera. In the epidemiological survey about PCM infection, 359 sera were analyzed by ELISA to detect IgG against CFA from *P. brasiliensis* B339, *P. americana* LDR3 and *P. lutzii* LDR2. Gp43 from B339 was used as a confirmatory test. These antigens were also tested after SMP treatment in order to reduce cross-reactions. ELISA reactivity: CFA/SMP-CFA in general 37.3/17.8%, B339 25.3/14.5%, LDR3 24.5/1.4%, LDR2 8.3/5.8%; gp43/SMP-gp43 7.2/4.7%. There were sera reactive with multiple species. IB of 37 selected-sera showed different patterns of recognition of gp43, gp70 and hMM. Residence in rural areas and professions related to soil handling were identified as risk factors. *P. brasiliensis* seems the prevalent species of the region, but 21 sera from people who declared to have only lived in Guarapuava reacted with *P. lutzii* LDR2. IgG anti-gp70 and -hMM were also investigated in reactive samples, but with low reactivity: gp70/hMM in general 6.4/6.7%. Use of SMP or purified antigens might not be suited for surveys about PCM infection in the evaluated region. Therefore, CFA-ELISA seems a better alternative. Although Guarapuava is a city of agricultural economy and is located in an endemic region for PCM, the low prevalence of PCM infection was expected, considering that the region had previous reports of lower endemicity of PCM disease than other areas of Paraná, but the general characteristics found are similar to other published works.

Keywords: Epidemiology. Immunoenzymatic assay. Immunoblot. *Paracoccidioides brasiliensis. Paracoccidioides lutzii.*

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ARTIGO B

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

µg	Microgramas
µL	Microlitros
AF	*Acute Form Paracoccidioidomycosis* (Paracoccidioidomicose aguda)
AMPDS	Ambulatório Municipal de Pneumologia e Dermatologia Sanitária de Guarapuava, PR (*Municipal Ambulatory of Pneumology and Sanitary Dermatology*)
Anti-CFA	Anticorpo contra a antígeno livre de célula (CFA)
Anti-gp43	Anticorpo contra a glicoproteína de 43 kDa
Anti-gp70	Anticorpo contra a glicoproteína de 70 kDa
Anti-hMM	Anticorpo contra os antígenos de alta massa molecular
AS	Antígenos Somáticos
CF	*Chronic Form Paracoccidioidomycosis* (Paracoccidioidomicose crônica)
CFA	*Cell Free Antigen* (Antígeno livre de células)
CFA-ELISA	ELISA utilizando CFA como antígeno
COU	Clínica Odontológica Universitária
DTH	*Delayed-type Hypersensitivity* (Hipersensibildiade do tipo tardia)
ELISA	*Enzyme-linked immunosorbent assay* (Ensaio imunoenzimático)
ExoAg	Exoantígeno de *Paracoccidioides* sp. (*Exoantigen*)
Fc	*Fragment, crystallizable* (Fragmento cristalizável)
gp43	Glicoproteína de 43 kDa
gp43-ELISA	ELISA utilizando gp43 como antígeno
gp70	Glicoproteína de 70 kDa
h	horas
H_2SO_4	Ácido sulfúrico
HC	Hospital das Clínicas (Ambulatório de Especialidades do Hospital Universitário)
hMM	*High Molecular Mass antigens* (Antígenos de alta massa molecular)
HU	Hospital Universitário
HURNP	Hospital Universitário da Região Norte do Paraná
IB	*Immunoblot*
IFM	*Institute of Food Microbiology,* da Universidade de Chiba (Japão). Antigo nome do *Medical Mycology Research Center*, utilizado como a abreviação oficial da coleção de cultura mundial de fungos patogênicos e actinomicetos.
IgE	Imunoglobulina E

IgG	Imunoglobulina G
IgG2	Imunoglobulina G subclasse 2
kDa	Quilodaltons
Ln	Logaritmo natural / neperiano
mg	miligramas
min	minutos
mL	Mililitros
mm	Milímetros
mM	Milimolar
MS	Ministério da Saúde do Brasil
MW	*Molecular weight* (peso molecular)
N	Normal
NHS	*Normal Human Serum* (soro humano normal)
nm	Nanômetros
NR	Grupo de participantes considerado não reagente
O.D.	*Optical density* (densidade ótica)
ºC	Graus Celsius
OPD	O*rtho-Phenylenediamine* (Orto-fenilenediamina)
PAS	Ácido periódico-Schiff
PBS	*Phosphate Buffered Saline* (Tampão salina fosfato)
PCM	Paracoccidioidomicose (*Paracoccidioidomycosis*)
PMSF	*Phenylmethylsulfonyl fluoride* (Fluoreto de fenilmetilsulfonil)
PS2	*Phylogenetic Species 2* (Espécie filogenética 2 do *P. brasiliensis*)
PS3	*Phylogenetic Species 3* (Espécie filogenética 3 do *P. brasiliensis*)
PS4	*Phylogenetic Species 4* (Espécie filogenética 4 do *P. brasiliensis*)
R	Grupo de participantes considerado reagente
S1	*Species 1* (Espécie 1 do *P. brasiliensis*, dividida em S1a e S1b)
SA	*Somatic antigens* (antígenos somáticos)
SD	*Standard Deviation* (Desvio padrão)
SDS-PAGE	*Polyacrylamide Gel Electrophoresis with Sodium Dodecyl Sulfate* (Eletroforese em gel de poliacrilamida com SDS)
SHN	Soro Humano Normal
SMP	*Sodium metaperiodate* (Metaperiodato de sódio)
SMP-CFA	ELISA utilizando CFA tratado com metaperiodato de sódio como antígeno
SMP-gp43	ELISA utilizando gp43 tratado com metaperiodato de sódio como antígeno
Th1	Linfócito T “helper” (auxiliar) 1

Th2	Linfócito T “helper” (auxiliar) 2
TMB	3’3’5’5-tetrametilbenzidina (*3,3,5,5-tetramethylbenzidine*)
UEL	Universidade Estadual de Londrina
UNIOESTE	Universidade Estadual do Oeste do Paraná
v/v	volume/volume
x *g*	força G (RCF, Força Centrífuga Relativa)

SUMÁRIO

1 INTRODUÇÃO

A paracoccidioidomicose (PCM) é uma micose sistêmica causada pelos fungos *Paracoccidioides brasiliensis* e *P. lutzii*, que agrupa as cepas anteriormente consideradas atípicas (TEIXEIRA et al., 2009). A PCM está distribuída ao longo da América Latina, com endemicidade muito variável, com maior prevalência de casos registrados no Brasil, seguido por Colômbia, Venezuela, Argentina e Equador (MARTINEZ, 2017). Poucos estados brasileiros adotaram a notificação compulsória da PCM, não sendo possível determinar a sua real frequência. Estima-se que no Brasil haja de 1 a 3 casos a cada 100.000 habitantes (COUTINHO et al., 2002; RESTREPO, TOBÓN; CANO, 2015), mas existem relatos de cidade, como Rondônia, com até 39 casos/100.000 habitantes (VIEIRA et al., 2014).

Paracoccidioides spp. são fungos termodimórficos. Em temperatura ambiente (25 °C) apresentam-se na forma de micélio, com crescimento lento em ágar Sabouraud dextrose (20-30 dias) na forma de colônias brancas. Ao exame microscópico, visualizam-se micélios septados finos, com esporos terminais ou intercalares. *In vivo* ou quando cultivados à 35-37 °C, transformam-se em leveduras com múltiplos brotamentos característicos como “roda de leme” e aspecto macroscópico cerebriforme (LACAZ et al., 2002). A diferenciação morfológica entre as espécies de *Paracoccidioides* ainda não é possível, mas há indícios de que os conídios de *P. lutzii* são geralmente mais alongados ou com formato de bastão, permitindo diferenciar ao menos esta espécie (TEIXEIRA et al., 2014; THEODORO et al., 2012; TURISSINI et al., 2017).

Apesar do ciclo biológico do *Paracoccidioides* spp. ainda não estar totalmente esclarecido, acredita-se que o solo seja seu habitat natural. Assim, ocorreria a contaminação do ser humano através da inalação de propágulos do fungo quando o solo é revolvido. No organismo do hospedeiro, ocorre a transformação para a forma de levedura e a disseminação por via linfática/hematogênica, quando não contidos pelo sistema imune (BAGAGLI et al., 2008). Estas leveduras poderão formar granulomas nos pulmões ou linfonodos que permanecerão latentes por toda a vida ou que evoluirão para PCM doença. Devido à disseminação pelos vasos, qualquer órgão pode ser infectado, o que acarreta numa grande variedade de sintomas (DA SILVA et al., 2016).

A Paracoccidioidomicose pode ser dividida em PCM infecção, na qual não

há sintomas de doença, mas há resposta imunológica positiva a antígenos de *Paracoccidioides* spp., ou PCM doença, que pode ser dividida em duas formas. A forma aguda ou juvenil soma de 5 a 25% dos casos e acomete indivíduos de até 30 anos, de forma homogênea entre os sexos e apresenta maior gravidade, com desenvolvimento de linfoadenomegalia, hepatoesplenomegalia, lesões cutâneas e em mucosas, envolvimento osteoarticular, com presença de febre, perda de peso e anorexia. A grande maioria dos casos de PCM (70 a 94%) é da forma crônica ou adulta, com preponderância de homens entre 30 e 60 anos, geralmente com comprometimento pulmonar, seguido de lesões de pele e mucosas (SHIKANAI-YASUDA et al., 2017).

O contágio costuma acontecer nas primeiras décadas de vida, mas o desenvolvimento da doença pode ocorrer muitos anos depois. Fatores ambientais, a virulência do fungo e a resposta imunológica do hospedeiro influenciam a forma da doença e a intensidade dos sintomas. Moradia em zona rural ou trabalhos ligados ao manejo do solo, como agricultura, terraplanagem, jardinagem, transporte de vegetais e construção civil, têm sido relatados como fatores de risco para o desenvolvimento de PCM (WANKE; LONDERO, 1994). A prevalência de PCM crônica é maior em homens do que em mulheres, em uma razão de até 22/1 (SHIKANAI-YASUDA et al., 2017). Os estrogênios conferem proteção contra a transformação de micélio para levedura durante a infecção (RESTREPO et al., 1984; SALAZAR et al., 1988; SHANKAR et al., 2011). A PCM também tem sido associada com baixas condições socioeconômicas, etilismo e tabagismo (SHIKANAI-YASUDA et al., 2017).

O diagnóstico laboratorial da PCM preconizado pelo Ministério da Saúde do Brasil (MS) é a visualização do fungo no material biológico, seja por microscopia direta, cultivo ou análise histopatológica (BRASIL, 2009). Entretanto, há dificuldade em se obter amostras (escarro, raspado de lesão, aspirado, etc.) ou mesmo em colher material do órgão afetado, como na PCM neurológica (DA SILVA et al., 2005; MARQUES DA SILVA et al., 2003). Existe a necessidade de ter pessoal treinado na execução da técnica, especialmente para a identificação das células com múltiplos brotamentos características de *Paracoccidioides* spp., além da dificuldade em isolar o fungo a partir do material biológico. Por isso, alternativas como os métodos imunológicos indiretos tem sido investigados, objetivando-se obter resultados mais rápidos em relação ao emprego de outras técnicas.

A imunodifusão é a técnica indicada pelo MS como auxiliar no diagnóstico

e acompanhamento do paciente com PCM, devido à facilidade de execução e baixo custo (BRASIL, 2009). Sua menor sensibilidade em relação aos outros métodos fez com que fossem estudados e aplicados o ensaio imunoenzimático (ELISA) ou o immunoblot (CAMARGO, 2008).

Várias técnicas para obtenção de antígenos de *Paracoccidioides* spp. tem sido utilizadas, destacando-se o exoantígeno (ExoAg), composto por antígenos liberados pelas células em meio líquido (CAMARGO et al., 1988), o *cell free antigen* (CFA), compostos por antígenos frouxamente ligados à superfície celular que se desprendem em meio aquoso (CAMARGO et al., 1991) e o antígeno somático (AS), obtido por rompimento das células fúngicas (BURGOS et al., 1985).

Os antígenos de *Paracoccidioides brasiliensis* mais pesquisados são os de 43 e 70 kDa, conhecidos como gp43 e gp70, respectivamente (CAMARGO, 2008). Estes já foram considerados marcadores de PCM doença por haverem relatos de detecção por quase 100% dos soros de pacientes avaliados em algumas pesquisas e por seus títulos diminuírem durante o tratamento com antifúngicos (BLOTTA; CAMARGO, 1993; CAMARGO; UNTERKIRCHER; TRAVASSOS, 1989; DA SILVA et al., 2004).

O antígeno imunodominante de *P. brasiliensis* é a gp43, presente na parede celular com função de adesina, capaz de ligar-se à laminina e fibronectina (DE OLIVEIRA et al., 2015). Sua expressão varia de acordo com a cepa, podendo deixar de ser produzida e posteriormente restaurada (BERZAGHI; DA SILVA; CAMARGO, 2005). Uma das principais diferenças genéticas encontradas entre *P. brasiliensis* e *P. lutzii* aparentemente está na sequência que codifica a gp43. Takayama et al. (2010) encontraram 89% de identidade para este gente entre as cepas *P. brasiliensis* Pb18 e *P. lutzii* LDR2. Além disso, a gp43 não é o principal antígeno produzido pelo *P. lutzii* e inclusive pode estar ausente em algumas cepas (BATISTA et al., 2010).

A gp70 de *P. brasiliensis* tem localização principalmente intracelular e pode ser um mecanismo de escape, pois inibe a liberação de óxido nítrico e peróxido de hidrogênio, e a fagocitose por receptores Fc e de manose (DE MATTOS GROSSO et al., 2003).

