FRANCIELE AYUMI SEMENCIO CHIYODA

ANTÍGENOS DE *PARACOCCIDIOIDES* SPP. NA URINA E SUA CORRELAÇÃO COM OS NÍVEIS SÉRICOS DE ANTICORPOS CONTRA *P. BRASILIENSIS* E *P. LUTZII* NA PARACOCCIDIOIDOMICOSE HUMANA

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

Orientadora: Profa. Dra. Eiko Nakagawa Itano

Londrina
2015

Catalogação elaborada pela Divisão de Processos Técnicos da Biblioteca Central da Universidade Estadual de Londrina

Dados Internacionais de Catalogação-na-Publicação (CIP)

C543a Chiyoda, Franciele Ayumi Semencio.
Antígenos de *Paracoccidioides* spp. na urina e sua correlação com os níveis séricos de anticorpos contra *P. brasiliensis* e *P. lutzii* na paracoccidioidomicose humana / Franciele Ayumi Semencio Chiyoda. – Londrina, 2015.
51 f. : il.

Orientadora: Eiko Nakagawa Itano.
Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina, Centro de Ciências Biológicas, Programa de Pós-Graduação em Patologia Experimental, 2015.
Inclui bibliografia.

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

CDU 616.993

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Londrina, 23 de abril de 2015.

Dedico este trabalho ao meu filho Francisco.

AGRADECIMENTOS

À minha orientadora professora doutora Eiko Itano Nakagawa pela inestimável generosidade, apoio e compreensão ao direcionar meu trabalho.

À Paula Leonello Álvares e Silva, Fabiana Filipin Rigobello, Tânia Maris Pedrini Soares da Costa, Adriane Lenhard Vidal, Fernando Cezar dos Santos, João Paulo Assolini, Cassia Reiko Takabayashi, Nilson de Jesus Carlos, Mári Sumigawa Kaminami, Luciene Airy Nagashima, Cláudia Yuri Akagi, Willian do Rosário Andrade Silva Cabral, Bruno Alexandre Sekiguchi, Shahzad Akbar Khan pela amizade e colaboração no desenvolvimento dessa dissertação.

Aos meus pais Mauro Shiguetoshi Chiyoda e Marcia Semencio, ao meu esposo Alfredo Pala Rodini, ao meu filho Francisco Semencio Chiyoda Pala Rodini, aos meus familiares e amigos que foram os maiores incentivadores para conclusão do Mestrado em Patologia Experimental.

Aos professores doutores Mário Augusto Ono e Elisa Yoko Hirooka, por aceitarem prontamente participar das bancas de qualificação e de defesa e pelas contribuições para correção deste trabalho.

À todos os Doscentes e colaboradores do Curso de Pós Graduação em Patologia Experimental da Universidade Estadual de Londrina, responsáveis pela minha formação acadêmica.

Aos discentes do curso e todos os colegas de turma que compartilharam suas experiências pessoais.

“Filho aproxime-se da sabedoria com toda sua alma, e com todas as suas forças observe os caminhos dela. Siga suas pegadas e a busque, quando você a alcançar não a deixe mais. A sabedoria se converterá em alegria para você”.

ECLESIÁSTICO 6-7

Este trabalho foi desenvolvido no Laboratório de Imunologia Aplicada, Departamento de Ciências Patológicas, Centro de Ciências Biológicas da Universidade Estadual de Londrina e contou com apoio financeiro da CAPES, PPSUS/Fundação Araucária, MEC PROEXT, PROEXT/UEL, PROPPG/UEL.

CHIYODA, Franciele Ayumi Semencio. **Antígenos de *Paracoccidioides* spp. na urina e sua correlação com os níveis séricos de anticorpos contra *P. brasiliensis* e *P. lutzii* na paracoccidioidomicose humana.** 2015. 51 f. Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2015

RESUMO

A paracoccidioidomicose (PCM) é uma micose sistêmica causada pela espécie fúngica *Paracoccidioides brasiliensis* (espécies cripticas S1, PS2, PS3 e PS4) e pela nova espécie, *Paracoccidioides lutzii*. O trabalho teve como objetivo determinar os níveis de antígenos totais de *Paracoccidioides* spp. e de gp43 (principal antígeno do *P. brasiliensis*) na urina de pacientes com PCM, correlacionando com os níveis de anticorpos séricos para *P. brasiliensis* (espécies críptica S1, cepa B339), (espécies críptica PS2, isolado LDR3) e *P. lutzii* (isolado LDR2). Na primeira etapa, foram utilizadas urinas de pacientes com suspeita de PCM (n= 44), para determinadação dos níveis de antígenos totais (CFA) foram utilizados anticorpos policlonais contra *P. brasiliensis* (B339) e contra *P. lutzii* (LDR2). Os níveis de gp43 foram determinados com anticorpos monoclonais contra gp43 de *P. brasiliensis* (ensaio imunoenzimático competitivo indireto- icELISA e de inibição- inhELISA), em paralelo procedeu-se a sorologia para PCM empregando ELISA indireto (iELISA) e imunodifusão (ID) com ExoAg B339. A positividade dos testes foi: 48% (anti-339) e 73% (anti-LDR2) de antígeno total na urina, 33,33% gp43 na urina, 87,5% sorologia por iELISA e 43,5% por ID. Não foi observada correlação entre o nível de antigeno total utilizando anticorpos anti-339 e anti-LDR2. Foram detectados casos de resultados negativos por sorologia (ELISA= 1, ID= 2) e com positividade para antigenos na urina. Na segunda etapa, foi calculada a correlação de Pearson (PCM n= 10) entre os níveis de antigenos totais ou de gp43 na urina e a reatividade sérica de IgG aos antígenos CFA ou de gp43 provenientes de *P. brasiliensis* S1, PS2 e *P. lutzii* (iELISA). Houve uma correlação positiva entre o nível de antígeno total determinado com anti-LDR2 e anticorpos a diferentes fontes de CFAs (S1, LDR2 e LDR3) (r >0,5), mas apenas com antigeno total determinado via anti-339 e reatividade de IgG sérica com CFA proveniente de LDR2 (r >0,5) e entre gp43 (µg/mL) e IgG anti-gp43 proveniente de LDR3 (r >0,5). Em conclusão, o diagnóstico serológico por iELISA seguido de ID é mais eficiente do que a detecção de antígenos urinários, mas o último pode aumentar a cobertura de diagnóstico, particularmente no caso de níveis baixos de anticorpos. A utilização de anticorpos contra o fungo LDR2 (*P. lutzii)* é mais eficiente na detecção de antígenos totais na urina que com anticorpos anti-Pb339 (*P. brasiliensis* S1) e as diferenças nas correlações entre os níveis de antígenos totais ou de gp43 com as reatividades de anticorpos a diferentes antígenos de *Paracoccidioides* spp. pode ser devido a diferenças entre as cepas infectantes.

Palavras-chave: Antigenemia. Antigenuria. Imunodiagnostico. Micose sistêmica.

CHIYODA, Franciele Ayumi Semencio. Antigens **of *Paracoccidioides* sp. in urine and its correlation with serum antibodies against *P. brasiliensis* and *P. lutzii* in human paracoccidioidomycosis.** 2015. 51 p. Master Thesis (Postgraduate degree in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2015.

ABSTRACT

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by *Paracoccidioides brasiliensis* (cryptic species S1, PS2, PS3 and PS4) as well as the new species, *Paracoccidioides lutzii*. This study evaluated the urinary *P. brasiliensis* total soluble antigens (CFA) and gp43 (main *P. brasiliensis* antigen) in patients with PCM, and correlate with the levels of serum antibodies against *P. brasiliensis* (S1= strain B339, PS2= clinical isolate LDR3) and *P. lutzii* (clinical isolate LDR2). First, urine from patients with PCM or suspected (n= 44) were evaluated for total antigens by using polyclonal antibodies against *P. brasiliensis* (S1= B339) and *P. lutzii* (LDR2) as well as gp43 levels by using monoclonal antibodies to *P. brasiliensis* gp43 (competitive indirect- icELISA and inhibition enzyme-linked immunosorbent assay- inhELISA), together with serology for PCM (indirect ELISA= iELISA and immunodiffusion= ID to B339 ExoAg). The positivity of the tests was: 48% (anti-339) and 73% (anti-LDR2) urinary total antigen, 33,33% urinary gp43, 87,5% serology by iELISA and 43,5% by ID. No correlation was observed between the total antigen level using anti-339 and anti-LDR2. Negative serology (iELISA= 1, ID= 2) and positive for antigens in the urine cases were detected. In the second step, Pearson's correlation was calculated (PCM n= 10) between the levels of urinary total antigens or gp43 and the sera reactivity CFA or gp43 derived from *P. brasiliensis* S1, PS2 and *P. lutzii* (iELISA). It was detected positive correlation between total antigen level determined with anti-LDR2 and IgG reactivity to all different CFAs sources (S1, LDR2, LDR3) ($r > 0.5$) but only with antigens levels determined by anti-339 and IgG reactivity to CFA from LDR2 ($r > 0.5$) and also only between gp43 (µg/mL) and IgG reactivity to gp43 from LDR3. Summering, the serological diagnosis by iELISA followed by ID is more efficient than detection of urinary antigens, but the latter could increase the diagnostic coverage, particularly in cases of low antibody levels. The use of antibodies against fungus LDR2 (*P. lutzii*) is more efficient than antibodies to Pb339 (*P. brasiliensis* S1) for urinary total antigens determinations and the differences in the correlations tests between the total antigen or gp43 levels and antibodies reactivity to different antigens of *Paracoccidioides* sp. may be due to differences in the infective strains.

