



UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA

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**“Patogenicidade e imunogenicidade de isolados clínicos
do complexo *Paracoccidioides brasiliensis*”**

Dissertação apresentada à Faculdade de Medicina, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Câmpus de Botucatu, para obtenção do título de Mestre em Doenças Tropicais.

Orientador: Prof. Dr. Rinaldo Poncio Mendes
Co-orientador: Prof. Dr. James Venturini

Botucatu

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“Não há melhor arma do que o conhecimento, e não há melhor fonte de conhecimento do que a palavra escrita” (Malala Yousafzai)

Resumo

Introdução. A correlação entre gravidade da paracoccidiodomicose e patogenicidade e imunogenicidade dos fungos causadores tem sido pouco investigada e foi o objetivo deste estudo. **Metodologia.** As cinco cepas Pb192, Pb234, Pb326, Pb417 e Pb531 foram identificadas pelo seqüenciamento da região Exon 2 da gp43. A patogenicidade foi determinada pelo cálculo da dose letal 50% (DL50%) e pela contagem do número de unidades formadoras de colônias, realizada na sexta semana pós-infecção de camundongos BALB/c. A imunogenicidade foi determinada pela avaliação da resposta imune humoral específica, utilizando-se a reação de imunodifusão dupla em gel de ágar e da imunidade celular, determinada pela concentração das citocinas interleucina -2, interleucina-10, interferon- γ , fator de necrose tumoral - α e do fator de crescimento do endotélio vascular, em tecido pulmonar. Quatro amostras clínicas foram recém-isoladas de pacientes com paracoccidiodomicose, provenientes da Região de Botucatu - os isolados Pb234 e Pb417, de pacientes com a forma crônica moderada; o Pb326 de um caso com a forma aguda grave; e o Pb531, de um caso com a forma crônica grave. As demais cepas Pb192, Pb01 e 8334 foram cedidas pelo laboratório de Moléstias infecciosas. **Resultados.** As cepas Pb417 e Pb326 agruparam-se às cepas identificadas como *P. brasiliensis* S1a, a Pb531 às *P. brasiliensis* S1b e as cepas Pb234 e Pb192 às cepas depositadas como *P. restrepiensis* (PS3). Os resultados demonstraram correlação direta entre patogenicidade do isolado e gravidade da doença. Os menores valores de DL50% foram observados em isolados dos casos graves da doença, Pb531 e Pb326. A cepa Pb531 foi considerada de alta virulência, Pb326 de virulência intermediária e os outros isolados Pb192, Pb234, Pb417, Pb01, 8334 de baixa virulência. Os níveis séricos de anticorpos só foram detectadas nos animais infectados com a cepa Pb326 na sexta semana pós-infecção. Nos dois primeiros estágios, 2ª e 4ª semana de infecção, observou-se uma distribuição razoavelmente uniforme da concentração pulmonar de citocinas e do fator de

crescimento do endotélio vascular; porém, a 6ª semana observou-se evidente predomínio da interleucina-10, com pequenas variações entre isolados. *Conclusões.* Observou-se correlação direta entre patogenicidade do isolado e gravidade do paciente e grande semelhança de imunogenicidade dos isolados; *P. restrepiensis* também se encontra na Região de Botucatu (Brasil) e não apenas na Colômbia.

Palavras-chave: paracoccidioidomicose, micoses sistêmicas, virulência, gravidade, modelo murino e seqüenciamento.

Summary

Introduction. The investigation of paracoccidioidomycosis and pathogenicity and immunogenicity of the provoking fungi has been little investigated and was the objective of this study. *Methodology.* As strains, Pb192, Pb234, Pb326, Pb417 and Pb531 were included by sequencing the Exon 2 region of gp43. The pathogenicity was determined by calculating the 50% lethal dose (LD50%) and by counting the number of colony forming units performed in the sixth week post-infection of BALB / c mice. Immunogenicity was determined by the humoral immune response, using the agar gel immunodiffusion reaction and the cellular immunity, determined the concentration of cytokines interleukin-2, interleukin-10, interferon- γ , tumor necrosis factor - and factor of vascular endothelial growth in lung tissue. Surgical has been associated with patients with paracoccidioidomycosis, derived from the Botucatu - the Pb234 and Pb417; the Pb326 of a case with a severe severe form; and Pb531, of a case with severe chronic form. The other strains Pb192, Pb01 and 8334 were transferred by the laboratory of Infectious Diseases. *Results.* Pb417 and Pb326 strains were grouped into the associated strains *P. brasiliensis* S1a, Pb531 to *P. brasiliensis* S1b and strains Pb234 and Pb192 to strains deposited as *P. restrepiensis* (PS3). The results demonstrate the pathogenicity of disease error and severity. The small LD 50 values were measured in the following cases: severe disease, Pb531 and Pb326. Pb531 strain was considered to be highly virulent, intermediate virulence Pb326 and other low virulence Pb192, Pb234, Pb417, Pb01, 8334. Serum antibodies were detected in animals infected with the Pb326 strain at the sixth post-infection week. In the first two stages, 2nd and 4th week of infection, we observed the presence of a coronary lymph node and a vascular endocrine growth factor; However, the 6th week observed a predominance of interleukin-10, with small averages between the results. Observing the pathogenicity of the weight and the severity of the immunogenicity of the

isolates; *P. restrepiensis* is also found in the Botucatu Region (Brazil) and not only in Colombia.