Os antígenos da região de alta massa molecular (≥ 150 kDa) ou *high molecular mass antigens* (hMM) apresentam um padrão de migração eletroforética difusa (PUCCIA et al., 1986) e compõe de 16 a 20% do CFA (FREDRICH et al.,

2010). Níveis mais elevados de IgG anti-hMM já foram identificados em pacientes com PCM crônica, mas não de IgE, sugerindo uma resposta Th1 e podendo servir como diferencial entre o quadro agudo e crônico (MARQUEZ et al., 2005). A imunização com hMM antes da infecção de camundongos com *P. brasiliensis* demonstrou um papel protetor dos anticorpos anti-hMM, servindo os níveis de IgG como um indicador prognóstico (PAVANELLI et al., 2007).

O balanço entre a resposta imunológica Th1/Th2 é importante no desenvolvimento da PCM, sendo o perfil celular (Th1) indicativo de resposta favorável a PCM, enquanto os altos níveis de anticorpos, característicos do perfil Th2, são correlacionados com quadros mais graves da doença (KASHINO et al., 2000; SINGER-VERMES et al., 1993). De forma contrária, a imunização com gp70 e hMM impediram ou amenizaram o desenvolvimento da doença em camundongos (DE MATTOS GROSSO et al., 2003; PAVANELLI et al., 2007), sugerindo-se que a resposta imune humoral também tenha algum papel protetor na PCM.

Acredita-se que 50% da população das áreas endêmicas entra em contato com o fungo, mas um número desconhecido desenvolve PCM infecção (reação sorológica/celular positiva na ausência de sintomas), sendo que poucas pessoas evoluem para PCM doença (SHIKANAI-YASUDA et al., 2006). A maioria dos levantamentos epidemiológicos utilizaram testes intradérmicos com paracoccidioidina ou gp43, com variação de positividade de 2 a 82% no Brasil (FAVA; FAVA NETTO, 1998; MARTINEZ, 2015). Soros de pacientes com outras micoses, como histoplasmose, aspergilose e lacaziose (doença de Jorge Lobo) podem apresentar reação cruzada (falso positivo) com antígenos de *Paracoccidioides* spp., como na contraimunoeletroforese, ELISA e immunoblot (CAMARGO, 2008), o que pode aumentar o número de pessoas consideradas positivas nestes inquéritos epidemiológicos.

A PCM já foi considerada a 8ª causa de morte por doença infecciosa ou parasitária predominantemente crônica ou recorrente no Brasil, com a maior taxa de mortalidade entre as micoses sistêmicas. Ela pode também ser considerada um problema de saúde pública no Brasil, pois costuma atingir a população em idade produtiva e pode deixar sequelas incapacitantes após a cura (COUTINHO et al., 2002).

Desde o começo da classificação das cepas de *Paracoccidioides* spp. através da origem geográfica (CALCAGNO et al., 1998), muito tem sido investigado

na tentativa de se identificar as semelhanças e diferenças das suas espécies. Atualmente acredita-se que o *P. brasiliensis* esteja distribuído em ao menos 5 clusters filogenéticos diferentes, S1a, S1b, PS2, PS3 e PS4 (MUÑOZ et al., 2016). Recentemente, Turissini et al. (2017) propuseram descrever estes *clusters* como novas espécies taxonômicas, uma vez que são muito diferentes considerando-se loci nucleares, mas com pouco fluxo genético mitocondrial. Sugeriu-se usar *P. brasiliensis* para S1, *P. americana* para PS2, *P. restrepiensis* para PS3 e *P. venezuelensis* para PS4.

Já o *P. lutzii* agrupa cepas consideradas atípicas quando comparadas ao *P. brasiliensis* e, até o momento, acredita-se que cause PCM principalmente nas regiões centro-oeste e norte do Brasil (THEODORO et al., 2012). O fungo *P. lutzii* já foi isolado no Paraná (TAKAYAMA et al., 2010) e estudo recente amplificou o DNA das duas espécies a partir de amostras de solo e aerossóis em regiões consideradas endêmicas para apenas uma das espécies (ARANTES et al., 2016), demonstrando possível sobreposição das espécies em uma mesma região. Sendo assim, torna-se importante ampliar as pesquisas para identificação da endemicidade de PCM em diferentes regiões, com estudos sobre o perfil imunológico, no intuito de identificar antígenos mais eficazes para o uso no imunodiagnóstico da PCM.

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3 OBJETIVOS

Objetivo geral

Estudar a reatividade de anticorpos da classe IgG e da subclasse IgG2 contra antígenos de diferentes espécies de *Paracoccidioides* em soro imune de coelho e em amostras de soro provenientes do estado do Paraná, de PCM doença e de PCM infecção.

Objetivos específicos

1. Avaliar os antígenos de *P. brasiliensis* B339 (S1), *P. americana* LDR3 (anteriormente *P. brasiliensis* PS2) e *P. lutzii* LDR2 por meio da reatividade aos epítopos de carboidratos de CFA por immunoblot e ELISA;

2. Pesquisar a presença de PCM infecção na população de Guarapuava, PR, utilizando antígenos de *P. brasiliensis* B339 (S1), *P. americana* LDR3 (PS2) e *P. lutzii* LDR2, por meio de:
   2.1. Obtenção de CFA e AS;
   2.2. Obtenção de antígenos específicos: gp70 e hMM;
   2.3. ELISA para IgG anti-CFA e CFA tratado com metaperiodato de sódio;
   2.4. ELISA para IgG anti-gp43 e gp43 tratada com metaperiodato de sódio;
   2.5. ELISA para detecção de IgG anti-gp70;
   2.6. ELISA para detecção de IgG anti-hMM;
   2.7. Avaliação do percentual de reatividade para cada espécie e comparação das intensidades das reações;
   2.8. Immunoblot para detecção de IgG anti-CFA;
   2.9. Avaliação do perfil dos participantes considerados reagentes.

4 ARTIGO A – ANÁLISE DA REATIVIDADE DE IgG/IgG2 A EPÍTOPOS DE CARBOIDRATO DE DIFERENTES ESPÉCIES DE *Paracoccidioides*.

Soluble antigens from *Paracoccidioides lutzii* strain LDR2 recognized by polyclonal antibodies to *P. brasiliensis*

ABSTRACT

After the split of *Paracoccidioides* into the new species *P. lutzii* in 2009, much has been researched on these strains previously considered atypical. One striking characteristic is its difference in the gp43 gene, which seems to influence its expression and recognition by sera from people with Paracoccidioidomycosis (PCM) caused by other species of *Paracoccidioides*, but still there is not a consensus on how far these phenotypic differences can affect PCM diagnosis. Current research investigated the recognition by polyclonal antibodies to *Paracoccidioides brasiliensis* of soluble protein/carbohydrate epitopes from *P. lutzii* LDR2, *P. brasiliensis* B339 (former S1) and *P. americana* LDR3 (former PS2). Cell free antigens (CFA) from these fungi were analyzed by silver and periodic acid-Schiff staining of SDS-PAGE and by immunoblot (IB) with rabbit IgG anti-*P. brasiliensis* Pb18 and a pool of PCM patients' sera. Additionally, CFAs were analyzed by IB and ELISA with the pool of PCM patients' sera after treatment with sodium metaperiodate (SMP) to oxidize carbohydrate epitopes. Antibodies from both sources recognized antigens from *P. lutzii* LDR2, mainly 70 kDa and > 150 kDa, while gp43 was not observed in IB or silver/PAS staining. In general, human IgG recognized SMP treated antigens less than untreated ones and this decrease was more evident with antigens from *P. lutzii* LDR2 in ELISA. IgG2 recognized mainly components of 70 and ≈200 to >250 kDa in *P. lutzii*, which decreased after SMP. In conclusion, polyclonal antibodies to *P. brasiliensis* Pb18 recognized antigens from *P. lutzii* LDR2, which did not express gp43 and, as *P. brasiliensis,* its major antigens present carbohydrate epitopes recognized by PCM patients' sera.

Keywords: Hyperimmune rabbit serum; Immunoblot; ELISA; Paracoccidioidomycosis.

Introduction

Paracoccidioidomycosis (PCM) is a systemic mycosis for long known to be caused by the thermodimorphic fungus *Paracoccidioides brasiliensis*, but recently atypical isolates were grouped into the new species *Paracoccidioides lutzii* (TEIXEIRA et al., 2009) and much has been published in order to establish its common and different characteristics with *P. brasiliensis*. Molecular evidence points out to larger differentiation within *P. brasiliensis*. This species is currently clustered into five distinct lineages: S1a and S1b (Brazil, Argentina), PS2 (Brazil, Venezuela), PS3 (Colombia) and PS4 (Venezuela) (MATUTE et al., 2006; MUÑOZ et al., 2016). Currently, evidence of low mitochondrial gene flow along with deep differences at nuclear loci point to additional division of *P. brasiliensis* into new species: *P. brasiliensis* only for S1, *P. americana* for PS2, *P. restrepiensis* for PS3 and *P. venezuelensis* for PS4 (TURISSINI et al., 2017).

PCM is still basically clinically diagnosed. The gold standard for laboratory diagnosis is the direct identification of fresh sputum or other clinical samples (SHIKANAI-YASUDA et al., 2017), but this demands experienced trained personnel. The use of serodiagnosis is a good alternative to confirm PCM diagnosis, especially when obtaining samples is hard, as in infection of deep and noble organs.

There are many methods to obtain proteins from *Paracoccidioides* spp. for serodiagnosis, as cell free antigens (CFA) which are loosely bound to cell surface (CAMARGO et al., 1991), exoantigens from liquid cultures supernatants (CAMARGO et al., 1988) and somatic antigens (SA) obtained by direct rupture of fungal cells (BURGOS et al., 1985). Many proteins of different molecular weights were already described for *P. brasiliensis*: 20-21, 43, 52, 55, 70 kDa and polydispersed high molecular mass antigens (hMM), of 278 to 466 kDa, but the main antigens used in serodiagnosis are gp43, gp70 and hMM (BLOTTA; CAMARGO, 1993; CAMARGO et al., 1991; CAMARGO; UNTERKIRCHER; TRAVASSOS, 1989; MARQUEZ et al., 2005; PUCCIA et al., 1986).

Batista Junior et al. (2010) demonstrated that exoantigens from *P. brasiliensis* 550B isolated from Mato Grosso state, located in the central-west region of Brazil, an area where *P. lutzii* prevails (TEIXEIRA et al., 2009), had low reactivity in immunodiffusion with sera from patients with PCM from southeast of the country. Meanwhile, Lenhard-Vidal et al. (2013) observed similar reactivity between *P.*

brasiliensis B339, *P. americana* LDR3 (*P. brasiliensis* PS2 at the time) and *P. lutzii* LDR2 in immunodiffusion with sera from Paraná, south of Brazil, a region of *P. brasiliensis* S1, according to Theodoro et al., (2012).

Del Negro et al. (1995) and Neves et al. (2003) have described cases of negative immunodiffusion in patients with confirmed PCM diagnosis, due to antibodies to carbohydrate epitopes, especially IgG2. Therefore, current research aimed at investigating protein/carbohydrate epitopes in soluble antigens from *P. lutzii* LDR2, *P. brasiliensis* B339 and *P. americana* LDR3 by immunoblot (IB) and immunoenzymatic assay (ELISA).

Materials and Methods

Antigens and antibodies

Fungi were maintained in yeast form on Sabouraud dextrose agar at 35 °C, subcultured every 5–7 days.

CFA was obtained as previously described (CAMARGO et al., 1991), modified by addition of 2.5 mM phenylmethylsulfonyl fluoride (PMSF, protease inhibitor). Protein content was determined in NanoDrop Lite UV-Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and aliquots were stored at -80 °C until use.

CFA for rabbit immunization was obtained from *P. brasiliensis* (S1) strain Pb18 (IFM 41621). CFAs for the use in IB and ELISA were prepared from *P. brasiliensis* strain B339 (former species S1; IFM 41630), *P. americana* strain LDR3 (former phylogenetic species PS2; IFM 54649) and *P. lutzii* strain LDR2 (IFM 54648) (TAKAYAMA et al., 2010).

First subcutaneous injection of the rabbit with CFA from *P. brasiliensis* Pb18 (200 µg) was performed with complete Freund's adjuvant (v/v), while second and third employed incomplete Freund's adjuvant, every two weeks. After bleeding, IgG was obtained using Protein G Sepharose® (P3296, Sigma Chemical Co., St. Louis, MO, USA) and IgG concentration was determined in NanoDrop Lite UV-Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

A pool of sera was obtained from samples of 30 men and 9 women, with chronic unifocal or multifocal PCM, not-yet treated or currently under treatment. These patients had been treated in different institutions from Paraná state, Brazil: University Hospital, Outpatients Clinic Center and University Dentistry Clinic from Londrina (HURNP / HC / COU – UEL); University Hospital of Western Paraná from Cascavel (HU/UNIOESTE); Municipal Laboratory from Foz do Iguaçu; Municipal Ambulatory of Pulmonology and Sanitary Dermatology from Guarapuava (AMPDS).

This research was approved by the Ethics Committees on Research Involving Human Beings and on the Use of Animals, from State University of Londrina, Paraná, Brazil.

SDS-PAGE and Immunoblot

After 10% SDS-PAGE in tris-glycine buffer, gels were stained for proteins by the silver method and for carbohydrates by the Periodic Acid-Schiff (PAS) staining protocol (JOHNSTONE; THORPE, 1982).

After separation in SDS-PAGE, proteins from CFAs were transferred overnight to nitrocellulose membranes (18 h, 23 v, 4 °C), along with a pre-stained molecular weight protein standard (Bio-Rad Precision Plus Protein™ Kaleidoscope cat. #161-0375, Bio-Rad Laboratories, Hercules, CA, USA). Unbound sites were blocked with PBS-5% skim milk-0.5% Tween-20 (1 h, room temperature) and membranes were cut into individual strips.