Keywords: Antigenemia. Antigenuria. Immunodiagnostic. Systemic mycosis.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

Anti-IgG	Anticorpo contra imunoglobulina G
Anti-gp43	Anticorpo contra glicoproteína de 43.000Da
BAL	Lavado bronco alveolar
BHI	Ágar infusão cérebro e coração
B339	Cepa da espécie críptica S1 de *P. brasiliensis*
CEI	Contraimunoeletroforese
CFA	Antígeno solúvel- cell free antigen
°C	Graus Celsius
Da	Daltons
DO	Densidade óptica
ELISA	Ensaio imunoenzimático
icELISA	Ensaio imunoenzimático de competição indireto
inhELISA	Ensaio imunoenzimático de inibição
gp 43	Glicoproteína de 43 kDa
h	Horas
HC	Hospital das Clínicas
HCl	Ácido clorídrico
HIV	Vírus da Imunodeficiência Humana
IB	Imunoblot
IC	Imunocomplexo
IFI	Imunofluorescência indireta
ID	Imunodifusão
IgG	Imunoglobulina G
kDa	Quilodaltons
LDR1	Isolado da espécie críptica *P. brasiliensis* PS2
LDR2	Isolado da espécie de *P. lutzii*
MM	Massa molecular
µg	Microgramas
µl	Microlitros
Mab	Anticorpo monoclonal (*monoclonal antibody*)
ml	Mililitros
mM	Milimol

min	Minuto
M	Mol
N	Normal
ng	Nanograma
NHU	Urina humana normal
nm	Nanômetro
NPCM	Neuroparacoccidioidomicose
OPD	O-phenilenediamina
PAS	Ácido periódico de Shiff
PBS	Solução fosfato salina tamponada
Pb	Espécie *Paracoccidioides brasiliensis*
Pb	18 Cepa da espécie críptica S1 de *Paracoccidioides brasiliensis* 18
PCM	Paracoccidioidomicose
PS2	Espécie críptica de *Paracoccidoides brasiliensis* PS2
PS3	Espécie críptica de *Paracoccidoides brasiliensis* PS3
PS4	Espécie críptica de *Paracoccidoides brasiliensis* PS4
P. lutzii	Espécie *Paracoccidioides lutzii*
PMSF	Fluoreto de fenilmetilsulfonil *(phenylmethylsulfonyl fluoride)*
PR	Paraná
RPM	Rotações por minuto
S1	Espécie críptica de *Paracoccidoides brasiliensis* S1
SDA	Ágar saboraud dextrose
TOM	Meio complexo enriquecido com suco de tomate
UEL	Universidade Estadual de Londrina

SUMÁRIO

INTRODUÇÃO

Paracoccidioidomicose é uma micose sistêmica granulomatosa, provocada pelo fungo patogênico do gênero *Paracoccidioides* spp*.,* sendo descrita pela primeira vez por Adolf Lutz em 1908. A PCM pode ser causada por *P. brasiliensis* (BRUMMER et al., 1993, NEGRONI, 1993) e *P. lutzii* (THEODORO et al., 2012; TEIXEIRA et al., 2013). *P. brasiliensis* é composta por um complexo de quatro espécies crípticas (S1, PS2, PS3 e PS4). A espécie críptica S1, é representada por Pb18 e Pb339, encontrada no Brasil, Paraguai, Argentina, Venezuela e Peru, sendo isolada em tatus (*Dasypus novemcinctus)*, pingüins e no solo das regiões endêmicas. A espécie PS2 apresenta menor potencial virulento, porém, capaz de provocar a PCM em humanos e em modelos animais, distribuídas no Brasil e Venezuela. A espécie PS3 foi isolada em humanos e em tatus (*Dasypus novemcinctus)*, encontrados na Colômbia (MATUTE et al., 2006). A espécie PS4 foi recentemente identificada em isolados clínicos na Venezuela (SALGADO-SALAZAR et al., 2010).

A espécie *P. lutzii* é considerada monofilética, encontrada com maior frequência nas regiões centro-oeste, sudeste e norte do Brasil e no Equador (CARRERO et al., 2008; TAKAYAMA et al., 2009 THEODORO et al., 2012; TEIXEIRA et al., 2013).

Trata-se de uma micose restrita às regiões subtropicais da América Latina, sendo endêmica no Brasil, Colômbia, Venezuela e Argentina (SAN-BLAS et al., 2002). A literatura relata 80% dos casos de PCM no território brasileiro, tornando o País o maior centro endêmico da doença, distribuídos nas regiões Sul, Sudeste e Centro Oeste (BRUMMER et al., 1993; BLOTTA; CAMARGO, 1993), e concentrados nos estados do Paraná, São Paulo, Rio Grande do Sul, Rio de Janeiro, Minas Gerais (COUTINHO et al., 2002), Mato Grosso do Sul (PANIAGGO et al., 2003), Mato Grosso e Goiás (CERRI et al*.,* 1998). No Paraná, são registrados os maiores índices de mortalidade do Sul do Brasil (COUTINHO et al*.*, 2002). No Brasil a taxa de mortalidade foi de 1,45 casos por milhão de habitantes entre 1980 e 1995 (FORNAJEIRO et al., 2005).

Paracoccidioides spp. é termodimórfico, sendo leveduriforme na temperatura entre 35 a 37^{o}C (LACAZ et al., 2002). Macroscopicamente, as colônias apresentam aspecto rugoso e cerebriforme, visíveis a partir de 10 a 15 dias de cultivo. Microscopicamente os conídios são células globosas, com múltiplos brotamentos-exoesporulações, lembrando um “leme de navio”, apresentam parede celular dupla e refringente. Em temperatura ambiente, apresenta-se na forma micelial ou filamentosa, com crescimento lento, após 20 a 30 dias de incubação entre 20 a 26°C, produzindo colônias brancas, pequenas e irregulares, cobertas por um curto micélio aéreo, composta por hifas finas e septadas, sendo esta, a provável forma encontrada no ambiente (BRUMMER; CASTANEDA; RESTREPO, 1993).

Inquéritos epidemiológicos indicam que 70% da população de áreas endêmicas já foram expostas a este agente etiológico dessa micose, todavia apenas uma pequena porção desse número apresente alguma manifestação clínica (RESTREPO, 1985; MARQUES et al., 2007)

A infecção possivelmente ocorre pela inalação de propágulos fúngicos aéreos (micelio) que passa por conversão para fase leveduriforme nos pulmões e linfonodos, induzindo uma resposta no hospedeiro caracterizada pela formação de granulomas. Estes podem permanecer dormentes ou progredir para a doença, disseminando para baço, fígado, pele e mucosas (BRUMMER et al., 1993, NEGRONI, 1993).

Contudo a manifestação da doença está relacionada à imunidade celular de cada hospedeiro (SHIKANAI-YASUDA et al., 2006). Em indivíduos imunocompetentes a lesão regride e o quadro evolui para a cura. A imunossupressão causada pelo HIV/AIDS promove a característica de doença oportunista à PCM (MARQUES et al., 2010). No entanto em alguns casos, o microrganismo permanece latente, possibilitando manifestações clínicas após anos da infecção (BRUMMER et al., 1993; POLONELLI et al., 2000). Observa-se persistência do quadro clínico, regularmente com mais de seis meses de duração (MARQUES et al., 2007). Podem ocorrer também lesões no aparelho genital (BROOKS; BUTEL; MORSE, 2000). O fungo é capaz de penetrar a

barreira hematoencefálica por mecanismos ainda não estabelecidos, causando a neuroparacoccidioidomicose (NPCM) (PEDROSO et al., 2009).

A PCM é usualmente diagnosticada por exames radiográficos para a detecção de lesões pulmonares (SPOSTO et al., 1994). O diagnóstico clínico é realizado por análise de amostras biológicas como escarros, a partir da observação do material clínico clarificado com KOH 20%. Outros exames clínicos como análise da cultura do fungo, pela coloração da prata (Gomori-Grocott) ou PAS - ácido periódico de Shiff são técnicas utilizadas no diagnóstico clínico (BEHTLEM et al., 1999).

Para o isolamento de *P. brasiliensis* são utilizados o ágar Sabouraud e BHI (FISHER; COOK, 2001). As desvantagens são o risco de contaminação e o tempo de incubação prolongado, podendo variar de uma a três semanas (SANO et al., 2001). O exame direto também apresenta baixa sensibilidade na visualização do fungo (SALINA et al., 1998). Exames histopatológicos com o emprego da coloração de hematoxilina-eosina e impregnação pela prata também são utilizados no diagnóstico da PCM, no qual é possível visualizar a célula mãe, circundada por inúmeras células filhas, característica das múltiplas exoesporulações do *P. brasiliensis* (LACAZ et al., 1991).

Para complementar o diagnóstico direto, é utilizado o diagnóstico indireto, utilizando métodos imunológicos de detecção de anticorpos para a confirmação da doença.

Os métodos de imunodifusão dupla (ID), contraimunoeletroforese (CIE), imunofluorescência indireta (IFI), ensaio imunoenzimático (ELISA) e imunoblot (IB) (SHIKANAI-YASUDA et al., 2006), são disponíveis em diferentes serviços de referência e que o título de anticorpos específicos anti-*P. brasiliensis* tem correlação com a gravidade das formas clínicas, sendo mais elevados na forma aguda-subaguda da doença.

O principal antígeno de diagnóstico é a glicoproteína de 43.000-Da (gp43), uma das principais moléculas antigênicas liberadas na infecção causada pelo fungo da espécie *P. brasiliensis*; soro de mais de 90% de paciente com PCM reagem com a gp43 em ensaios de imunodifusão (ID), e 100% dos soros foram positivos em ensaios de *immunobloting* (BLOTTA; CAMARGO, 1993; GÓMEZ et al., 1997; SOMENSI et al., 1997).

Valle et al. (2001), estudando 245 pacientes não tratados, obtiveram 90,2% de sensibilidade no teste de ID empregando substrato filtrado de cultura na fase leveduriforme, concentrada de pool de cepas de *P. brasiliensis*.

O diagnóstico por ELISA apresenta sensibilidade de até 100%, porém apresenta especificidade menor que a ID, o que exige uma criteriosa padronização e interpretação de resultados positivos (DEL NEGRO; PEREIRA; ANDRADE, 2000; SHIKANAI-YASUDA et al., 2006), ensaios sorológicos podem apresentar reação cruzada com epítopos do *Histoplasma capsulatum* e até mesmo apresentar falso negativo para pacientes imunocomprometidos (PUCCIA; TRAVASSOS, 1991).

A quantificação da glicoproteína de 43-kDa de *P. brasiliensis* no soro por inhELISA é um método sensível e pode ser utilizado no monitoramento de pacientes com PCM em tratamento (MARQUES DA SILVA et al., 2004). A metodologia inhELISA para detecção de gp43 em amostras de fluído de lavado bronco alveolar (BAL) mostrou a alta sensibilidade e especificidade (100%) do ensaio, uma vez que o fluido BAL é extremamente diluído (MARQUES DA SILVA al., 2003).

Em suma, a sorologia é amplamente utilizada para o monitoramento do paciente em tratamento, todavia ocorrem resultados falso- negativos, por baixo nível de anticorpos ou pelos altos níveis de anticorpos que permanecem mesmo após um período longo de tratamento, dificultando o critério de cura (MARQUES et al., 2004; SHIKANAI-YASUDA et al., 2006). Diante disso, métodos de detecção de antígenos utilizando diferentes amostras biológicas estão sendo introduzidos. A antigenúria proporciona benefícios quanto à antigenemia, pois o material clínico pode ser coletado de forma simples, não invasiva e em abundante volume amostral. A coleta da urina é menos invasiva em relação às técnicas tradicionais como a punção venosa. Todavia existem poucos trabalhos de pesquisa de antígenos na urina. A detecção de antígenos do fungo na urina contribuiria com a rapidez no diagnóstico da PCM de forma não invasiva. Como a PCM é altamente incapacitante e apresenta diversas comorbidades, o diagnóstico precoce evitaria a progressão da doença. Além disso, considerando a variabilidade genética dos agentes da PCM, o estudo

envolvendo resposta imunológica a estes diversos agentes poderia contribuir com uma melhor compreensão da patogênese da PCM.

OBJETIVOS

1 OBJETIVO GERAL

Determinar níveis de antígenos totais e de gp43 em urina de pacientes com paracoccidioidomicose e correlacionar com os níveis séricos de anticorpos aos antígenos de *P. brasiliensis* e *P. lutzii.*

2 OBJETIVOS ESPECIFICOS

2.1 Determinar níveis de antígenos totais de *Paracoccidioides* spp. em amostras de urina de pacientes utilizando anticorpos policlonais anti-*P. brasiliensis* e anti-*P. lutzii.*

2.2 Analisar os níveis de gp43 em urina de pacientes com PCM ou suspeita de PCM.

2.3 Avaliar a correlação dos níveis urinários de antígenos totais de *Paracoccidioides* spp. com os títulos séricos de IgG anti-*P. brasiliensis* determinados por ELISA, em pacientes com PCM ou suspeita de PCM.

2.4 Avaliar a correlação dos níveis urinários de gp43 com os títulos séricos de IgG anti-*P. brasiliensis* determinados por ELISA, em pacientes com PCM ou suspeita de PCM.

2.5 Avaliar a correlação dos níveis urinários de antígenos totais de *P. brasiliensis* com os títulos séricos obtidos por imunodifusão, em pacientes com PCM ou suspeita de PCM.

2.6 Avaliar a correlação dos níveis urinários de gp43 com os títulos séricos obtidos por imunodifusão, em pacientes com PCM ou suspeita de PCM.

2.7 Avaliar a correlação dos níveis urinários de antígenos totais com a reatividade sérica de IgG anti-antígenos solúveis provenientes de *P. brasiliensis* B339 (S1), LDR3 (PS2) e LDR2 (*P. lutzii*), em pacientes com PCM.

2.8 Avaliar a correlação dos níveis urinários de gp43 com a reatividade sérica de IgG anti-antígenos solúveis provenientes de *P. brasiliensis* B339 (S1), LDR3 (PS2) and LDR2 (*P. lutzii*), em pacientes com PCM.

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ARTIGO: Antigens levels of *Paracoccidioides* sp. in urine and its correlation with serum antibodies against *P. brasiliensis* and *P. lutzii* in human paracoccidioidomycosis

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ABSTRACT

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by *Paracoccidioides brasiliensis* (S1, PS2, PS3 and PS4) as well as the new species, *Paracoccidioides lutzii*. This study was designated to evaluate the urinary *P. brasiliensis* total soluble antigens (CFA) and gp43 (main *P.brasiliensis* antigen) in patients with PCM, and to correlate with the levels of serum antibodies against *P. brasiliensis* (S1= B339, PS2= LDR3) and *P. lutzii* (LDR2). First, urine from patients with PCM or suspected (n= 44) were evaluated for total antigens by using polyclonal antibodies to *P. brasiliensis* (S1= B339) and *P. lutzii* (LDR2) as well as gp43 levels by using monoclonal antibodies to *P. brasiliensis* gp43 (competitive indirect and inhibition enzyme-linked immunosorbent assay= icELISA and inhELISA), together with serology for PCM (indirect ELISA= iELISA and immunodiffusion= ID to B339 ExoAg). The positivity of the tests was: 48% (anti-339) and 73% (anti-LDR2) urinary total antigen, 33,33% urinary gp43, 87,5% serology by iELISA and 43,5% by ID. No correlation was observed between the total antigen level using anti-339 and

anti-LDR2. Negative serology (iELISA= 1, ID= 2) and positive for antigens in the urine cases were detected. In the second step, Pearson's correlation was calculated (PCM n= 10) between the levels of urinary total antigens or gp43 and the sera reactivity CFA or gp43 derived from *P. brasiliensis* S1, PS2 and *P. lutzii* (iELISA). It was detected positive correlation between total antigen level determined with anti-LDR2 and IgG reactivity to all different CFAs sources (S1, LDR2 and LDR3) (r >0.5) but only with antigens levels determined by anti-339 and IgG reactivity to CFA from LDR2 (r >0.5) and also only between gp43 (µg/mL) and IgG reactivity to gp43 from LDR3. Summering, the serological diagnosis by iELISA followed by ID is more efficient than detection of urinary antigens, but the latter could increase the diagnostic coverage, particularly in cases of low antibody levels. The use of antibodies to fungus LDR2 (*P. lutzii*) is more efficient than antibodies to Pb339 (*P. brasiliensis* S1) for urinary total antigens determinations and the differences in the correlations tests between the total antigen or gp43 levels and antibodies reactivity to different antigens of *Paracoccidioides* sp. may be due to differences in the infective strains

Key Words: Antigenemia, antigenuria, immunodiagnostic, systemic mycosis.

1 INTRODUCTION

Paracoccidioidomycosis (PCM), caused by a dimorphic fungus, is one of the most important systemic mycoses in Latin America (BRUMMER et al., 1993). According to McEwen et al. (1995), approximately 10 million people are infected with this fungus, of which 2% may develop the disease. This development of disease is dependent on factors such as the virulence of the pathogen, genetic susceptibility and the immune status of the host (FRANCO 1987; SAN-BLAS et al., 2002).

PCM is caused by two fungal species *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii*. The cryptic species *P. brasiliensis* is composed by of a complex named S1, PS2, PS3 and PS4. The S1 cryptic species are represented by Pb18 or Pb339, is distributed in Brazil, Paraguay, Argentina, Venezuela and Peru, while PS3 has been described only in Colombia. PS2 has less potential virulence, but all three pathogenic species are capable of causing the PCM in humans and in animal models (MATUTE et al., 2006).