CFAs (12.5 mg/mL) were analyzed by IB (7.5% SDS-PAGE) with purified rabbit IgG (1/10), anti-rabbit IgG peroxidase labeled antibody (Sigma A1949, 1/1,000) and detection with 3,3,5,5-tetramethylbenzidine (Zymed TMB).

In the other IB with human antibodies, CFAs (5 mg/mL) were analyzed after 10% SDS-PAGE. The influence of carbohydrate epitopes was investigated by periodate oxidation of vicinal hydroxyl groups on sugar to dialdehydes at acid pH (WOODWARD; YOUNG; BLOODGOOD, 1985). Membrane strips were washed with 50 mM sodium acetate buffer (pH 4.5) and treated with sodium metaperiodate (SMP: 0.5, 1, 2.5, 5 and 10 mM; only 2.5 mM in IgG2 test) in acetate buffer (1 h, room temperature, in the dark). Control strips were incubated with acetate buffer alone. Reaction was interrupted with sodium borohydride (50 mM; 30 min, room

temperature, in the dark). Strips were incubated with the pool of PCM patients' sera (1/20; 2 h, 35 °C) and then with anti-human IgG peroxidase labeled antibody (Sigma A6029, 1/2,000; 1.5 h, 35 °C). To detect IgG2, unconjugated monoclonal anti-human IgG2 mouse ascites fluid was used (Sigma I9513, 1/500), followed by Biotin-SP anti-mouse IgG (H+L) (Jackson ImmunoResearch 115-065-030, 1/20,000; 1.5 h, 35 °C) and Streptavidin-HRP (Jackson ImmunoResearch 016-030-084, 0.01 µg/mL; 1.5 h, room temperature). Detection was performed using TMB.

Bands from gels and membranes were visually identified, also relying on pixel intensity (peaks and valleys) obtained in Image Lab™ v. 6.0.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Molecular weights were automatically calculated in this software by point-to-point method (semi-log).

ELISA

Ninety-six wells plates were coated with 25 µg/mL CFA in carbonate-bicarbonate buffer pH 9.6 (100 µL/well; 1 h, 35 °C and 18 h, 4 °C). After blocking of unbound sites (PBS-5% skim milk-0.5% Tween-20; 1 h, room temperature), microplates were washed with 50 mM sodium acetate buffer (pH 4.5) and were treated with 10 mM SMP in acetate buffer or acetate buffer alone (1 h, room temperature, in the dark). Reaction was interrupted with sodium borohydride (50 mM; 1 h, room temperature, in the dark). The pool of PCM patients' sera was applied to the plates at 1/200 dilution in PBS-0.05% skim milk (2 h, 35 °C). The following steps were the same as in IB, but the reaction was evidenced by o-phenylenediamine (OPD) substrate solution (20 min, interrupted with 50 µL/well of 4N H_2SO_4). Absorbance at 492 nm was measured in a Multiskan EX Reader (Labsystems, Helsinki, Finland) and results were expressed in optical density units (O.D.).

Results were analyzed in GraphPad Prism v. 6.01 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Unpaired T-test was employed to compare readings with and without SMP treatment of the same antigen/antibody, while Ordinary One-way ANOVA with Tukey's multiple comparison test was employed to compare results among investigated strains within the same conditions (same antigen/antibody and with or without SMP treatment). Results were deemed significant at p-value ≤ 0.05.

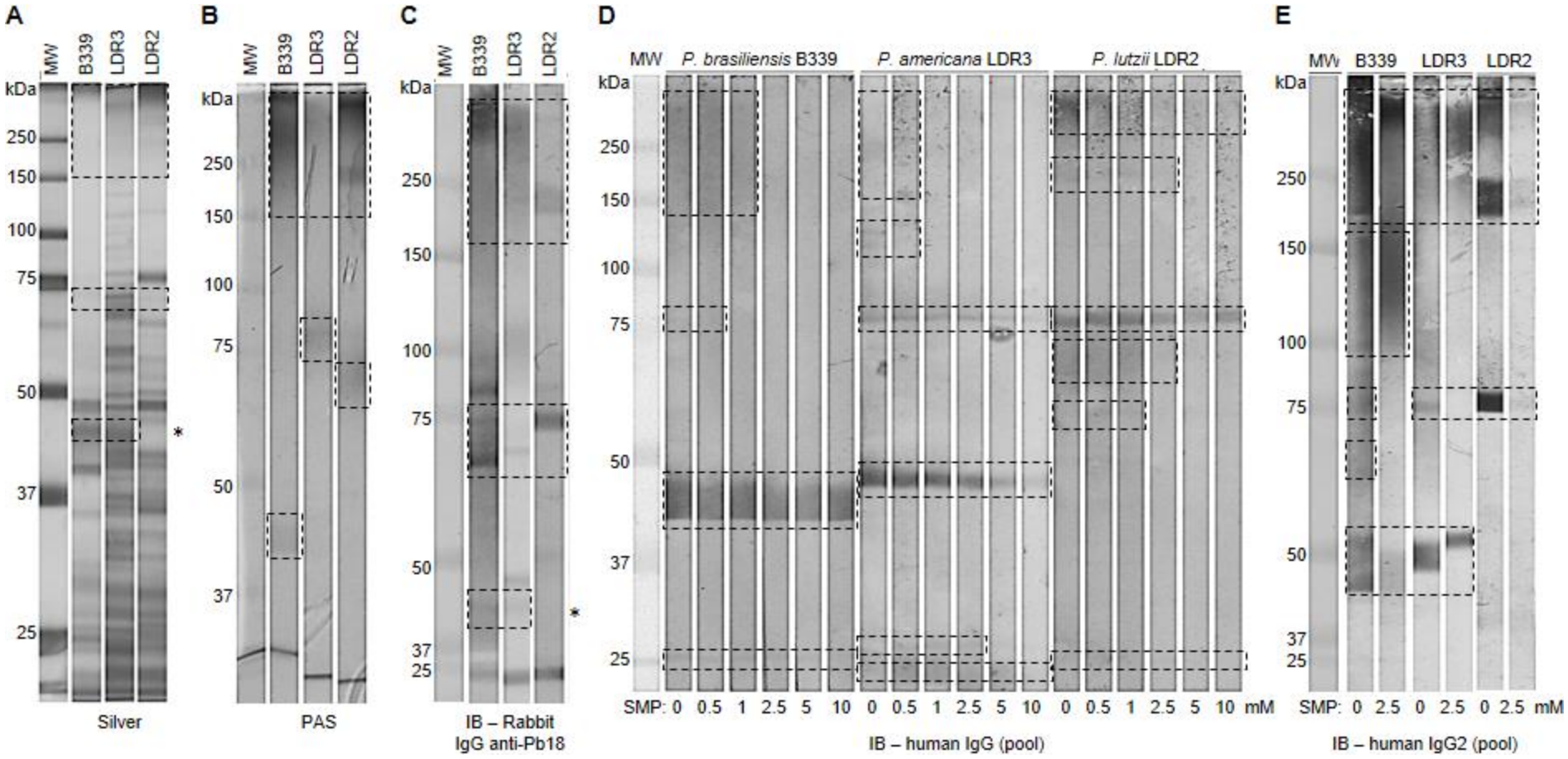
Results

Figure 1A shows the many proteins that compose CFA, highlighting the absence of the 43 kDa antigen in CFA from *P. lutzii* LDR2. High molecular mass antigens (hMM, ≥ 150 kDa) seem the most glycosylated area colored in PAS and the 43 kDa band also did not appear in CFA from *P. lutzii* LDR2 (Figure 1B). Rabbit IgG (Figure 1C) recognized many molecules, highlighting the antigens of clinical interest: gp43, gp70 and hMM. Gp43 was absent in CFA from *P. lutzii* LDR2 in IB with rabbit and human IgG (Figure 1C and 1D-strip SMP 0). Besides, this strain had hMM composed of more compact bands (≈200 to 250 kDa) than the area of polydispersed reactivity from *P. brasiliensis* B339 or *P. americana* LDR3, but all were heavily glycosylated.

The reaction with PCM patients' antibodies can be seen in Figure 1 D and E. Stained carbohydrates (Figure 1B) correlate well to areas which decreased their IgG reactivity after SMP treatment and strong bands with IgG2 corroborate their reactivity is largely due to presence of carbohydrates.

Gp70 from *P. lutzii* LDR2 was strongly recognized by rabbit IgG and by the pool of human sera, which indicates greater amounts in CFA from this species, stronger recognition of protein epitopes or stronger resistance to SMP oxidation.

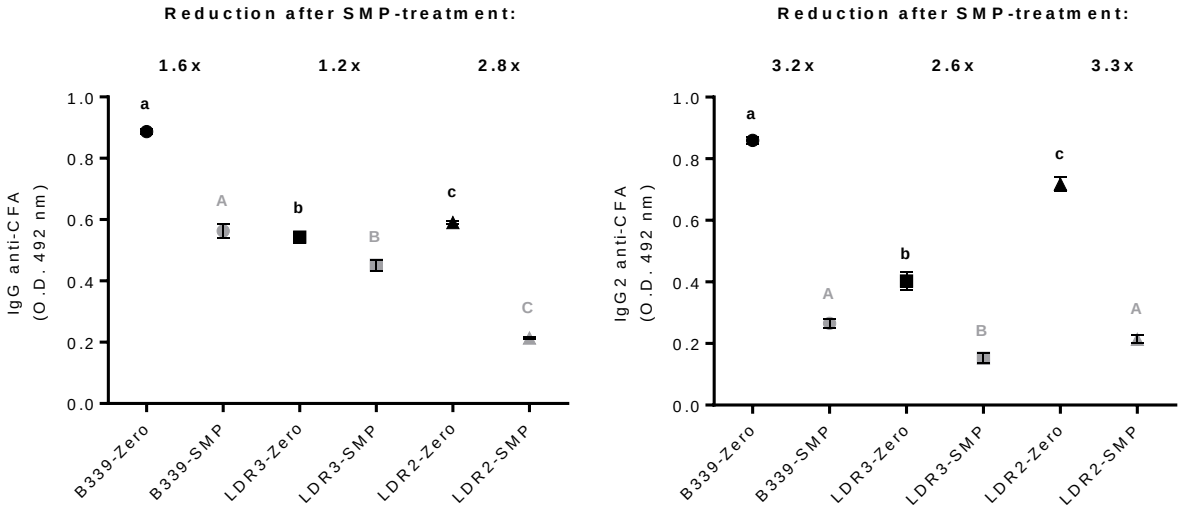
Figure 1 – Analyses of (A) proteins and (B) carbohydrates from CFA of *P. brasiliensis* B339, *P. americana* LDR3 and *P. lutzii* LDR2. (C) Immunoblot with rabbit IgG anti-CFA from *P. brasiliensis* Pb18. Dashed rectangles in A/B/C indicate areas of the major antigens of *Paracoccidioides* spp. (hMM, gp70, while gp43* was absent in *P. lutzii* LDR2). Influence of carbohydrates epitopes was investigated using CFA treated with SMP and a pool of PCM patients' sera, demonstrating the reactivity of (D) IgG and (E) IgG2. Dashed rectangles in D/E indicate areas of reactivity identified by computer analysis.



*: absence of gp43 in *P. lutzii* LDR2. CFA: cell free antigens. IB: immunoblot. PAS: periodic acid-Schiff. SMP: sodium metaperiodate

Results of IgG and IgG2 in ELISA are shown in Figure 2.

Figure 2 – ELISA with CFA of *P. brasiliensis* B339, *P. americana* LDR3 and *P. lutzii* LDR2 using a pool of 39 sera from chronic form PCM patients, with or without treatment with 10 mM SMP, expressed in optical density units (492 nm).



Significant unpaired T-Tests for all strains before and after SMP-treatment (black vs. grey). Different letters (lowercase or uppercase) indicate significant results between strains tested in the same conditions in Ordinary One-way ANOVA with Tukey's multiple comparison test. CFA: cell free antigens. PCM: Paracoccidioidomycosis. SMP: sodium metaperiodate.

Presence of carbohydrates in CFA influenced ELISA results because there was significant statistical differences for all strains before and after treatment with SMP, either for IgG or IgG2. Results most affected by the presence of carbohydrates were from *P. lutzii* LDR2. By contrast, carbohydrates in CFA from *P. americana* LDR3 seemed to have little influence in this strain, as was also seen in IB results. There was significant difference between the three species in the same conditions, except between *P. brasiliensis* B339 and *P. lutzii* LDR2 for IgG2 after SMP treatment.

Discussion

The greatest intensity of IgG-CFA-ELISA was seen with *P. brasiliensis* B339. This was expected because this species (former phylogenetic species S1) is supposed to be the most prevalent in the region from where the sera were obtained (MUÑOZ et al., 2016). Even though *P. brasiliensis* and *P. americana* have

distributions overlaps and are close in the phylogenetic scale (TURISSINI et al., 2017), *P. americana* LDR3 presented lower readings. Their major antigen must be gp43, as can be seen in IB, but PCM patients' sera also reacted with many other antigens, including those from *P. lutzii* LDR2.