The PCM it is classified as infection and disease, which is then subdivided into acute (AF) and chronic form (CF). CF is more frequent in male older than 40 years, and it ranges from a benign and localized (unifocal) to a severe and disseminated (multifocal) disease (FRANCO et al., 1987; BLOTTA et al.,1993; BRUMMER et al., 1993; MCEWEN et al., 1995).

Due to the wide range of clinical manifestations, laboratory diagnosis is important for the diagnosis and follow-up establishment. Depending on the site of the infection, direct visualization of the fungus in clinical and biopsy specimens or its isolation by culture has been used for diagnosis (CAMARGO et al., 1988). But more frequently is performed serological test for detection of antibody against gp43, main *P. brasiliensis* antigen recognized by virtually all sera from infected patients, followed by gp70 recognized by 96% of sera from PCM patients (BLOTTA; CAMARGO, 1993). Moreover, methods for detection or determination of antigen levels in biological fluids have been developed. They are considered more effective than detection of antibodies for diagnosis in immunocompromised patients, as well as more efficient in monitoring treatment (GOMEZ et al., 1997; SALINA et al., 1998; MARQUES DA SILVA et al., 2006)

Current study determined the levels of *P. brasiliensis* total soluble antigens and gp43 levels in urine of patients with PCM and correlated them with serum antibodies to *P. brasiliensis* (S1 and PS2) and *P. lutzii* antigens.

2 MATERIAL AND METHODS

2.1 Samples

For first group it were used serum and urine samples from 44 patients suspected of PCM and 10 negative control samples and in the second group 10 chronic PCM patients (unifocal and multifocal) not-yet treated or currently under treatment. A total of 44 samples were used (obtained between 2002 to 2014), 9 from female and 35 from male with a mean age of 52 years old, treated at Clinics Hospital from at Londrina State University Clinical Hospital (Londrina, state of Paraná, Brazil).

2.2 Fungal strains

Yeast cells were maintained by sub-culturing at 35ºC with 5-day intervals and were used for antigen production. The strains were: (1) *P. brasiliensis* strain B339, cryptic species S1 (typical; IFM 41630) (MATUTE et al., 2002); (2) *P. brasiliensis* LDR3, cryptic species PS2 (typical; IFM 54649) (TAKAYAMA et al., 2010) and (3) *P. lutzii* LDR2 (Pb01-like; IFM 54648) (TAKAYAMA et al., 2010).

2.3 Cell-free antigen (CFA)

CFA was obtained from 3 strains (B339; LDR3 and LDR2) according to the method described by Camargo et al. (1991), modified by addition of 2.5 mM phenylmethylsulfonyl fluoride (PMSF) protease inhibitor to the supernatant, which was stored at -80 ºC until use.

2.4 Exoantigen (ExoAg) and gp43

A lyophilized ExoAg from *P. brasiliensis* B339 was prepared from a yeast-phase culture of *P. brasiliensis* according to Camargo et al. (1998) and gp43 was purified as previously described (MARQUES DA SILVA et al., 2003). The protein concentration was determined using the Folin method with bovine serum albumin as the standard, and stored at -80ºC.

2.5 Inhibition ELISA for detection of *P. brasiliensis* gp43 in urine

Inhibition-ELISA (inhELISA) was performed as previously described by Marques da Silva et al. (2003). Briefly, inhibition standard curve was constructed by adding different of *P. brasiliensis* gp43 (from 1 ng up to 30 µg/mL) in 100 µL of normal urine and then adding 100 µL of the standardized concentration of monoclonal antibody (17BmAb) anti-gp43 (10 µg/mL). Urine samples (100 µL) were added to 100 µL of MAb anti-gp43. Normal urine was used as a negative control. Polystyrene plates (Corning Costar Co., Corning, USA) were coated with 500 ng of gp43 in carbonate buffer (pH 9.6) per well (100 µL/well) for 18h at 4ºC. After, the plates were blocked by incubation with 200 µL per well of 1% bovine serum albumin in PBS, for 1 h at 37ºC; washed 3 times and then 100 µL from inhibition standard curve, samples and controls were added per well and allowed to stand for 2 h at 37ºC. After washing 3 times, 100 µL of anti-mouse IgG-peroxidase labeled antibody (Sigma, Sigma Chemical Co., St. Louis, MO, USA) was added to each well at 1:4000 dilution, and the plates were incubated for 1 h at 37ºC. After further washings, the reaction was developed with a solution of *o*-phenylenediamine (OPD) (Sigma) and H_2O_2. The reaction was stopped with 4N H_2SO_4 after 10 min of incubation in the dark. Optical densities (O.D.) were measured at 492 nm on a Multiskan EX microplate reader (Labsystems, Helsinki, Finland). Concentrations of gp43 in the urine were calculated based on the inhibition standard curve.

2.6 Indirect competitive ELISA (icELISA) for detection of *P. brasiliensis* CFA in urine.

Polystyrene plates (Corning Costar Co., Corning, USA) were coated with 25 µg/mL of CFA in carbonate buffer (pH 9.6) per well (100 µL/well) for 18h at 4ºC. The plates were blocked as described above. After washing, 50 µL of standard solution, samples and controls were incubated with an equal volume of polyclonal antibody against CFA produced in rabbit (25 µg/mL) for 3 h at 37ºC. After a new cycle of washes, 100 µL anti-rabbit IgG-peroxidase labeled antibody (A1949, Sigma Chemical Co., St. Louis, MO, USA) were added to each well at 1:4000 dilution and incubated for 1,5h at 37°C, followed by the addition of OPD and the other steps as described in the previous item. The concentrations of *P. brasiliensis* CFA in the urine were calculated based on the standard curve (6 ng – 100 µg/mL), plotting percentage of binding and the log of CFA concentration.

The percentage of binding was calculated from equation: Binding %= (A+/ A-) x 100, where that A+ was the mean absorbance in the presence of sample or CFA standard and A- was the mean absorbance in their absence. The cutoff point (3.16) was fixed as the mean plus two standard deviations of the negative control readings, and CFA levels expressed as µg protein/ml.

2.7 PCM serology using indirect ELISA and Immunodiffusion

Polystyrene plates (Corning Costar Co., Corning, USA) sensitized with ExoAg from *P. brasiliensis (*B339) and *P. brasiliensis* (LDR2) and was incubated in duplicates with serum samples (diluted 1/100, 1/200, 1/400, 1/800) and then with conjugate goat anti-human IgG-peroxidase (Sigma A-8775 Sigma Chemical Co., St. Louis, MO, USA) at 1:4000 dilution (100µL/well). The reaction was evidenced by addition of OPD substrate solution and the absorbance read at 492nm in a Multiskan EX Reader (Labsystems, Helsinki, Finland). Only titers above 1/200 were considered significant, according to previous standardization. For the immunodiffusion (ID), glass slides (2.5 x 7.5 cm) were covered with a 3.0 mm thick layer of 1% agar according with seven wells (one central and six peripheral). The *P. brasiliensis* ExoAg was applied to the central well, and samples not diluted and diluted from 1:2 up to 1:32 were applied in the surrounding wells. The samples were incubated for 24 h and stained with starch black (amido black).

2.8 Indirect ELISA for IgG anti-CFA and anti-gp43 from *P. brasiliensis* (S1), PS2 (LDR3) and *P. lutzii* (LDR2)

High affinity 96 wells high-affinity 96-well polystyrene plates (Costar, Corning Incorporated, Corning, NY, USA) were coated with CFA according to Lenhard – Vidal et al. (2013) or gp43 from each *Paracoccidioides* sp. at 1µg/mL (100µL/well). Unbound sites were blocked with PBS-Tween-5%PBS-Tween 5 % skim milk and plates incubated (2h, 35ºC) with serum samples diluted 1:400. Anti-human IgG-peroxidase labeled antibody (Sigma A6029 Sigma Chemical

Co., St. Louis, MO, USA) was added at 1:4000 dilution and incubated again for 1.5h followed by the addition of OPD as described in the previous items.

2.9 Statistical analysis

Mann Whitney test was used for comparisons between "two samples", one-way ANOVA was performed with the Tukey test for multiple comparisons. Significance was established at p value ≤ 0,05. The Pearson correlation was performed adopting positive correlation when r ≥ 0.5. Statistical analyzes were performed using GraphPad Prism software v. 6:01 for Windows *(GraphPad Software, La Jolla California USA, www.graphpad.com).* The construction of the standard curve and the calculation of concentrations were performed using Microsoft Excel 2010 software (Microsoft Office Professional Plus 2010).

3 RESULTS

3.1 CFA, gp43 levels in urine samples and correlation analysis

The standard inhibition curve (Figures 1A, 2A) built by the variations in antigen concentration measured of the OD values for CFA by using polyclonal antibodies to *P. brasiliensis* (S1= B339) (R^2= 0,9717) and using polyclonal antibodies to *P. lutzii* (LDR2) (R^2= 0.9846), were employed to determine the concentration of total antigens from *Paracoccidoides* sp. in each patient sample tested. The levels of total soluble antigens determined by antibodies to *P. brasiliensis* (Figure 1B) and by antibodies to *P. lutzii* (Figure 2B) levels were expressed as µg protein/ml, were higher in urine of patients suspected of PCM (n= 44) than health control (n=10) $P<0.05$. No correlation between total soluble antigens determined by antibodies to *P. brasiliensis* and to *P. lutzii* was observed (r= 0,2254) (Figure 3). The levels of gp43, were higher in urine of patients suspected of PCM (n= 44) than health control (n= 10) $P<0.05$. No correlation between CFA and gp43 levels was observed (r= 0.0936) (Figure 6).

3.2 *Paracoccidoides* sp. total antigens and gp43 levels in urine of patients presenting distinct serum titer by ELISA or ID and correlation analysis, as previously established, the significant titer for ELISA using ExoAg is 1/200.

Figure 4 show the levels of total antigens (CFA) determined by using polyclonal antibodies to *P. brasiliensis* (S1= B339) in urine of patients suspected of PCM (n= 44) who presented different titers of antibodies anti-ExoAg from *P. brasiliensis* in ELISA (Figure 4A) and by ID (Figure 4B). Figure 5 show the levels of total antigens (CFA) determined by using polyclonal antibodies to *P. lutzii* (LDR2) in urine of patients suspected of PCM (n= 44) who presented different titers of antibodies anti-ExoAg from *P. brasiliensis* in ELISA (Figure 5A) and by ID (Figure 5B). Figure 7 show the levels of gp43 in urine of patients suspected of PCM (n= 44) who presented different titers of antibodies anti-ExoAg from *P. brasiliensis* in ELISA (Figure 7A) and in ID (Figure 7B). No correlation was observed in all sub-groups of distinct titer ELISA (considering O.D. at 492nm in each titer) with gp43 levels ($r < 0.5$). Data not shown.

3.3 Pearson's correlation analysis between urine *Paracoccidoides* sp. total antigens levels and serum IgG reactivity to CFA from *P. brasiliensis* B339 (S1), CFA from LDR3 (PS2) and CFA from LDR2 (*P. lutzii*) in PCM patients

A significant positive correlation was observed with urinary *Paracoccidoides* sp. total antigen level determined by using antibodies to *P. lutzii* (LDR2) and serum IgG reactivity to CFA from *P. brasiliensis* B339 (S1) ($r= 0.5972$), from *P. brasiliensis* LDR3 (PS2) ($r= 0.6100$) and from LDR2 (*P. lutzii*) ($r= 0.7586$) (Figure 8). A significant positive correlation was observed only with urinary *Paracoccidoides* sp. total antigen level determined by using antibodies to *P. brasiliensis* B339 and serum IgG reactivity to CFA from LDR2 (*P. lutzii*) ($r= 0.5731$) (Figure 9) in group of PCM patients (n= 10).

3.4 Pearson's correlation analysis between urine gp43 levels and serum IgG anti-gp43 from *P. brasiliensis* B339 (S1), gp43 from LDR3 (PS2) and gp43 from LDR2 (*P. lutzii*) in PCM patients

No correlation between CFA and gp43 levels was observed (r= -0,2909) (Figure 10). A significant positive correlation was observed only with urinary gp43 level and serum IgG reactivity to gp43 from *P. brasiliensis* LDR3 (PS2) (r= 0.5428) and not with *P. brasiliensis* B339 (S1) (r= 0.1828) or with LDR2 (*P. lutzii*) (r= 0.4397) PCM patients (n= 10) (Figure 11).

4 DISCUSSION

There were some limitations in serology by antibody detection in PCM, due to some cases of false negative results, which depends on the immune status of the patient or otherwise cases of persistence of antibodies for a long period after treatment, making it hard to establish diagnosis or to define a cure criteria. In this context, the tests to detect circulating antigens have been introduced for diagnostic purposes, and particularly for monitoring treatment (MENDES GIANNINI et al., 1994; SALINA et al., 1998; MARQUES et al., 2004). Among these, urine samples were useful in determining systemic antigens levels because the clinical material can be collected easily and abundantly. Besides, urine collection is certainly less invasive than traditional techniques, such as venipuncture. However, there is few data in the literature about antigenuria in PCM.

In the current research, *Paracoccidioides* sp. total soluble antigens levels were evaluated in urine of patients with PCM or suspect of PCM by using polyclonal antibodies to *P. brasiliensis* B339 and antibodies to *P. lutzii* (LDR2) by icELISA. As not expected, higher positivity (73%) was observed when it was used antibodies to LDR2 than when used antibodies to B339 (48%) (Figures 1, 2). In Brazil, *P. brasiliensis* S1 is the most widely distributed species, including our south region and on the other hand, *P. lutzii* occurs predominantly, though not exclusively, in the western central region of Brazil (TEIXEIRA et al., 2009; THEODORO et al., 2012). Salina et al. (1998) found 75% of positivity by using anti-total antigen of *P. brasiliensis.* Therefore in accordance to these authors if we consider only positivity detected by antibodies to LDR2.

The presence of *P. brasiliensis* gp43 in urine has been demonstrated by immunoblotting in a patient with the acute form of PCM (MENDES GIANNINI et

al., 1994) or in concentrated urine samples from patients with chronic PCM (SALINA et al., 1998). The authors considered gp43 and gp70 as important markers for monitoring the treatment of PCM due to a correlation of their decrease with clinical improvement after antifungal treatment and their increased reactivity in samples collected during relapses.

The levels of urinary gp43 of this study, determined by inhibition ELISA, resulted in a 33,33% positivity (Figure 6), lower than CFA, as expected considering the presence of other components in CFA preparations (CARLOS et al., 2014) that could have reacted with the antibodies used in this assay. Meanwhile, this result is lower than the one cited by Camargo (2008), which had a mean gp43 of 8.65 µg/mL in PCM patients' urine, with 87.5% of sensitivity. Our lower percentage of positive urines for gp43 is possibly due to the heterogeneity of PCM patients included in the current study (as various stages of treatment and the inclusion of suspected patients).

According to the literature data, gp43 level in either serum or urine decrease with efficient treatment (MENDES GIANNINI et al., 1989; SALINA et al., 1998; MARQUES et al., 2004), and most of our patients were under treatment.

As expected, there was no correlation between the urinary levels of total antigens or gp43. This is due to the involvement of many others antigens in urine samples, such as gp87, gp70 and others (GÓMEZ et al., 1997; SALINA et al.,1998). It can be also explained by different PCM agents, which are described as low or high gp43 producers (MACHADO et al., 2013).

Even though the analysis were carried out using different groups of patients with diverse antibodies titers by ELISA or ID, there were no differences in the levels of urinary CFA (determined by anti-B339 or anti-LDR2) or gp43 between groups. This possibly results from the heterogeneity of patients presenting low or high levels of antibodies. Antibody levels decrease with treatment (CAMARGO, 2008) and high antibody levels are associated with disease severity in human PCM or in experimental animal models (MENDES-GIANNINI et al., 1990; SINGER-WORMS et al., 1993). Thus, it would be ideal to work with a homogeneous group of patients, but this was not possible due to the reduced number of subgroups of PCM patients.

Interestingly, in this study patients with high levels of total urinary antigens or gp43 had negative PCM serology by iELISA and ID. This can be explained by early disease stage (prior to antibodies production), infection by a different PCM agent used in the tests or immune suppressed patient, which requires further study. There are reports in the literature of some PCM agents which produce low levels of gp43 (MACHADO et al., 2013) and about low levels of antibodies to *P. brasiliensis* in cases of patients with PCM associated with other diseases, such as HIV infections, cancer and transplantation (SHIKANAI-YASUDA; MARQUES, 1994). Although the percentage of total antigen positivity with anti-B339 antibody was smaller, as mentioned above, the use of antibody allowed the detection of three cases of negative serology by ID (Figure 4) while with anti-LDR2 only one case was detected (Figure 5). The highest detection was observed with gp43 with 4 ID negative cases (Figure 7). Together with of the results already we discussed earlier, suggests that for greater efficiency in diagnosis is important to use varieties of antibodies.

The presence of anti-gp43 IgG suggests the presence of gp43 in the corresponding infectious agents, we calculated correlations between urinary gp43 or CFA levels and serum IgG reactivity with CFA or gp43 from *P. brasiliensis* B339 (S1), LDR3 (PS2) and *P. lutzii* (LDR2). There was a positive correlation for gp43 in urine and anti-gp43 IgG of *P. brasiliensis* LDR3 (PS2) and total urine antigen level and IgG to total antigens from *P. lutzii* (LDR2), but not for B339 (S1), considered as possibly the most frequent in our region by LENHARD-VIDAL et al. (2013). But the best correlation was observed with total antigen level determined with antibodies to LDR2 and reactivity with all distinct sources of CFA (Figure 9). This suggests the possible involvement of various agents of PCM in our group of patients, including *P. lutzii*, which requires further study.

The investigation of urinary antigens is interesting because they are soluble and free, which may represent more clearly the real level of circulating antigens in PCM patients. In addition, in the free form they could act more efficiently as a virulence factor, what requires investigations. Gp43 acts as a laminin receptor, favouring the adhesion, colonization and propagation of the pathogen (VICENTINI et al., 1994; GESZTESI et al., 1996; MENDES-GIANNINI

et al., 2006) and like gp70, it presents immunosuppressive effect on lymphocytes and macrophages *in vitro* (BERNARD et al., 1997; GROSSO et al., 2003). In the serum, they may also be found free and as immune complexes (IC) (UNTERKIRCHER et al., 1996), which will depend on the host immune response. In the IC form, its virulence sites may be blocked, but it can also cause tissue damage by deposition of immune complexes in tissues.