Until the grouping of atypical isolates in the new species *P. lutzii*, gp43 was the most studied glycoprotein of *P. brasiliensis* (CAMARGO, 2008; DE OLIVEIRA et al., 2015). Takayama et al. (2010) showed the gene sequence of gp43 from strain LDR2 of *P. lutzii* had an identity of 98.9 and 89.0% with *P. lutzii* Pb01 and *P. brasiliensis* Pb18, respectively. Protein expression from *Paracoccidioides* sp. is dependent on strain and culture conditions, as they present different virulence levels, especially concerning gp43 (BERZAGHI et al., 2005). Besides, the main characteristic that differentiates *P. lutzii* from *P. brasiliensis* is the low production or even absence of gp43 (BATISTA JUNIOR et al., 2010; BERZAGHI et al., 2005). In the current research, gp43 was not detected among the antigens from *P. lutzii* LDR2, evidenced by silver/PAS staining and no recognition by immune serum against *P. brasiliensis* or by PCM patients' sera. As *P. lutzii* LDR2 probably did not express gp43 at the time this CFA was produced, additional investigations of different culture conditions (medium, time, temperature, etc.) are important to find out if this strain can eventually produce this important serodiagnosis antigen and whether it will be equally recognized by sera from patients with PCM caused by *P. brasiliensis* and *P. lutzii*.

In the work of Gegembauer et al. (2014), patients with PCM caused by *P. brasiliensis* (confirmed by immunodiffusion with antigens from *P. brasiliensis* B339) were not able to recognize antigens from *P. lutzii*. At this work, IgG from a rabbit immunized with *P. brasiliensis* Pb18 and antibodies from patients with PCM probably caused by *P. brasiliensis* because they are from the state of Paraná, Brazil (THEODORO et al., 2012) recognized various antigens from *P. lutzii* LDR2. There is a previous report of antigenic differences among *P. lutzii* strains isolated from Central-West and North regions of Brazil (GEGEMBAUER et al., 2014). Hence, it is expected that *P. lutzii* LDR2 may also present a distinct reactivity pattern from the already described strains of *P. lutzii*.

P. lutzii LDR2 employed in the current research was isolated from a PCM patient living in Northern Paraná (TAKAYAMA et al., 2010), a region known as endemic for *P. brasiliensis* (MUÑOZ et al., 2016). Recent research using Nested PCR and *in situ* hybridization techniques demonstrated the presence of both *P.*

brasiliensis and *P. lutzii* in soil and aerosol samples from endemic and non-endemic areas of PCM in Southeastern, Midwestern and Northern regions of Brazil (ARANTES et al., 2016). This demonstrates *P. lutzii* may be widely distributed throughout Brazil, so more characteristics still need to be investigated and, especially, which factors would influence its potential to cause disease mainly in a determined region and sometimes with distinct characteristics from what is still known about other species of *Paracoccidioides*, especially *P. brasiliensis.*

As SMP oxidizes carbohydrate and hence reduces binding of antibodies to carbohydrate epitopes, overall antibody reactivity was usually higher with whole antigens than with SMP treated antigens, as also demonstrated by Ferreira et al. (2008). As expected, increasing concentrations of SMP caused greater decrease of antibody reactivity in IB. In ELISA, *P. lutzii* LDR2 had the largest decreases, probably because CFAs from *P. brasiliensis* and *P. americana* suffered larger influence of non-carbohydrate epitopes from gp43, as most PCM sera react essentially with peptides and only a few owe up to 45% of their ELISA reactivity to carbohydrate epitopes (PUCCIA; TRAVASSOS, 1991). By contrast, antigens of 70 kDa and hMM from *P. lutzii* LDR2 remained evident in IB even at maximum concentration of SMP (10 mM). These epitopes may be recognized by other IgG subclasses than IgG2 or may be periodate-resistant carbohydrates.

CFAs from different strains of *Paracoccidioides* spp. are constituted of 16-20% of high molecular mass (hMM) antigens (FREDRICH et al., 2010). Puccia et al. (1986) first described this area of polydispersed components and mainly chronic PCM patients' sera recognized it in IB (MARQUEZ et al., 2005). These antigens influenced the intensity of CFA reaction in ELISA with PCM patients' sera, becoming an important serological marker (LENHARD-VIDAL et al., 2013). Here, *P. brasiliensis* B339 had a large area of hMM with heterogeneous migration heavily stained in PAS, hence heavily glycosylated, and a lighter area for *P. americana* LDR3, while *P. lutzii* LDR2 had two separate bands (≈200 to 250 kDa). Thus, hMM is a potential antigen for differentiation of *Paracoccidioides* species, which requires additional studies.

A possible specific pattern of recognition to differentiate the evaluated species was found after treatment of CFAs with 2.5 mM SMP (Figure 1D): only gp43 for *P. brasiliensis* B339, gp43+gp70 for *P. americana* LDR3 and gp70+hMM for *P. lutzii* LDR2. This may be a possible way to determine the infecting strain using PCM patients' sera and needs to be further investigated.

Although IgG2 is known to most commonly bind to carbohydrate epitopes, Von Gunten et al. (2009) reported that other human IgG subclasses can act as anti-carbohydrate antibodies. Sugar oxidation was already employed by Neves et al. (2003) to identify responses to carbohydrates epitopes, mainly by IgG2, of sera from patients with negative immunodiffusion, while there was only a small decrease in reactivity of IgG2 from patients with acute PCM who had positive immunodiffusion, indicating that IgG2 antibodies could also be directed against non-carbohydrate epitopes. In accordance to the present PAS stained gel and human IgG-IB, IgG2 from PCM patients recognized antigens of ~70 kDa and hMM from *P. lutzii* LDR2, which underwent greater reactivity reduction than that seen with IgG. *P. brasiliensis* B339 and *P. americana* LDR3 antigens, especially hMM and gp43, were also strongly reactive with IgG2. Differently from *P. lutzii*, IgG2 still had strong reaction after SMP treatment of antigens from *P. brasiliensis* B339 and *P. americana* LDR3. If these carbohydrate epitopes recognized by IgG2 are resistant to oxidation with SMP will require additional studies. As expected, the ratio of IgG2 reactivity decrease in ELISA of untreated vs. SMP-treated CFAs was larger than IgG, which is in accordance to the mostly anti-carbohydrate nature of this IgG subclass. Differences between IB and ELISA might be due to participation of other components less evident in IB, but which are sensitive to SMP, to relative larger amounts of periodate-resistant epitopes or to differences in exposure of antigens in each system.

Puccia and Travassos (1991) demonstrated that probably more than 85% of reaction with gp43 involved peptide epitopes, while cross-reaction was attributed to periodate-sensitive carbohydrate epitopes. Taborda and Camargo (1994) employed periodate oxidation of *P. brasiliensis* antigens to avoid cross-reaction with sera from patients with other mycosis. Although cross-reactivity can be eliminated by establishment of a good cutoff value, as there was a strong reaction with carbohydrates of *P. lutzii* LDR2, these antigens should be investigated concerning cross-reactivity.

This is the first report of carbohydrates influence in antigens of *P. lutzii*, demonstrated by staining in PAS and great decrease after SMP treatment. Currently, *P. lutzii* is usually identified by molecular biology methods to investigate the gp43 gene. It is hard to isolate *Paracoccidioides* sp. from human patients' samples or sometimes to obtain a good sample from a patient, as when deep organs are affected. Therefore, it becomes important to find other biological markers useful in

indirect diagnosis. These results point to the existence of some differences between antigens of *P. lutzii* LDR2 and *P. brasiliensis* B339 or *P. americana* LDR3., but they need to be further investigated if they can be used as biological markers.

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5 ARTIGO B – DETECÇÃO DE CASOS DE PARACOCCIDIOIDOMICOSE INFECÇÃO NA POPULAÇÃO DE GUARAPUAVA, PR

IgG reactivity profile to *Paracoccidioides* spp. antigens from sera of people with Paracoccidioidomycosis Infection in Guarapuava, PR, Brazil

ABSTRACT

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by the thermodimorphic fungi *Paracoccidioides* spp. As the disease is known to affect mostly men over 40 years old who previously worked handling soil, cities of agricultural economy are prone to have more cases of PCM infection. This condition is usually asymptomatic, despite a detectable immune reaction to *Paracoccidioides* spp. antigens. Current research aimed to evaluate sera from volunteers from an inland city of Paraná state, Southern Brazil, an endemic region of PCM. Guarapuava is located in state's South-Central region, has ≈170,000 inhabitants (14,000 in rural areas) and agricultural/timber-based economy. Immunoenzymatic assay (ELISA) was employed to evaluate 359 serum samples, to detect IgG against soluble antigens (cell free antigens, CFA) from *P. brasiliensis* B339 (S1), *P. americana* LDR3 (former *P. brasiliensis* PS2) and *P. lutzii* LDR2. Gp43 from B339 was used as a confirmatory test. Reduction of cross-reactions was sought by treatment with sodium metaperiodate (SMP-CFA, SMP-gp43). Epidemiological profile of participants was assessed by questionnaire. ELISA reactivity was: CFA/SMP-CFA in general 37.3/17.8%, B339 25.3/14.5%, LDR3 24.5/1.4%, LDR2 8.3/5.8%; gp43/SMP-gp43 7.2/4.7%. There were sera reactive with multiple CFAs. Immunoblot of 37 selected-sera showed different patterns of recognition of antigens. There was no differences concerning gender, age, education, residence only in the region of Guarapuava, alcohol consumption or presence of symptoms of PCM. Residence in rural areas and profession related to soil were found to be risk factors for the presence of PCM infection. The low prevalence found was expected, because the region had previous reports of lower endemicity than other areas of Paraná. The results indicate that *P. brasiliensis* seems to be the prevalent strain of the region, but there were 21 sera from people who declared to have only lived in Guarapuava, which reacted with *P. lutzii* LDR2. Purified antigens might not be suited for epidemiological surveys, so CFA-ELISA with whole antigens from *Paracoccidioides* spp. seems a better alternative for serological screening.

Keywords: ELISA, immunoblot, *Paracoccidioides americana*, *Paracoccidioides brasiliensis*, *Paracoccidioides lutzii*.

Introduction

Paracoccidioidomycosis (PCM) is a systemic mycosis of many different clinical manifestations, but usually presents pulmonary involvement and skin/mucosae lesions. It is restricted to Latin America, but it has a heterogeneous distribution. Colombia, Ecuador, Venezuela and Brazil, especially central west, southeast and south regions of this country, gather the majority of cases (MARTINEZ, 2017). Incidence of PCM is estimated in 1-3 cases/100,000 inhabitants per year, but higher or lower numbers are expected according to the known endemicity of each area (COUTINHO et al., 2002).

PCM was known to be caused by two different species of *Paracoccidioides*: *P. brasiliensis*, composed of at least five phylogenetic clusters, named S1a, S1b, PS2, PS3 and PS4 (MATUTE et al., 2006; MUÑOZ et al., 2016); and *P. lutzii*, which gathers strains formerly considered atypical (TEIXEIRA et al., 2009). Recently, evidence of low mitochondrial gene flow but of deep differences at nuclear loci led to the proposal of new formally described taxonomic species, maintaining only S1 as *P. brasiliensis sensu stricto* and replacing PS2 by *P. americana*, PS3 by *P. restrepiensis* and PS4 by *P. venezuelensis* (TURISSINI et al., 2017).

When an individual is exposed to the inhalation of *Paracoccidioides* spp. propagules, granulomatous lesions in lungs and lymph nodes are established, which may remain latent for long periods and later evolve to PCM disease or remain quiescent (DA SILVA et al., 2016). Chronic form (CF) of the disease accounts for 74-96% of all cases, and affects mainly men with 30 to 60 years-old, who were involved in activities directly related to soil handling. Acute form (AF) of the disease affects both genders equally and usually occurs in children, adolescents and young adults, but can occur between 30 and 40 years-old (SHIKANAI-YASUDA et al., 2017). Besides disease forms, PCM is also classified as PCM infection, when the individual has none or minor symptoms of the disease, but has a detectable reaction to *Paracoccidioides* spp. antigens. This corresponds to the so-called subclinical forms of other systemic mycoses (WANKE; LONDERO, 1994).

It is estimated that 50% of the population living in endemic areas is exposed to the fungus, but only few of these people will develop PCM disease (SHIKANAI-YASUDA et al., 2006) and an unknown number will remain with PCM

infection for the rest of their lives. Researches that investigated reactions to *P. brasiliensis* antigens in people not diagnosed with PCM disease have showed a wide variation of reactivity, ranging from 2 to 82% in Brazil using various sources of antigens (MARTINEZ, 2015).

Serological techniques employing antigen-antibody reactions became very useful in the past years of PCM research, and the main antigens studied are glycoproteins of 43 and 70 kDa, known as gp43 and gp70 (CAMARGO, 2008). Because *P. lutzii* may produce low amounts of gp43 or even not at all (BATISTA JUNIOR et al., 2010; BERZAGHI; DA SILVA; CAMARGO, 2005), this antigen may not be adequate for diagnosis in areas where *P. lutzii* is endemic. Presence of antibodies directed to *P. brasiliensis* antigens, usually gp43, constituted a good indicator of PCM, especially when deep or delicate organs are affected and biopsy or culture are not possible (DA SILVA et al., 2016). The same indirect methods can be applied to detect PCM infection.

PCM disease was already declared the eighth most common cause of death from predominantly chronic or recurrent types of infectious and parasitic diseases in Brazil, and the fifth in the state of Paraná. PCM also had the highest mortality rate among systemic mycoses, rendering a major health problem in Brazil (BITTENCOURT; DE OLIVEIRA; COUTINHO, 2005; COUTINHO et al., 2002). Most cases of PCM occur during the most productive stage of life and its sequels can cause work disability, what makes this disease a public health issue. PCM disease still is not of mandatory reporting, so it is not possible to establish its real distribution and to implement preventive or surveillance measures. Therefore, detection of PCM infection can aid in this planning.

Current research aimed to evaluate the presence of PCM infection in the population of Guarapuava, PR, a city in an endemic area of Brazil, using different antigens of three species of *Paracoccidioides* spp. ELISA was the elected method, because it does not depend on further compliance of volunteers after collection of blood samples to evaluate the final results, as happens with when delayed-type hypersensitivity (DTH) reactions are employed.

Materials and Methods

Participants and region of study

South-central region of Paraná State (south of Brazil) consists of 24 cities with economy based on agriculture and logging. Guarapuava is its largest city, located 257 km from the state capital, Curitiba (coordinates: 25º 23' 26" S, 51º 27' 15" W; altitude 1098 m). According to the Köppen classification, the region has Cfb climate, with a moderate, temperate and humid climate, mean annual precipitation of 1800 to 2000 mm, without significant difference in rainfall volume between seasons (IAPAR, 2000). Its winters presents frost and snow, with average annual temperature of 16.8 °C (average maximum of 36 and minimum of 6.8 °C) (GUARAPUAVA, 2016).

In the 2010 demographic census, Guarapuava had a total population of 167,328 individuals, of whom 14,335 lived in rural areas (IBGE, 2010). Considering the total population of the city and the fact that many researches about PCM infection had less than 35% of positive results, a minimum of 349 people (95% confidence level), needed to be investigated so the sampling would be representative of the inhabitants of the municipality (SANTOS, 2015). In this research, 359 individuals were included. Volunteers were randomly invited to join the project in medical laboratories and universities of the city, favoring a more representative sampling of the regional population. After participants provided free and clarified consent, they filled out a form composed of questions about: epidemiological data (age, gender, education), cities of previous residence, aspects already known to correlate with development of PCM disease (profession, alcohol consumption, smoking habits), diseases of differential diagnosis of PCM and main symptoms of PCM disease.

Negative controls consisted of twenty-seven samples of normal human sera (NHS; 10 men, 17 women), from volunteers who did not have signs of PCM disease and were previously non-reactive in ELISA with exoantigens from *P. brasiliensis* B339. Positive control sera were from 39 CF PCM patients (30 men, 9 women). Samples were from the biological samples bank of the Laboratory of Applied Immunology, State University of Londrina, Londrina, PR, Brazil. Current research was approved by the Ethics Committee on Research Involving Human Beings from State University of Londrina.

Antigens preparation

Antigens were produced from *P. brasiliensis* B339 (IFM 41630, former phylogenetic species S1), *P. americana* LDR3 (IFM 54649, former *P. brasiliensis* phylogenetic species PS2), and *P. lutzii* LDR2 (IFM 54648). This *P. lutzii* strain was chosen because it was isolated from a patient living in north region of Paraná, Brazil (TAKAYAMA et al., 2010). Fungi were subcultured in the yeast form (35 °C) on Sabouraud dextrose agar, every 5-7 days.

Cell free antigens (CFA) were obtained according to the modified method of Camargo et al. (1991). Briefly, yeast mass was scrapped from agar surface into conical centrifuge tubes containing phosphate buffered saline (PBS, 0.15 M, pH 7.4), 2.5 mM phenylmethylsulfonyl fluoride (PMSF, protease inhibitor) and 0.02% thimerosal. After vortex mixing and centrifuging (3600 x g and then with 15000 x g, 20 min each, 4 °C), supernatants were collected, protein concentration was determined at 280 nm in NanoDrop Lite UV-Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and soluble antigens were stored at -80 °C until use. Gp43 from *P. brasiliensis* B339 was kindly provided by Dr. Zoilo Pires de Camargo.

ELISA to detect IgG against CFA and gp43

Initial tests employed ninety-six well plates coated with CFA (CFA-ELISA) from *P. brasiliensis* B339, *P. americana* LDR3 and *P. lutzii* LDR2 at 25 µg/mL (100 µL/well; 1 h, 35 °C and 18 h, 4 °C). Subsequent tests of sera positive for any CFA employed high affinity polystyrene plates coated with 1 µg/mL of gp43 from *P. brasiliensis* B339, as a confirmatory test (gp43-ELISA). Unbound sites were blocked with PBS-5% skim milk-0.5% Tween-20 (1 h, room temperature). To avoid cross-reaction of sera with carbohydrate epitopes, microplates were washed with 50 mM sodium acetate buffer (pH 4.5), half of them were treated with 10 mM sodium metaperiodate (SMP) in acetate buffer (1 h, room temperature, in the dark) (SMP-CFA, SMP-gp43), while the other half was incubated with just acetate buffer. Reaction was interrupted with sodium borohydride (50 mM; 1 h, room temperature, in the dark).

For all ELISAs, serum samples were analyzed in duplicates at 1/200 dilution in PBS-0.05% skim milk (2 h, 35 °C). Anti-human IgG-peroxidase labeled antibody was added at 1/4,000 dilution (1.5 h, 35 °C; A6029, Sigma Chemical Co., St. Louis, MO, USA). O-phenylenediamine (OPD) substrate solution was allowed to react for 20 minutes and reaction was interrupted with 50 µL/well of 4N H_2SO_4. Absorbance at 492 nm was measured in a Multiskan EX Reader (Labsystems, Helsinki, Finland). Antibody levels were expressed in optical density units (O.D.) and mean blank reading of each plate was subtracted from samples. In order to better compare the results, the mean reading from a positive control serum (patient with diagnosed PCM) was used to equalize readings of different microplates. Cutoff value was determined for each test as the mean plus two standard deviations (SD) of the NHS readings in O.D., grouping participants in reactive (R) and non-reactive (NR).

Immunoblot

All sera reactive in gp43-ELISA were tested with CFA from *P. brasiliensis* B339. Sera reactive in CFA-ELISA with the three strains and six sera reactive in SMP-CFA of *P. lutzii* LDR2 after SMP treatment were also tested with the according strains. A pool of 39 serum samples from CF PCM patients was used as a positive control. CFAs (5 mg/mL) from *P. brasiliensis* B339, *P. americana* LDR3 and *P. lutzii* LDR2 were diluted v/v with mercaptoethanol sample-buffer (Bio-Rad Laboratories, Hercules, CA, USA) and boiled for 3 minutes. Soluble antigens from CFA were separated in 10% SDS-PAGE in tris-glycine buffer (pH 8.1; 120 v), along with a pre-stained molecular weight protein standard (Precision Plus Protein™ Kaleidoscope™, #161-0375, Bio-Rad Laboratories, Hercules, CA, USA). Gels were transferred to nitrocellulose membranes (18 h, 23 v; 1h, 60 v), blocked (1 h, room temperature) and cut into individual strips. Next, they were incubated with sera (1/25; 2 h, 35 °C), Biotin-SP anti-human IgG (H+L) (1/80,000; 1.5 h, 35 °C; #109-065-003, Jackson ImmunoResearch Inc., West Grove, PA, USA) and Streptavidin-HRP (0.05 µg/mL; 1.5 h, room temperature; #016-030-084, Jackson ImmunoResearch Inc., West Grove, PA, USA). Detection was performed with 3,3,5,5-tetramethylbenzidine (TMB) (Zymed #00-2019, Zymed, San Francisco, CA, USA).

Bands from membranes were visually identified, also relying on pixel intensity (peaks and valleys) obtained in Image Lab™ v. 6.0.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Molecular weights (MW) were automatically calculated in this software by point-to-point method (semi-log).

Statistical Analysis

Participants were divided into reactive (R) and non-reactive (NR) by the cutoff established with NHS results. After Kolmogorov-Smirnov normality test, the following analyses were carried out: (1) Wilcoxon test or Friedman's One-Way ANOVA with Dunn's multiple comparison test to compare the same sera with and without SMP treatment (paired samples of gp43 and CFA, respectively); (2) Kruskal Wallis' One-Way ANOVA with Dunn's multiple comparison test to compare three groups of different sizes (NHS x NR x R; Reactive sera: B339 x LDR3 x LDR2). Chi-square test of association was used to evaluate if there was a link between categorical variables from the questionnaire, followed by odds ratio with a 95% confidence interval. Results were deemed significant at p-value ≤ 0.05. Pearson's correlation (Ln transformed data) was defined as a strong correlation when r ≥ 0.75. Statistical analyses were performed in GraphPad Prism v. 6.01 for Windows (GraphPad Prism Software, La Jolla California USA, www.graphpad.com) or IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA).

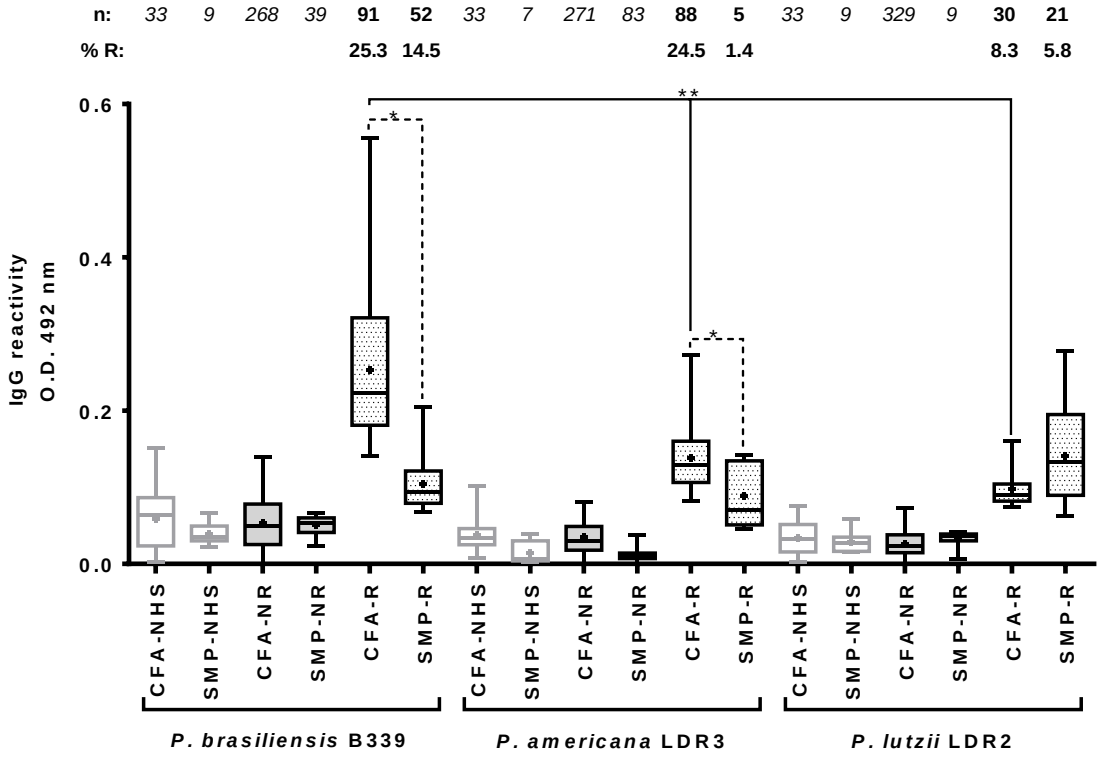
Results

Reactivity with CFA

Out of the 359 samples analyzed, 134 (37.3%) were considered reactive in CFA-ELISA with at least one of the strains tested and this number decreased to 64 (17.8%) after SMP treatment. Figure 1 shows the number of samples per group and reaction intensities with CFA and CFA treated with SMP, classifying participants in NR and R. There were significant statistical differences for each CFA between R/NR and R/NHS (p-value ≤ 0.01), but not between NR/NHS. When reactive samples from

CFA-ELISA were compared to their corresponding SMP-CFA test (CFA x SMP of each strain), there were significant differences for B339 and LDR3 (p-value ≤ 0.0001), also seen clearly by reduction in the number of reactive samples with the two strains. CFA-ELISA readings from the R groups were statistically different between strains (p-value ≤ 0.001), but this was not observed after SMP treatment.

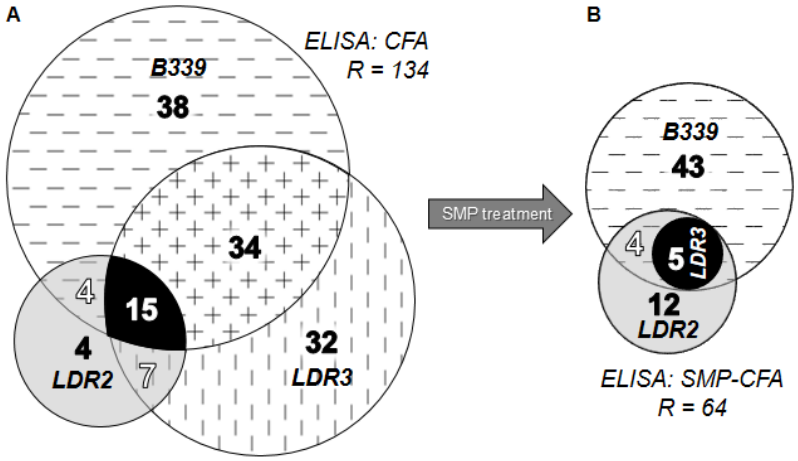
Figure 1 - ELISA to detect IgG against soluble antigens (CFA) or SMP-treated CFAs (SMP) from *P. brasiliensis* B339, *P. americana* LDR3 and *P. lutzii* LDR2 of 359 sera (1/200) from Guarapuava, PR, Brazil: reactivity rate and readings (O.D. at 492 nm).



+: mean of each test. *: p-value ≤ 0.0001. **: p-value ≤ 0.001. CFA: cell free antigen. NHS: normal human sera (negative control). NR: non-reactive (below the cutoff of the test, established as mean + 2 SD of the NHS results). R: reactive (above cutoff). SMP: sodium metaperiodate.

Figure 2 demonstrates that some of the 134 reactive sera were responsive to one or more strains (A). After treatment with SMP (B), this number decreased, but there were still multiple-reactive samples.