Considering the existence of several PCM agents, it becomes important to investigate the various antigens in urinary secretion and if they are dependent or not on the PCM agent. These new data could help to better understand the pathogenesis and to develop a more effective diagnosis and monitoring of treatment of PCM.

In conclusion, the antibodies detection by iELISA followed by ID is more efficient than detection of urinary antigens, but the latter could increase the diagnostic coverage, particularly in cases of low antibody levels.

No correlation between CFA and gp43 levels suggests that some antigens present or the absence of gp43 in CFA contributes as antigens in urine and this may be due to differences in the infective strains, which requires further research.

Summering, the serological diagnosis by iELISA followed by ID is more efficient than detection of urinary antigens, but the latter could increase the diagnostic coverage, particularly in cases of low antibody levels. The use of antibodies against fungus LDR2 (*P. lutzii*) was more efficient than antibodies against Pb339 (*P. brasiliensis* S1) for urinary total antigens determinations and the differences in the correlations tests between the total antigen or gp43 levels and antibodies reactivity to different antigens of *Paracoccidioides* sp. may be due to differences in the infective strains.

5 ACKNOWLEDGMENTS

This work was supported by PPSUS/Fundação Araucária/PR, CAPES, MEC/PROEXT.

6 DISCLOSURES

The authors have no financial conflict of interest.

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FIGURES

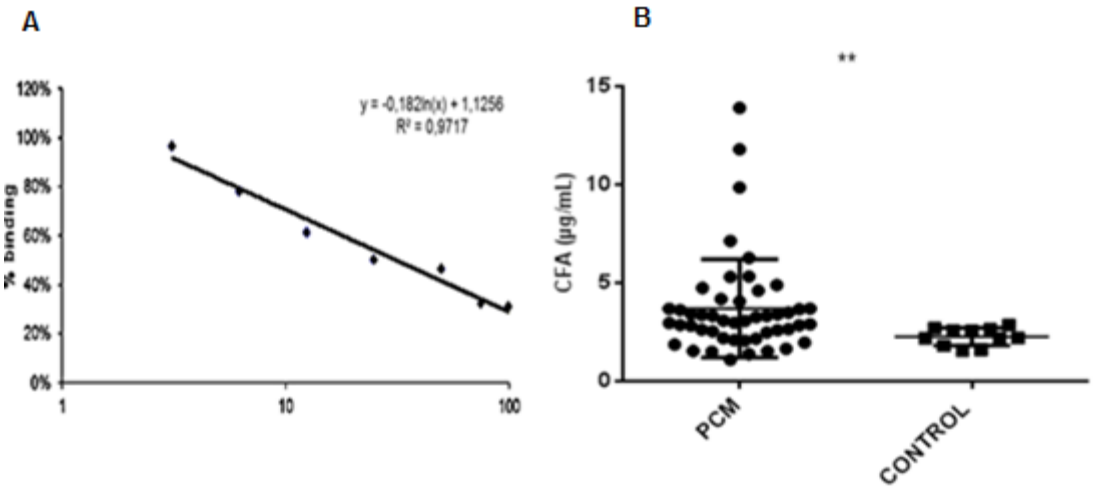

Figure 1. Indirect competitive ELISA for total soluble *Paracoccidioides* sp. antigens in urine, using immune serum against *P. brasiliensis* B339. 1A. Standard inhibition curve by the variations in antigen concentration of the OD values for CFA (R^2= 0,9717) was used to determine the concentration of CFA in each patient urine sample. 1B. PCM: patients suspected of PCM and PCM patients versus negative control (P<0.05).

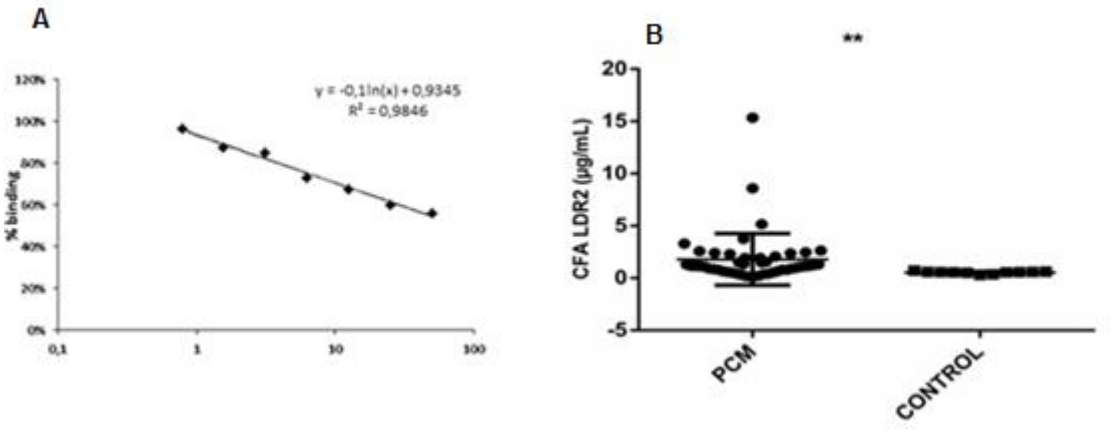

Figure 2. Indirect competitive ELISA for total soluble *Paracoccidioides* sp. antigens in urine, using immune serum against *P. lutzii* (LDR2).

2A. The standard inhibition curve by the variations in antigen concentration of the OD values for CFA (R^2= 0,9846) was used to determine the concentration of CFA in each patient urine sample. 2B. PCM: patients suspected of PCM and PCM patients versus negative control (P<0.05).

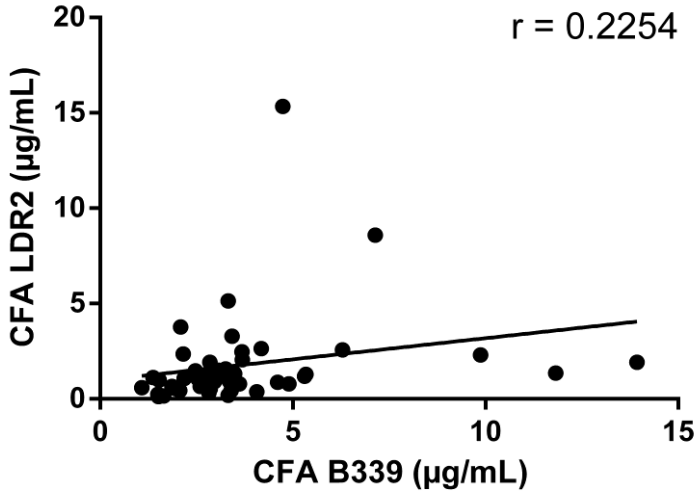

Figure 3. Pearson's correlation analysis between urine total soluble *Paracoccidioides* sp. antigens using immune serum against *P. brasiliensis* B339 and *P. lutzii* (LDR2). Indirect competitive ELISA for total soluble *P. brasiliensis* antigens in urine CFA levels by using immune serum against *P. brasiliensis* B339 (CFA B339 µg/mL) and immune serum IgG anti- *P. lutzii* (CFA LDR2 µg/mL). Total soluble urinary antigens levels expressed as µg protein/ml in chronic PCM patients or suspected of PCM determined by indirect competitive ELISA by using immune serum IgG anti-*P. brasiliensis* B339 (CFA B339 µg/mL) and immune serum IgG anti-*P. lutzii* (CFA LDR2 µg/mL). No correlation between CFA levels determined by using immune serum against *P. brasiliensis* B339 and *P. lutzii* (LDR2) was observed (r= 0,2254).

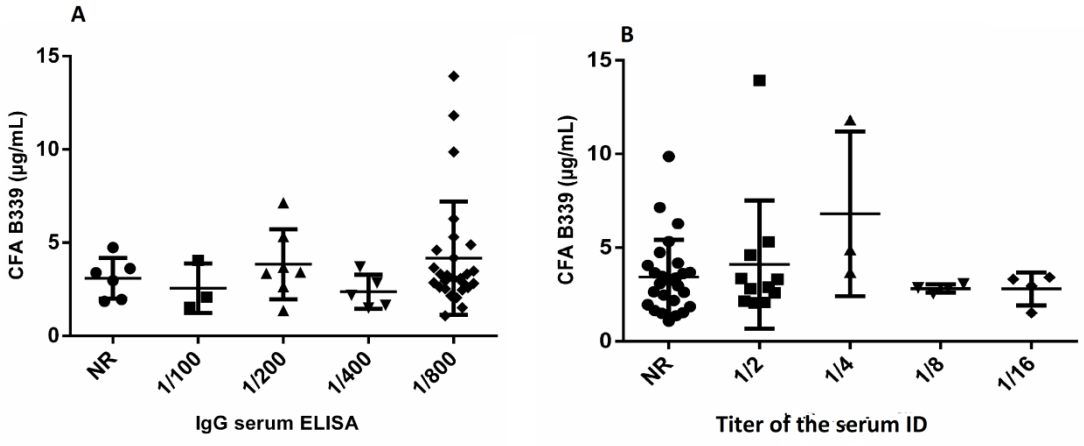

Figure 4. *Paracoccidioides* sp. CFA urinary levels determined by immune serum against *P. brasiliensis* B339 (4A) by icELISA and serological titer determined by immunodiffusion (4B). CFA urinary levels by icELISA expressed in µg/ml and ID results by using *P. brasiliensis* Exo Ag expressed as: (NR-no reactivity) n=23 and titer (1/2) n=10, (1/4) n=5, (1/8) n=5 and (1/16) n=3 in PCM patients or suspected. No serum sample reactive in dilution 1/32 was detected. NR x (1/2) x (1/4) x (1/8) or (1/16) (P>0.05).