Figure 2 – Venn diagram illustrating the number of reactive participants in ELISA with native CFA (A) and SMP-CFA (B) from *P. brasiliensis* B339, *P. americana* LDR3 and *P. lutzii* LDR2, demonstrating overlapping reactivity of some participants with different strains.



CFA: cell free antigen. SMP: sodium metaperiodate. R: reactive samples.

Further investigation of participants: reactivity to gp43

Because reactivity to gp43 is still used as a PCM marker in areas of *P. brasiliensis* endemicity, samples were subjected to ELISA with gp43 and gp43 treated with SMP. Out of 359 samples, 26 (7.2%) were reactive to gp43 and this number decreased to 17 (4.7%) after SMP treatment. Means and standard deviations from the R group were 0.090 ± 0.049 and 0.063 ± 0.022 for gp43 and SMP-gp43, respectively. There were significant statistical differences between R and NR/NHS (p-value ≤ 0.0001), but no differences between NR and NHS. Comparison of the 26 samples reactive to gp43 and their corresponding SMP-gp43 ELISA resulted in p-value ≤ 0.0001. Pearson's correlation of participants reactive with both native and SMP-treated gp43 (n = 17) was 0.894, considered strong.

Immunoblot of selected reactive sera

Thirty-seven participants were chosen to be further investigated by Immunoblot because they were: (1) reactive in CFA-ELISA with the three tested strains (there wasn't sufficient sample to test two of these sera); (2) reactive with any CFA and with gp43 and; (3) still reactive with CFA from *P. lutzii* after SMP treatment. Figure 3 illustrates their results.

Twenty-seven participants were able to recognize gp43 from *P. brasiliensis* B339, of which 23 also reacted with the polydispersed area of hMM (Figure 3B). Meanwhile, gp43 from *P. americana* LDR3 was not recognized by any participant, just hMM (Figure 3C). Five sera recognized two bands of hMM along with ≈75 kDa band of *P. lutzii* (Figure 3D), which is the same profile of the pool of PCM patients' sera (Figure 3A, strip LDR2).

Participants profile

Answers to the questionnaire are organized in Table 1.

Figure 3 – Immunoblot of 37-selected sera with CFA from *P. brasiliensis* B339 (B), *P. americana* LDR3 (C) and *P. lutzii* LDR2 (D). (A) Positive control employed a pool of chronic PCM patients' sera.

Rectangles indicate areas of reactivity. NR: non-reactive. 1-13: sera reactive with the three investigated CFAs.

Table 2 – Answers to the questionnaire applied to the participants, concerning epidemiological data and information related to development of PCM disease. Participants were divided into non-reactive (NR) and reactive (R) according to CFA-ELISA results.

Question		Total	NR	R	R x NR: Statistically significant?
Gender	Female	187 (52.1%)	119 (52.9%)	68 (50.7%)	Ns
	Male	172 (47.9%)	106 (47.1%)	66 (49.3%)	
Age (years-old)	Min. / Max.	18 / 86	18 / 86	18 / 84	Ns
	Mean ± SD	46.84 ± 18.30	46.06 ± 18.12	48.16 ± 18.68	
Age groups (n)	18-29	84	57	27	Ns
	30-39	53	30	23	
	40-49	55	38	17	
	50-59	56	36	20	
	60-69	69	41	28	
	70-79	34	17	17	
	80-89	8	6	2	
Education*	≤ Elementary	80 (36.7%)	52 (36.6%)	28 (36.8%)	Ns
	> Elementary	138 (63.3%)	90 (63.4%)	48 (63.2%)	
Always lived in Guarapuava / south central region of Paraná?	N	115 (32%)	71 (31.6%)	44 (32.8%)	Ns
	Y	244 (68%)	154 (68.4%)	90 (67.2 %)	
Reactive with *P. brasiliensis* B339	N	---	---	36 (39.6%)	Ns
	Y			55 (60.4%)	
Reactive with *P. americana* LDR3	N	---	---	25 (28.4%)	Ns
	Y			63 (71.6%)	
Reactive with *P. lutzii* LDR2	N	---	---	9 (30.0%)	Ns
	Y			21 (70.0%)	
Lived in rural areas?	N	176 (49%)	120 (53.3%)	56 (41.8%)	p-value = 0.034 OR 1.592 (CI 1.034-2.451)
	Y	183 (51%)	105 (46.7%)	78 (58.2%)	
Worked handling soil?	N	200 (55.7%)	135 (60.0%)	65 (48.5%)	p-value = 0.034 OR 1.592 (CI 1.034-2.451)
	Y	159 (44.3%)	90 (40.0%)	69 (51.5%)	
Smokes?*	Never had	282 (79.5%)	167 (74.9%)	115 (871 %)	p-value = 0.006 OR 0.441 (CI 0.244-0.797)[1]
	Nowadays	37 (10.4%)	30 (13.5%)	7 (5.3%)	
	Not anymore	36 (10.1%)	26 (11.7%)	10 (7.6%)	
Alcohol consumption*	N	241 (67.9%)	148 (66.4%)	93 (70.5%)	Ns
Frequency during the week	1x	88 (24.8%)	54 (24.2%)	34 (25.8%)	
	2x	17 (4.8%)	13 (5.8%)	4 (3.0%)	
	3x	9 (2.5%)	8 (3.6%)	1 (0.8%)	
Treated disease(s) of differential diagnosis of PCM?	N	336 (93.6%)	213 (94.7%)	123 (91.8%)	Ns
	Y	23 (6.4%)	12 (5.3%)	11 (8.2%)	
Tuberculosis		7 (2.0%)	3 (1.3%)	4 (3%)	Ns
Leprosy		3 (0.8%)	1 (0.4%)	2 (1.5%)	
Leishmaniasis		0	0	0	
Chronic pulmonary disease		5 (1.4%)	3 (1.3%)	2 (1.5%)	
Neoplasia		8 (2.2%)	5 (2.2%)	3 (2.2%)	
Had any symptom of PCM disease?	N	288 (80.2%)	184 (81.8%)	104 (77.6%)	Ns
	Y	71 (19.8%)	41 (18.2%)	30 (22.4%)	
Participants with multiple symptoms		*30 (8.3%)*	*14 (6.2%)*	*16 (11.9%)*	Ns
Participants with only one symptom		*41 (11.4%)*	*27 (12%)*	*14 (10.4%)*	
Weight loss		16	9	7	Ns
Lymph node enlargement		8	3	5	
Wounds which don't heal		5	4	1	
Hoarseness		21	10	11	
Dyspnea		25	14	11	
Productive cough		25	14	11	
Dry cough		18	8	10	

* Not all participants provided information. CI: confidence interval. Min.: minimum. Max.: maximum. N: no. Ns: not significant. OR: odds ratio. SD: standard deviation. Y: yes. Percentages in NR and R columns relate to the total number of non-reactive (225) and reactive (134) samples in CFA-ELISA or to the total number of answered questionnaires (*).

[1] There was no statistical difference between readings (O.D. 492 nm) of smokers (current + former) and non-smokers within R or NR groups (Mann-Whitney).

Discussion

Not much about PCM infection and its evolution to PCM disease has been studied and, so far, epidemiological surveys have been conducted mainly using DTH reactions with whole antigens (paracoccidioidin) or gp43, especially from *P. brasiliensis* (FAVA; FAVA NETTO, 1998; FORNAJEIRO et al., 2005; MARQUES et al., 2013). Because high levels of antibodies anti-*Paracoccidioides* and a lack of cell-mediated immunity have been associated with PCM disease (KASHINO et al., 2000; MARQUES MELLO et al., 2002), detection of circulating antibodies becomes more interesting than the use of DTH tests.

Total PCM infection prevalence regardless of strain (37.3%) is close to other epidemiological surveys conducted in the same state (Paraná), which had varying results. Botteon et al. (2002) analyzed sera by ELISA of 700 blood donors from rural and suburban areas of Londrina (Northern Paraná) and found a reactivity rate of 21% of IgG anti-ExoAg and gp43 from *P. brasiliensis* B339, which fell to 12.8% after adsorption with *Histoplasma capsulatum* antigens and to 12.3% with *Leishmania amazonensis*. Maluf et al. (2003) found a reactivity of 27% among blood donors from Northwestern Paraná, also by ELISA with ExoAg. Meanwhile, Fornajeiro et al. (2005) presented 43% of positive DTH reactions with gp43 among workers from the alcohol industry also from the northwest region of the state.

The number of reactive samples to CFA from *P. brasiliensis* B339 and *P. americana* LDR3 was very similar (91 vs. 88), but O.D. readings were higher with *P. brasiliensis* B339. By contrast, only few samples (30) reacted with soluble antigens from *P. lutzii* and in lower intensity (Figure 1). This was fairly expected because isolates from Southern Brazil are mainly *P. brasiliensis* S1 (MUÑOZ et al., 2016).

Because carbohydrate epitopes have been shown to cause cross-reaction with sera from people with other mycoses (histoplasmosis, aspergillosis, Jorge Lobo's disease) (CAMARGO; UNTERKIRCHER; TRAVASSOS, 1989), CFA was treated with SMP. This involves periodate oxidation of vicinal hydroxyl groups on sugar to dialdehydes, but not all carbohydrate epitopes are sensitive to periodate cleavage (WOODWARD; YOUNG, BLOODGOOD, 1985). As expected, all percentages and readings became lower, so probably many participants had antibodies directed to carbohydrate epitopes or had cross-reactions. After SMP treatment, there was no statistical differences between CFAs, indicating common

epitopes possibly remained after carbohydrate withdrawal, which yielded closer intensity of reactions.

Differently from a previous report of no recognition of antigens from *P. lutzii* by sera from patients with PCM caused by *P. brasiliensis* (GEGEMBAUER et al., 2014), current research showed reactivity with *P. lutzii* LDR2 antigens, although at lower rates than with *P. brasiliensis*, just as described by Lenhard-Vidal et al. (2013) with sera from patients with PCM probably caused by *P. brasiliensis*.

DNA sequences from both *P. brasiliensis* and *P. lutzii* were isolated from soil from Southeastern, Midwestern and Northern regions of Brazil, demonstrating possible overlapping distribution of the species (ARANTES et al., 2016). Ninety out of 134 reactive participants declared to have lived exclusively in the region of Guarapuava and, from these, 21 and 15 reacted with CFA and SMP-CFA from *P. lutzii*, respectively, indicating possible presence of *P. lutzii* in the region or the presence of epitopes in common with the strain prevalent in the region.

CFA from *P. lutzii* LDR2 had the smallest reduction in the number of reactive sera after SMP treatment, indicating reaction to these antigens did not depend much on carbohydrate epitopes. The opposite is suggested about *P. americana* LDR3, which had a great decrease, implying larger cross-reactivity. Although *P. brasiliensis* and *P. americana* have overlapping distributions and look close in the phylogenetic scale (TURISSINI et al., 2017), it seems these strains have differences between CFAs' immunogenic antigens and, perhaps, absence of common immunodominant epitopes. Therefore, antigens from *P. americana* LDR3 may not be suitable for use in PCM infection diagnosis in this region, while *P. brasiliensis* S1 seems better suited. However, five samples were reactive with CFA from the three strains before and after SMP treatment, indicating at least some common protein epitopes between strains.

Many previous researches employed gp43 from *P. brasiliensis* B339 and, therefore, it was chosen to be used as a confirmatory test for PCM infection in this research. Positivity rate with gp43 in Guarapuava (7.2%) was 2.6 times lower than in Londrina, PR (BOTTEON et al., 2002) and even lower after SMP treatment (4.7%). Gp43 is considered a marker of active disease because its titers decreased during antimycotic therapy (CAMARGO; UNTERKIRCHER; TRAVASSO, 1989). Therefore, these antibodies levels are probably low during PCM infection. In immunoblot, 27 out of 34 sera reacted with gp43.

Even though SMP treatment of antigens has been shown to decrease cross-reactivity, its use may not be ideal in epidemiological surveys about PCM infection, because it might reduce reactivity that is expectedly low and generate false negative results. Neves et al. (2003) reported SMP use may impair assay sensitivity because some people with proven PCM produced low-avidity IgG2 anti-*P. brasiliensis*, whose reaction in ELISA was eliminated after oxidation of carbohydrates. Likewise, Puccia and Travassos (1991) demonstrated that carbohydrate epitopes accounted for up to 45% of total reactivity with gp43 in ELISA. Albeit ELISA can be less specific for PCM diagnosis, the use of a good serum dilution and cutoff helps to diminish this cross-reactivity (CAMARGO; UNTERKIRCHER; TRAVASSOS, 1989). Here, non-reactive sera (those below cutoff) had readings much smaller than reactive sera and had no statistical difference from NHS (Figure 1), so this might be enough to differentiate cross-reactivity.

Besides the presence of at least four isoforms of gp43 from *P. brasiliensis* that are not equally recognized by PCM patients' sera (SOUZA et al., 1997), its expression depends on strain and culture conditions, may cease after subculture or is even absent in some strains of *P. lutzii* (BATISTA JUNIOR et al., 2010; BERZAGHI; DA SILVA; CAMARGO, 2005). As gp43 was not detected in CFA from *P. lutzii* LDR2 (unpublished results – see *"Artigo A"*), it was not evaluated. Absence of gp43 in CFA from *P. lutzii* LDR2 likely affected its tests with sera from PCM disease patients, as already suggested by Lenhard-Vidal et al. (2013). Thus, it became interesting to test other antigens from the investigated strains that had good reactions with participants IgG (Figure 3), but the number of reactive sera with gp70 or high molecular mass antigens (hMM, > 150 kDa) in ELISA was very low (Supplementary material).

The distribution by gender and age found in this study was very similar to the profile of the city of Guarapuava (IBGE, 2010), indicating good randomization in the selection process. Researches relating sociodemographic and life style information with PCM development are mainly about PCM disease patients and not PCM infection. The majority of PCM disease patients have contact with rural areas, whether by profession, residence, or both. Smoking and large intake of distilled alcoholic beverages have also been correlated to PCM disease (MARTINEZ, 2017). In the current research, PCM infection in Guarapuava did not seem to present the same characteristics of PCM disease, because there was no statistical differences between R and NR groups concerning age, education, alcohol consumption,

presence of diseases of differential diagnosis of PCM or common symptoms of PCM disease. The majority of participants were invited to join this research while seeking assistance in medical laboratories of Guarapuava, thus prone to show any of these unspecific symptoms also present in PCM disease.

Most cases of CF PCM disease occur in men, while AF affects both genders equally. This is explained by the protective effect of female estrogens, which prevents mycelia-to-yeast transition (RESTREPO et al., 1984). According to Wanke and Londero (1994), there is no gender difference in primary PCM infection and the same was seen here. On the other hand, other epidemiological surveys from Paraná had 80 and 98% of reactive samples from men (BOTTEON et al., 2002; FORNAJEIRO et al., 2005).

Smoking habits odds ratio was a surprising result, because it indicated those who smoke or had smoked were more prone to be in the NR group, even though smoking was already associated with a 14-fold greater risk of developing PCM disease (DOS SANTOS et al., 2003). Effects of passive smoking cannot be discarded for those who stated they did not smoke, as environmental tobacco fume has been shown to impact on many aspects of human health (CAOS et al., 2015). The overall low number of smokers in both groups (R and NR) was a possible statistical bias, because there was no statistical difference between smokers and non-smokers readings (O.D. at 492 nm) within each group. A greater number of smokers would be required to improve this analysis and conclusions.

The biological cycle of *Paracoccidioides* sp. is still not fully known. Possibly, people are infected by inhalation of propagules present in soil (BAGAGLI et al., 2008), so those who have lived in rural areas or have worked with close contact to soil usually have greater chances of developing PCM disease (SHIKANAI-YASUDA et al., 2017). Nonetheless, a single contact with the fungus may be enough to develop PCM disease (BUCCHERI et al., 2016). Here, not much more than half of reactive participants declared to have lived in rural areas or had already worked handling soil, but there was 1.6 times more chance reactive sera would have these profiles. In the work of Fornajeiro et al. (2005), 80% of reactive sera were from rural residents. In surveys conducted in small cities, it must be considered that boundaries between rural and urban areas are very dubious, so probably all individuals are eventually exposed to the fungus, either during leisure or because of dust carried with the wind (MALUF et al., 2003).

Mortality coefficient of South-Central Paraná was the second lowest in the state between 1980 and 1995 (BITTENCOURT; OLIVEIRA; COUTINHO, 2005), even though the region has a strong agricultural history. The low reaction rates of the current research are fairly expected, because the number of PCM disease cases was considered low in Guarapuava during a 25-year period: 1.52 cases/year (unpublished results – see *"Apêndice C"*). Perhaps local environment of Guarapuava may contribute to a low presence of the fungus in soil. *P. brasiliensis* is known to prefer acidic soils, tropical or subtropical humid forests with abundant watercourses, with temperatures from 10 to 28 °C and altitudes of up to 1,000 m above sea level (MORENO-RESTREPO, 1994). Most cases of PCM disease caused by *P. lutzii* are from the Central West region of Brazil (TEIXEIRA et al. 2014), an area with tropical climate, hot and rainy climate. Therefore, weather conditions in Guarapuava (see *Participants and region of study*) differ from the ones described as best for development of *Paracoccidioides* spp. Sampling in different cities and soils from this region is an interesting aspect to be further addressed.

Environment factors, host's immune response, fungus virulence and amount of inhaled propagules account for successful establishment of the infection and future intensity of PCM disease symptoms (BUCCHERI et al., 2016). This may also prove right for PCM infection development, allowing greater or smaller numbers of infected people within an endemic region. Isolation of strains from soil or from people with diagnosed PCM who have exclusively lived in the region of Guarapuava could enlighten this question.

Most of the previous epidemiological surveys about PCM infection employed paracoccidioidin in DTH tests or ELISA with gp43 from *P. brasiliensis*, so current research reinforces the indication of ELISA with whole antigens from *Paracoccidioides* spp. in future researches.

Conclusions

Current research had low reactivity rates of possible PCM infection in Guarapuava, PR, Brazil. Participants' reactivity was stronger with antigens from *P. brasiliensis* B339, followed by *P. americana* LDR3 and *P. lutzii* LDR2. Probably there are common protein epitopes between the three investigated strains. PCM infection

risk factors in the region were past/present residence in rural areas and soil-related professions.

It is important to highlight 21 participants declared they had lived solely in this region of Paraná and were reactive with CFA from *P. lutzii* LDR2. For this reason, we do not recommend determination of infecting strain based solely on detection of specific antibodies, but on adoption of more specific methods, such as DNA amplification. The use of a combination of antigens from different strains may improve the detection of PCM infection.

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Supplementary material – Reactivity with gp70 and hMM

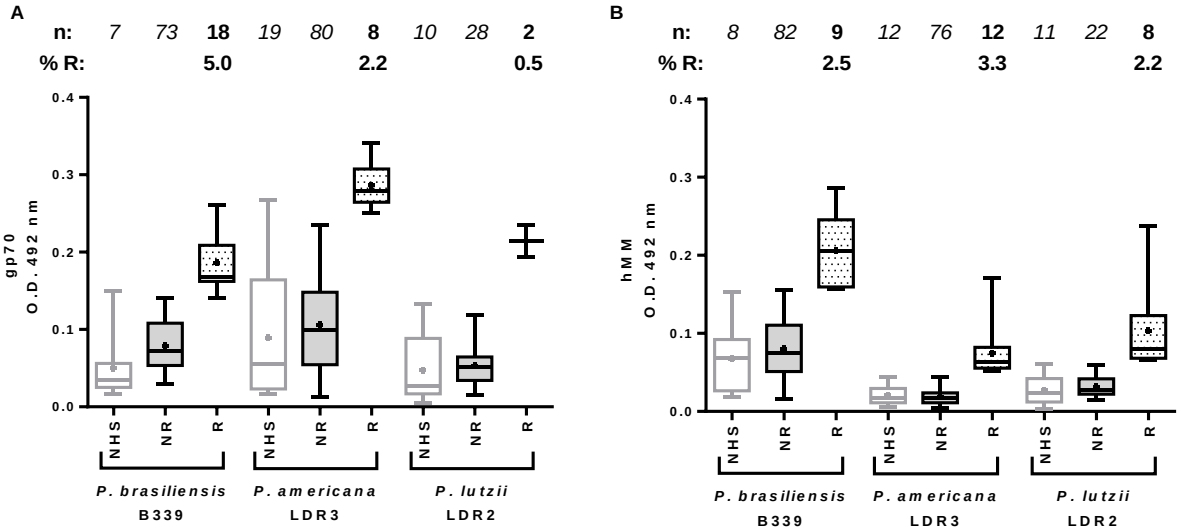
Besides gp43, other immunogenic proteins of different molecular weights have been described for *P. brasiliensis*: 20-21, 43, 52, 55, 70 kDa and polydispersed high molecular mass antigens (hMM), from 278 to 466 kDa (BLOTTA; CAMARGO, 1993; CAMARGO; UNTERKIRCHER, TRAVASSOS, 1989; MARQUEZ et al., 2005; PUCCIA et al., 1986).

Gp70 was obtained from somatic antigen (SA), as reported by Rigobello et al. (2013) while hMM was purified from CFA according to Lenhard-Vidal et al. (2013).

ELISA with antigens at 1 µg/mL was performed only with the corresponding reactive CFA strain for each serum (see *ELISA to detect IgG against CFA and gp43*).

Overall reactivities were 23 (6.4%) and 25 (6.7%) participants with gp70 and hMM, respectively. Figure S1 shows reaction intensities with gp70 (A) and hMM (B). There were significant statistical differences between R and NR/NHS (p-value ≤ 0.01), but not between NR and NHS. Readings from R groups were statistically different between *P. brasiliensis* B339 and *P. americana* LDR3 for both antigens. It was not possible to perform statistical analysis with the R group of gp70 from *P. lutzii* LDR2, because only two participants were reactive to this antigen.

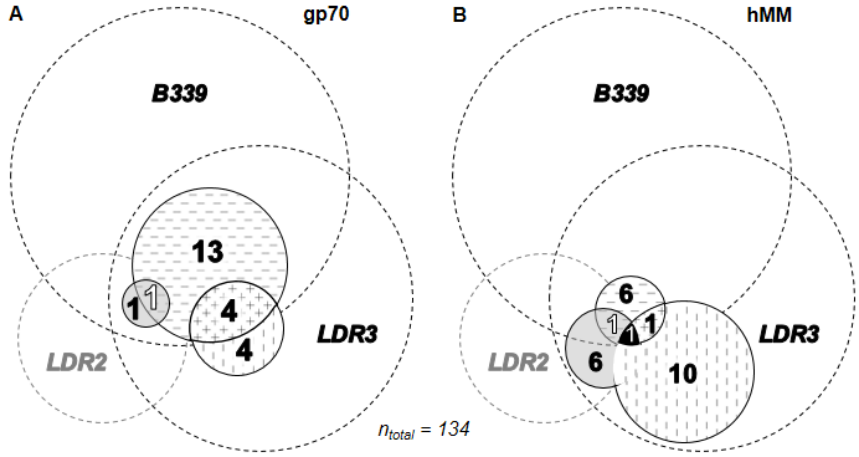
Figure S1 - ELISA to detect IgG against gp70 (A) and hMM (B) from *P. brasiliensis* B339, *P. americana* LDR3 and *P. lutzii* LDR2 of sera (1/200) from Guarapuava, PR, Brazil, previously reactive in CFA-ELISA: reactivity rate and readings (O.D. at 492 nm).



+: mean of each test. hMM: high molecular mass antigens (> 150 kDa). NHS: normal human sera (negative control). NR: non-reactive (below the cutoff of the test). R: reactive (above cutoff). Percentages refer to total number of participants (n = 359).

Although the number of reactive samples was low, there were still some participants who were reactive to multiple strains (Figure S2).

Figure S2 – Venn Diagrams illustrating the number of reactive participants in ELISA with gp70 (A) and hMM (B) from *P. brasiliensis* B339, *P. americana* LDR3 and *P. lutzii* (LDR2), still showing reactivity overlaps among strains.



Obs.: dashed circles represent the number of reactive samples for each strain in CFA-ELISA.

Gp70 from *P. brasiliensis* is mainly intracellularly localized (DE MATTOS GROSSO et al., 2003), so techniques employing rupture of cells to release SA were more suited to its production from *P. brasiliensis* (RIGOBELLO et al., 2013). However, CFA from *P. lutzii* LDR2 presented larger amounts of gp70 than SA (unpublished results). Even though gp70 from *P. lutzii* was strongly detected by the pool and five participants' sera (Figure 3), only two participants were reactive in ELISA, probably due to differences in presentation in different systems. As gp70 can be considered a marker of active disease (CAMARGO; UNTERKIRCHER; TRAVASSOS, 1989; DA SILVA et al., 2004), low antibodies levels can be expected during PCM infection. Similarities and differences between gp70 expressed by different species will require further study.

The same area of polydispersed high molecular weight components described by Puccia et al. (1986) and Marquez et al. (2005) has influenced the intensity of CFA reaction with PCM patients' sera and the reaction was stronger with hMM from *P. brasiliensis* B339 than with *P. lutzii* LDR2 (LENHARD-VIDAL et al., 2013). Greater reaction intensity in ELISA with hMM from *P. brasiliensis* B339 (Figure S1) was possibly due to the larger area of diffuse electrophoretic migration (> 150 kDa), if compared to smaller, more delimited bands from *P. lutzii* LDR2 in the same

region, besides the previously discussed geographic distribution of species. As hMM is partially composed of carbohydrate epitopes (unpublished results – see *"Artigo A"*), this may also influence cross reactivity with other fungi.

To our knowledge, this is the first report about antibodies anti-gp70 and anti-hMM from people with possible PCM infection, but these antigens do not seem suitable for use in PCM infection diagnosis.

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CONCLUSÕES GERAIS

Artigo A: anticorpos policlonais à *P. brasiliensis* (IgG de coelho anti-Pb18 e IgG/IgG2 de pacientes com PCM crônica) foram capazes de reconhecer antígenos de *P. lutzii* LDR2. Destaca-se que:

a) Áreas mais intensas da coloração de carboidratos corresponderam as regiões afetadas pela oxidação de carboidratos, sendo as glicoproteínas de alta massa os antígenos mais afetados.
b) Sobre *P. lutzii*:
    i. Ausência de gp43 no CFA avaliado;
    ii. Importante reação com antígenos de ≈ 75 kDa e de alta massa molecular (bandas mais delimitadas com > 200 kDa)
    iii. Área hMM diferente do encontrado com *P. brasiliensis* ou *P. americana* (duas bandas ao invés de área difusa de migração eletroforética);
    iv. Efeito mais forte do SMP, possivelmente devido a maior concentração de açúcares ou maior reatividade cruzada;
    v. Não foi possível identificar antígeno específico como seu marcador.

Artigo B: em relação à PCM infecção em Guarapuava, PR, houve:

a) Reatividade decrescente à *P. brasiliensis* > *P. americana* > *P. lutzii*.
    ✓ Misturas de antígenos de diferentes espécies poderão aumentar a cobertura de detecção PCM infecção em estudos epidemiológicos.
b) Baixa prevalência, se considerados os antígenos tratados com SMP.
    ✓ Sabendo-se que o SMP pode causar resultados falso negativos em pacientes com PCM doença, sugere-se utilizar antígenos totais sem tratamento com SMP para detecção de PCM infecção.
c) Baixa reatividade aos antígenos de *P. lutzii*:
    ✓ A espécie não deve ser nativa/predominante da região, mas indica a sua presença na região ou de epítopos em comum com as outras espécies.