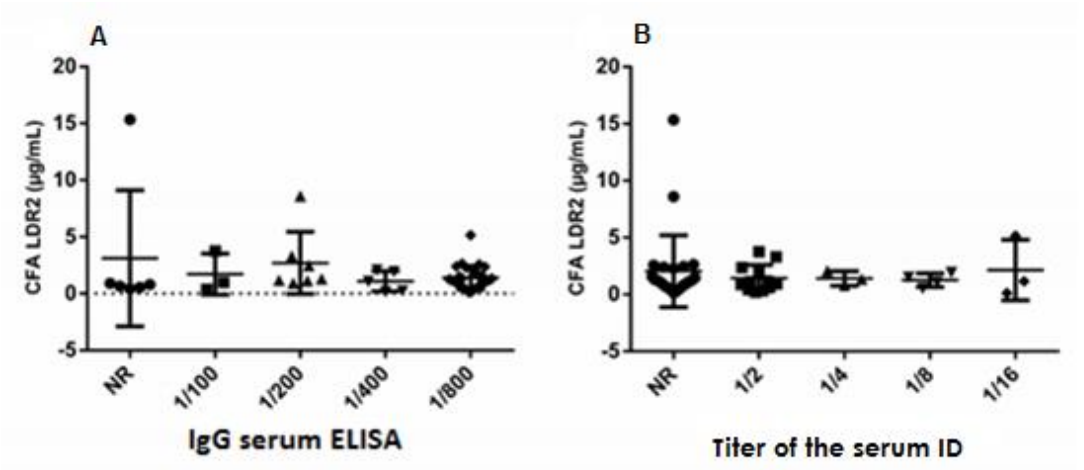

Figure 5. *Paracoccidioides* sp. CFA urinary levels determined by immune serum against *P. lutzii* (LDR2), and serological titer determined by ELISA (5A) and immunodiffusion (5B). Serological titer to ExoAg determined by ELISA (5A), expressed as: (NR-no reactivity) n=6 and titer 100 (1/100) n=3, 200 (1/200) n=7, 400 (1/400) n=5, ≥ 800 (1/800) n=23 in PCM patients or suspected. NR x (1/100) x (1/200) x (1/400) or (1/800) (p>0.05). Serological titer to ExoAg determined by immunodiffusion= ID (5B) expressed as NR (no reactivity) n=23 and titer (1/2) n=10, (1/4) n=5, (1/8) n=5 and (1/16) n=3 in PCM patients or suspected. No serum sample reactive in dilution 1/32 was detected. NR x (1/2) x (1/4) x (1/8) or (1/16) (P>0.05) in PCM patients or suspected.

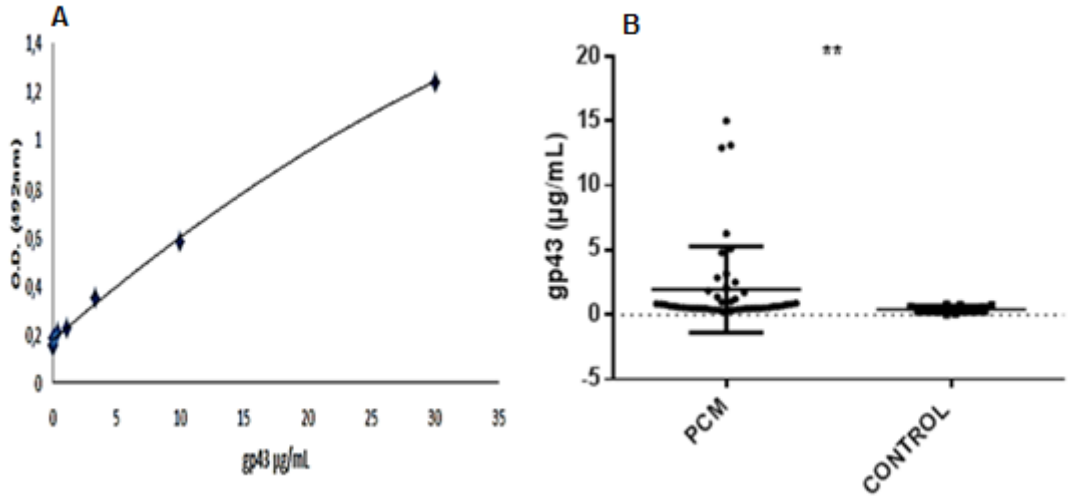

Figure 6. Inhibition ELISA for detection of *P. brasiliensis* gp43 in urine. 6A. The standard inhibition curve by the variations in antigen concentration of the OD values for gp43 (R^2= 0,9989) was used to determine the concentration of gp43 in patient urine samples. 6B. PCM= patients suspected of PCM and PCM patients x negative control (P<0.05).

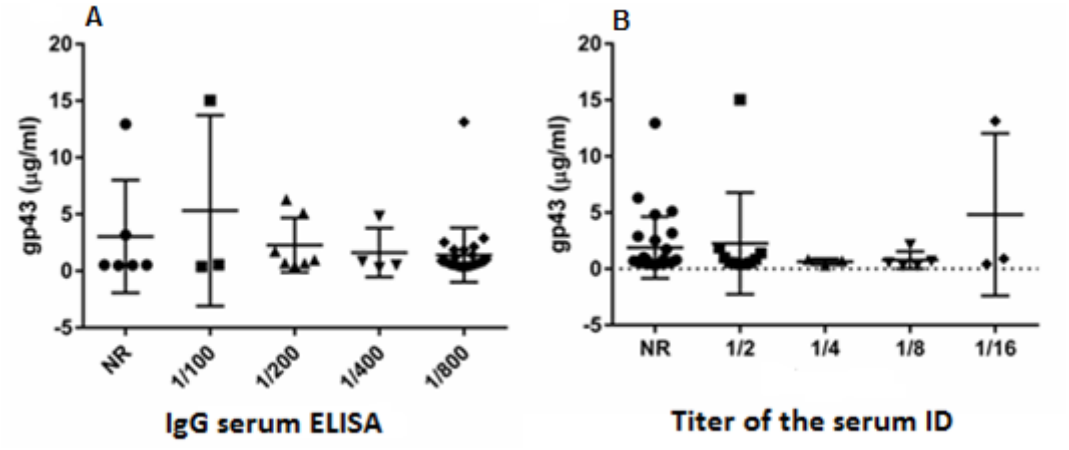

Figure 7. Gp43 urinary levels by inhELISA (µg/ml), and serological titer determined by ELISA (7A) and immunodiffusion (7B).

Serological titer to ExoAg determined by ELISA (7A), expressed as: NR (no reactivity) n=6 and titer 100 (1/100) n=3, 200 (1/200) n=7, 400 (1/400) n=5, ≥ 800 (1/800) n=23, in PCM patients or suspected. NR x (1/100) x (1/200) x (1/400) or (1/800) (P>0.05). Serological titer to ExoAg determined by immunodiffusion= ID (7B) expressed as NR (no reactivity) n=23 and titer (1/2) n=10, (1/4) n=5, (1/8) n=5 and (1/16) n=3, in PCM patients or suspected.

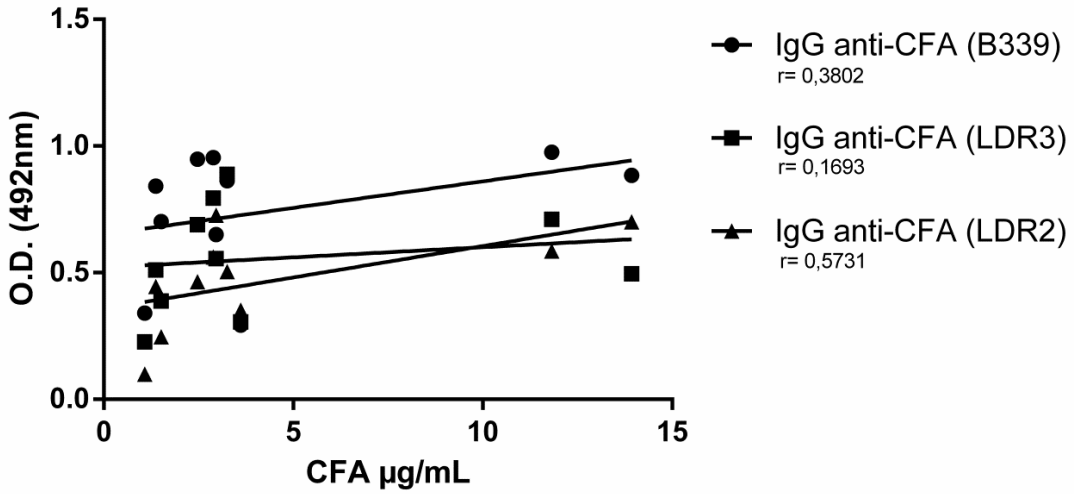

Figure 8. Pearson's correlation analysis between urine CFA *Paracoccidioides* sp. antigens levels, determined by immune serum

against *P. brasiliensis* B339 and serum IgG to P*. brasiliensis* B339 (S1), LDR3 (PS2) and LDR2 (*P. lutzii*).