APÊNDICES

APÊNDICE A – TCLE da pesquisa de PCM infecção

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Título da Pesquisa:

DIAGNÓSTICO LABORATORIAL/MONITORAMENTO DE TRATAMENTO DA PARACOCCIDIOIDOMICOSE NA REGIÃO NORTE E OESTE DO ESTADO DO PARANÁ

Prezado(a) Senhor(a):

Gostaríamos de convidá-lo(a) a participar da pesquisa acima intitulada, realizada pelo Laboratório de Imunologia Aplicada do Departamento de Ciências Patológicas/Centro de Ciências Biológicas da Universidade Estadual de Londrina, com cooperação de outras instituições do estado do Paraná. O objetivo da pesquisa é tentar melhorar as técnicas/exames usados para diagnosticar a paracoccidioidomicose, um tipo de doença que pode causar lesões principalmente em pulmão, pele e boca, mas também em qualquer outro lugar do corpo. Esta pesquisa avaliará a hipótese de que pessoas de diferentes localidades do estado podem ter diferentes resultados nos exames de laboratório feitos para esta doença.

1. PARTICIPAÇÃO NA PESQUISA. A sua participação é muito importante e ela se dará da seguinte forma: a coleta de urina, sangue e saliva será realizada nas instituições participantes desta pesquisa por pessoas especializadas. Todos os materiais usados são descartáveis e estarão devidamente esterilizados. Caso você tenha a forma pulmonar da doença, será solicitada também a amostra de escarro ou se houverem lesões de pele/mucosa, seu dentista/médico poderá fornecer uma amostra da biópsia. Os materiais coletados serão utilizados para obtenção de anticorpos, antígenos ou de fungos. Após a realização deste projeto, os materiais coletados ficarão à disposição para utilização em outras pesquisas sob coordenação e gerenciamento do Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina, desde que esta instituição venha futuramente apresentar um Regulamento Institucional para sua utilização. Todos os projetos que utilizarem estes materiais posteriormente a esse projeto deverão ser submetidos ao Comitê de Ética em Pesquisa para reanálise e consentimento da pesquisa a ser realizada. O(a) senhor(a) também será convidado à preencher um questionário com perguntas que poderão ajudar a entender o desenvolvimento da doença, tendo direito de recusar-se a responder qualquer pergunta que possa lhe causar constrangimento.

2. DESISTÊNCIA DA PARTICIPAÇÃO NA PESQUISA. Lembramos que a sua participação é voluntária: você tem a liberdade de não querer participar, de não responder perguntas do questionário e pode desistir de participar na pesquisa a qualquer momento, mesmo após ter cedido suas amostras ou respostas, sem nenhum ônus ou prejuízo para você. Se este for o caso, é só entrar em contato que os seus dados não serão utilizados nesta pesquisa.

3. CONFIDENCIALIDADE. Todas as informações que você fornecer ou que sejam conseguidas pelos exames realizados ou pelo preenchimento do questionário serão utilizadas somente para os fins desta pesquisa e serão tratadas com o mais absoluto sigilo e confidencialidade. Sua participação ficará em segredo e o seu nome não aparecerá em nenhum lugar, nem quando os resultados da pesquisa forem apresentados, de modo a preservar sua identidade.

4. BENEFÍCIOS. Informamos que o senhor não pagará nem será remunerado por sua participação, além de que o senhor não terá prejuízo. Por outro lado, caso já seja portador da doença, obterá o benefício de ter um diagnóstico da paracoccidioidomicose mais sensível, portanto mais eficaz (melhor), além de ter uma metodologia adicional para o acompanhamento do tratamento. Se for voluntário sem a doença e seus resultados dos exames forem positivos para paracoccidioidomicose, você será contatado pela equipe de pesquisa para novos esclarecimentos e será instruído a procurar um médico especialista. Indiretamente, os achados desta pesquisa poderão gerar formas de diagnóstico mais eficazes e rápidos da doença paracoccidioidomicose.

5. POSSÍVEIS RISCOS E DESCONFORTOS. Os procedimentos desta pesquisa apresentam um risco mínimo, com desconforto leve. A coleta de sangue pode trazer algum desconforto como uma pequena dor na hora da punção ou o desenvolvimento de hematoma (mancha roxa) no local da coleta de sangue, que desaparece dentro de alguns dias. A coleta dos outros materiais não costuma causar nenhum tipo de desconforto por não serem obtidos de forma invasiva. Se sentir qualquer tipo de constrangimento ao responder o questionário, poderá recusar-se a preenche-lo. Se você precisar de alguma orientação ou encaminhamento, por se sentir prejudicado por causa da pesquisa, ou se sofrer eventualmente algum dano decorrente da mesma, o pesquisador se responsabiliza pela reparação e pela assistência integral, imediata e gratuita.

6. ESCLARECIMENTOS. Caso você tenha dúvidas ou necessite de maiores esclarecimentos pode nos contatar: **Profa. Dra. Eiko Nakagawa Itano**, E-mail: itano@uel.br Endereço: Universidade Estadual de Londrina, CCB – Depto. de Ciências Patológicas, Laboratório de Imunologia Aplicada; Rodovia Celso Garcia Cid, PR 445 Km 380, Campus Universitário, CEP 86051-980, Londrina – PR; Telefone: (43) 3371-4469 ou (43) 9995-3488. Você também pode procurar o Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina: LABESC – Laboratório Escola, no Campus Universitário, telefone 3371-5455, e-mail: cep268@uel.br.

7. CONCORDÂNCIA NA PARTICIPAÇÃO. Se o(a) senhor(a) estiver de acordo em participar, deverá preencher e assinar este **Termo de Consentimento Livre e Esclarecido** que se segue em duas vias, sendo que uma ficará com você.

Pelo presente instrumento que atende às exigências legais, o Sr.(a) _____

_____, portador da identidade _____, declara que, após leitura minuciosa do *Termo de Consentimento Livre e Esclarecido*, teve oportunidade de fazer perguntas e esclarecer dúvidas que foram devidamente explicadas pelos pesquisadores, ciente dos serviços e procedimento aos quais será submetido e, não restando quaisquer dúvidas a respeito do lido e explicado, firma seu CONSENTIMENTO LIVRE E ESCLARECIDO em participar voluntariamente desta pesquisa. E, por estar de acordo, assina o presente Termo.

_____, _____ de _____ de 20_____.

Assinatura do participante _____
(ou impressão dactiloscópica)

Assinatura do pesquisador responsável _____

DADOS DO PARTICIPANTE

Data de Nascimento ____ / _____ / _____ Sexo ☐ Feminino ☐ Masculino

Telefone (__) _____ Celular (__) _____

Endereço _____

Bairro _____ Cidade _____

APÊNDICE B – Questionário da pesquisa sobre PCM infecção

QUESTIONÁRIO DA PESQUISA: **Diagnóstico laboratorial/monitoramento de tratamento da Paracoccidioidomicose na região norte e oeste do estado do Paraná**

Participante _____ Sexo: ( ) F ( ) M

Data de nascimento ___ / ___ / ___ Data da coleta __/__/____

1) Qual é a sua escolaridade?
( ) não frequentei a escola
( ) Ensino fundamental (antigo 1º grau) incompleto
( ) Ensino fundamental (antigo 1º grau) completo
( ) Ensino médio (antigo 2º grau) incompleto
( ) Ensino médio (antigo 2º grau) completo
( ) Ensino superior (faculdade) incompleto
( ) Ensino superior (faculdade) completo
( ) Pós-graduação incompleta
( ) Pós-graduação completa

2) Sempre morou em Guarapuava ou região?
( ) Sim ( ) Não

2a) Em quais lugares e qual idade tinha?

_____

_____

3) Já morou em zona rural? ( ) Não ( ) Sim

3a) Qual idade tinha? _____

4) Já trabalhou lidando com o solo?
( ) Não ( ) Agricultor(a)
( ) Jardineiro(a) ( ) Terraplanagem
( ) Pedreiro ( ) Outro: _____

4a) Qual idade tinha? _____

5) É fumante?
( ) Não
( ) Sim, fumo em média ____ cigarros ou ____ maços por dia, desde os ____ anos.
( ) Fumei por ____ anos, mas não fumo há ____ anos.

6) Consome bebidas alcóolicas? (aguardente, cerveja, etc.)
( ) Não
( ) Sim, uma vez por semana
( ) Sim, duas vezes por semana
( ) Sim, três ou mais vezes por semana

7) Possui animais no local onde mora?
( ) Não ( ) Galinha
( ) Cachorro ( ) Passarinho
( ) Gato ( ) Outros: _____

8) Tem algum tipo de alergia?
( ) Não ( ) Alergia cutânea (pele)
( ) Asma ( ) Rinite / Sinusite / Conjuntivite
( ) Alergia alimentar

9) Sua alergia acontece por causa de:
( ) Ácaros
( ) Pólen
( ) Animais: _____
( ) Alimentos: _____
( ) Remédios: _____
( ) Outros: _____

10) Já teve ou tratou alguma das seguintes doenças?
( ) Tuberculose
( ) Hanseníase
( ) Leishmaniose
( ) Doença nos pulmões (Doença Pulmonar Obstrutiva Crônica, DPOC)
( ) Tumor / câncer → Local do corpo: _____
( ) Não tive ou tratei nenhuma destas doenças.

11) Apresenta de forma persistente nos últimos meses algum destes sintomas?
( ) Grande perda de peso
( ) Inchaço de linfonodos (“ínguas”)
( ) Feridas no corpo ou boca que não saram
( ) Rouquidão
( ) Falta de ar (dispneia)
( ) Tosse com secreção (catarro)
( ) Tosse seca
( ) Nenhum dos sintomas listados.

Gostaria de fazer alguma outra observação? _____

*Visto do participante:*_____

APÊNDICE C – Resumo do artigo sobre PCM doença em Guarapuava

Esta pesquisa foi realizada por Caroline Geovana Bensberg, como Trabalho de Conclusão de Curso de Biomedicina na Faculdade Campo Real (Guarapuava, PR), sob orientação da Prof.ª Ms. Adriane Lenhard-Vidal.

PARACOCCIDIOIDOMICOSE EM GUARAPUAVA-PR:
LEVANTAMENTO EPIDEMIOLÓGICO

RESUMO

A Paracoccidioidomicose (PCM) é uma micose sistêmica causada por *Paracoccidioides* spp. Acredita-se que o contágio ocorra pela inalação de propágulos do fungo quando o solo é revolvido. A PCM pode se apresentar de forma aguda, uma situação mais grave que ocorre em crianças e jovens, ou de forma crônica, que ocorre em adultos e soma praticamente 90% dos casos. A PCM é restrita à América Latina, sendo endêmica no sul do Brasil. O objetivo desta pesquisa foi avaliar epidemiologicamente os casos de PCM doença no município de Guarapuava-PR. Foram utilizados dados dos prontuários de pacientes atendidos no Ambulatório Municipal de Pneumologia e Dermatologia Sanitária (AMPDS), que centraliza o tratamento de PCM no município. Avaliaram-se os pacientes registrados entre 1990 e 2016, coletando-se características gerais dos pacientes, como sexo, idade e características clinicas importantes. De 41 prontuários analisados, 39 foram confirmados como PCM, gerando uma média de 1,5 casos/ano, com a maior frequência no ano de 2003 (10 casos), seguido de 2007 (5 casos). O gênero masculino representou 74,4%, com uma proporção de 2,6 homens para cada mulher. A idade dos pacientes variou de 33 à 87 anos, com média de 54,3 ± 13,9 anos, com metade dos pacientes com idades entre 50 e 69 anos. A maioria dos pacientes (34) eram moradores da zona urbana no momento de diagnóstico. Sobre escolaridade, havia registro de analfabetismo apenas em três prontuários. Sobre profissão, houve relatos de trabalho como auxiliar de produção (2), doméstica, dona de casa, pedreiro, comerciante, lavrador e trabalhador de extração florestal. Havia relato apenas de um paciente etilista e um tabagista. As manifestações clínicas foram problemas respiratórios (tosse/dispneia 34, lesão pulmonar 3) e lesões de mucosa (5), ou seja, 100% dos pacientes apresentaram a forma crônica da doença. O exame laboratorial de pesquisa de fungos foi realizado ao menos uma vez para todos os pacientes. Houve baixa presença de co-morbidades: 3 casos de tuberculose, 4 casos de hanseniano, 1 caso de leishmaniose e sem presença de infecção por HIV. O tempo de tratamento dos pacientes teve uma média de 16 meses de duração até sua alta por cura. Como tratamento, 65,8% dos pacientes fizeram uso de Sulfametoxazol + Trimetoprim seguido de Itraconazol, 21,1% fizeram uso apenas de Sulfametoxazol + Trimetoprim e 13,1% apenas o uso de Itraconazol. Apenas um paciente fez uso de Anfoterecina B, seguido pelo uso de Itraconazol. Mesmo Guarapuava sendo uma cidade de economia basicamente agrícola e estar situada em região endêmica para PCM, a prevalência da doença foi pequena no período analisado, sendo as características gerais da doença semelhantes à outros trabalhos publicados. Ressalta-se a necessidade de padronização no preenchimento dos prontuários com informações sociodemográficas e de hábitos do paciente que podem auxiliar no diagnóstico e acompanhamento do paciente, além de permitir estudos epidemiológicos mais completos no futuro.

Palavras-chave: Epidemiologia; *Paracoccidioides* spp.*;* Micose Sistêmica.