CFA urinary levels expressed as µg protein/ml in PCM patients (n=10) and serum IgG anti- CFA from *P. brasiliensis* B339 (S1), CFA from LDR3 (PS2) and CFA from LDR2 *(P. lutzii),* expressed as O.D. at 492nm. Correlation between CFA levels and IgG to CFA from LDR2 *(P. lutzii)* (r >0.5) and no correlation between gp43 levels and IgG to CFA from *P. brasiliensis* B339 (S1) or CFA from LDR3 (PS2) (r <0.5) were observed.

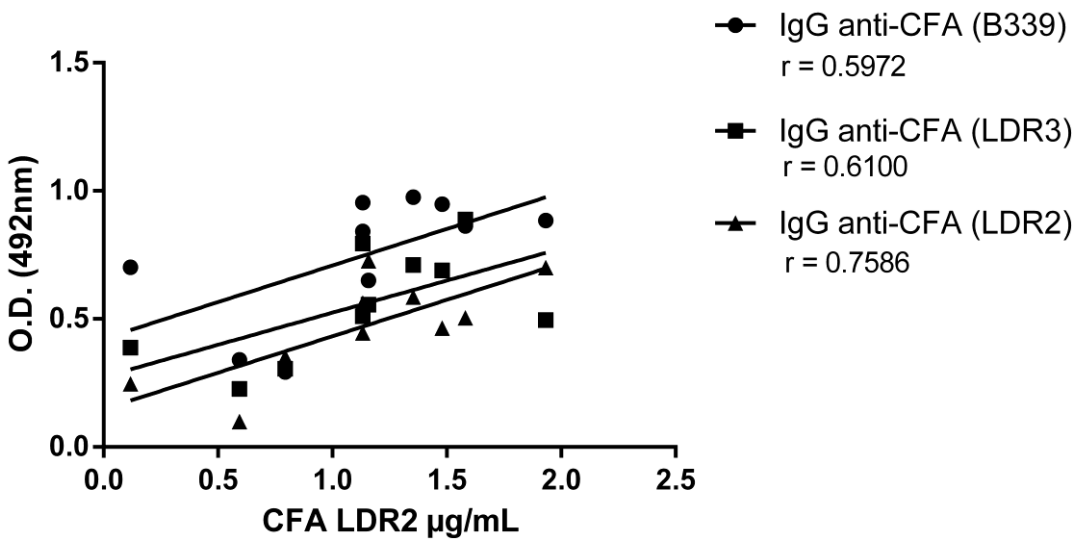

Figure 9. Pearson's correlation analysis between urine CFA *Paracoccidioides* sp. antigens levels, determined by immune serum against *P. lutzii* (LDR2) and serum IgG to P*. brasiliensis* B339 (S1), LDR3 (PS2) and LDR2 (*P. lutzii*).

CFA urinary levels expressed as µg protein/ml in PCM patients (n=10) and serum IgG anti- CFA from *P. brasiliensis* B339 (S1), CFA from LDR3 (PS2) and CFA from LDR2 *(P. lutzii),* expressed as O.D. at 492nm. Correlation between CFA levels and IgG to CFA from LDR2 *(P. lutzii)* (r >0.5) and no correlation between gp43 levels and IgG to CFA from *P. brasiliensis* B339 (S1) or CFA from LDR3 (PS2) (r <0.5) were observed.

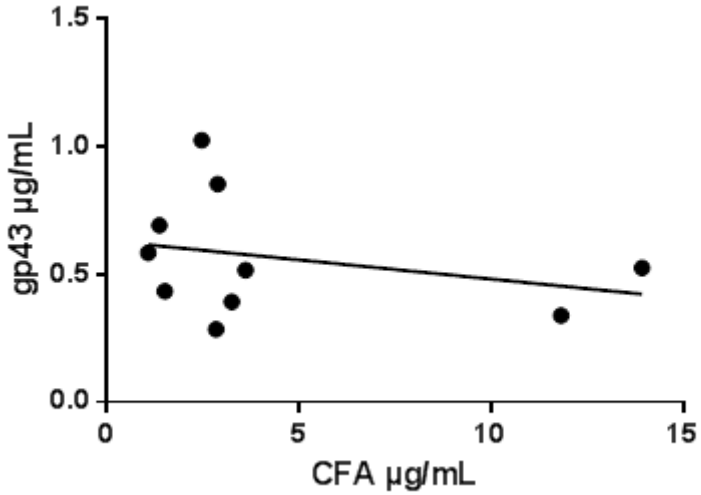

Figure 10. Pearson's correlation analysis between urine gp43 and CFA levels.

Gp43 and CFA urinary levels expressed as µg protein/ml in (n=10) chronic patients of PCM. No correlation between CFA and gp43 levels was observed (r= -0,2909).

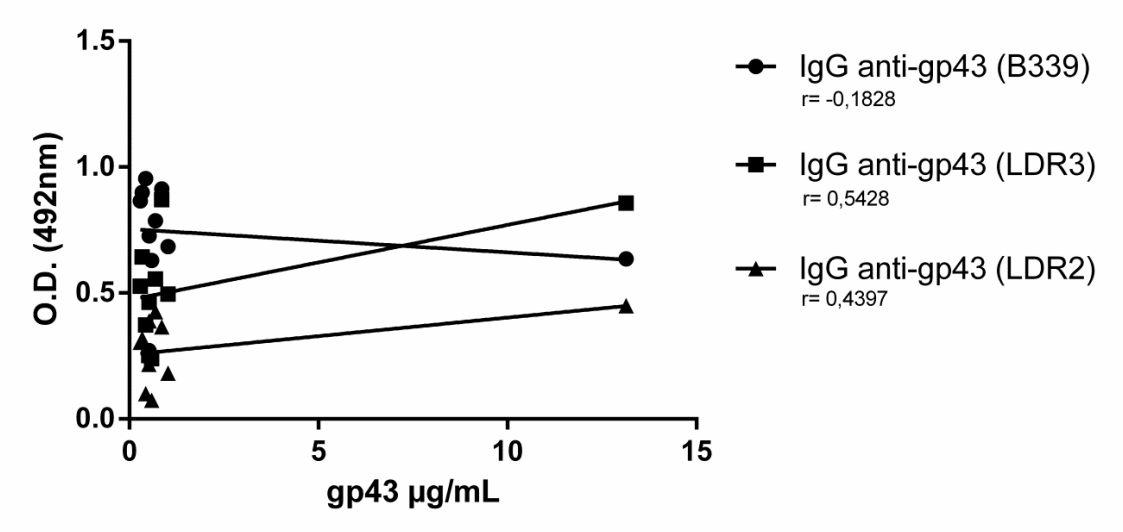

Figure 11. Pearson's correlation analysis between urine gp43 levels and serum IgG anti- ***P. brasiliensis*** **B339 (S1), LDR3 (PS2) and LDR2 (*P. lutzii*).**

Gp43 urinary levels expressed as µg/ml in PCM patients (n=10) and serum IgG anti-CFA from *P. brasiliensis* B339 (S1), CFA from LDR3 (PS2) and CFA from

LDR2 *(P. lutzii),* expressed as O.D. at 492nm. Correlation between gp43 levels and IgG to CFA from *P. brasiliensis* LDR3 (PS2) (r >0.5) and no correlation between gp43 levels and IgG to CFA from *P. brasiliensis* B339 (S1*)* or CFA from LDR2 *(P. lutzii)* (r <0.5) were observed.

CONCLUSÕES GERAIS

- O diagnóstico de PCM baseado em níveis de anticorpos a *P. brasiliensis* foi mais eficiente que a de detecção de antígenos na urina.
- ELISA foi mais eficiente que imunodifusão na detecção de anticorpos a *P. brasiliensis* para fins de diagnóstico da PCM.
- A detecção de antígenos totais de *P. brasiliensis* é mais eficiente que a de detecção de gp43 para fins de diagnóstico da PCM, mas requer estudos adicionais de reatividade cruzada com outros fungos.
- A utilização de anticorpos anti-*P. lutzii* é mais eficientes que anticorpos anti-*P. brasiliensis* na determinação de níveis de antígenos totais na urina.
- A inclusão de metodologia de detecção de antígenos aos testes sorológicos pode aumentar a detecção, permitindo o diagnóstico de casos de PCM com baixos níveis de anticorpos.
- A correlação positiva entre os níveis urinários de gp43 ou de CFA com os níveis séricos de IgG a CFA de *P. brasiliensis* LDR3 (PS2) ou mesmo proveniente de outra espécie (CFA de LDR2= *P. lutzii),* sugere infecção por agentes diferentes de *P. brasiliensis* S1, requerendo estudos adicionais.