Key words: paracoccidioidomycosis, systemic mycoses, virulence, severity, murine model and sequencing.

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Capítulo I – Introdução

I. INTRODUÇÃO

1. Aspectos do complexo *Paracoccidioides brasiliensis* e da Paracoccidioidomicose.

A paracoccidioidomicose (PCM) é micose sistêmica granulomatosa, causada por fungos termo-dimórficos do complexo *Paracoccidioides brasiliensis* e *Paracoccidioides lutzii*,^(1,2) que é confinada à América Latina e endêmica em área que se estende do México à Argentina,⁽³⁾ e apesar de incompletos, os dados indicam maior incidência dessa micose no Brasil, onde é diagnosticada com grande frequência no Estado de São Paulo.⁽⁴⁾

Foi descrita pela primeira vez por Adolpho Lutz ⁽⁵⁾ em 1908, que encontrou, em lesões bucais de dois pacientes de São Paulo, fungos esféricos, com morfologia diferente da revelada pelo *Coccidioides immitis*. As principais manifestações clínicas dos pacientes incluíam amolecimento dos dentes e comprometimento de mucosa oral, laringe, linfonodos, glândula salivar e traquéia. Lutz registrou que o exame histopatológico desses pacientes revelava reação tuberculóide, células gigantes, células epitelióides e fungos com exo-esporulação. Ele também isolou o fungo em cultivo, demonstrou a existência das fases micelial em cultivo à temperatura ambiente e leveduriforme em pacientes infectados, e reproduziu a doença em cobaias.

Essa micose foi inicialmente chamada de Blastomicose Sul-Americana (BSA) ou Doença de Lutz, Splendore e Almeida, entre outras denominações.⁽⁶⁾ Del Negro et al. (1954) ⁽⁷⁾ já utilizava o termo paracoccidioidomicose, mas sua oficialização se deu em 1972, no First Pan American Symposium on Paracoccidioidomycosis, realizado em Medellín, Colômbia, única denominação utilizada por Ayello em sua conferência de abertura, na qual abordou aspectos históricos dessa micose sistêmica.⁽⁸⁾

Matute et al. (2006)⁽⁹⁾ avaliaram a variação genética do *Paracoccidioides*

brasiliensis através de um estudo filogenético por genealogia “multi loci”, identificando três espécies crípticas, S1, PS2 e PS3, sugerindo a criação do complexo *Paracoccidioides brasiliensis*.

Soares et al. (1995)⁽¹⁰⁾ caracterizaram sete isolados Pb01, 2052, 1684, S, G, 662 e 7455 de *Paracoccidioides brasiliensis* através do *Random Amplified Polymorphic DNA* (RAPD); o isolado Pb01, obtido do Instituto de Patologia Tropical e Saúde Pública da Universidade Federal de Goiás (Goiânia, Brasil) e o isolado 7455 apresentaram um coeficiente de similaridade genética de 35% quando comparados aos outros isolados.

Teixeira et al. (2009)⁽¹¹⁾ identificaram um grupo de 16 isolados - 7455, 6810, 3171, 218, 717, 189, RAJ2, 694, 57, 351, 61, 84, 397, 769, 133 e 206 que se agrupavam ao Pb01 e que se diferenciavam das espécies crípticas S1, PS2 e PS3. Esses isolados constituíram o cluster “Pb01-like”, que passou a constituir uma nova espécie, que recebeu a denominação *Paracoccidioides lutzii*, em homenagem ao médico Adolpho Lutz. A seguir ocorreu a identificação de mais uma espécie críptica PS4.⁽¹¹⁾

Turissini et al. (2017)⁽¹²⁾ propuseram adotar uma nova nomenclatura para as quatro espécies filogenéticas pertencentes ao complexo *Paracoccidioides brasiliensis*: *P. brasiliensis strict sensu* (S1), *P. americana* (PS2), *P. restrepiensis* (PS3) e *P. venezuelensis* (PS4) e o *Paracoccidioides lutzii*. A espécie críptica S1 é amplamente distribuída em seis países - Brasil, Argentina, Paraguai, Uruguai, Peru e Venezuela; a PS2 ocorre na Venezuela e no Brasil e pode ser encontrada na mesma região da S1; a PS3 é restrita à Colômbia e a PS4 à Venezuela. A Região Centro – Oeste do Brasil é a que apresenta a maior prevalência de *P. lutzii*.⁽¹¹⁾

Uma das principais características desse fungo imperfeito é o dimorfismo térmico. Em temperatura ambiente apresenta-se na fase filamentosa, sua forma

infectante, que ocorre na natureza, com parede rica em β -1,3 - glucana. Em tecidos humanos e em culturas mantidas a 37°C apresenta-se na fase leveduriforme, sua forma patogênica, com parede rica em α -1,3 - glucana.⁽¹³⁾

Apesar de existirem áreas endêmicas bem definidas para este patógeno, o nicho ecológico de sua fase sapróbia continua mal caracterizado, devido ao pequeno número de vezes em que o fungo foi isolado do ambiente ^(14,15), ao longo período de latência da doença ⁽¹⁶⁾ e ao pequeno número de relatos da doença em animais domésticos, como em cães.⁽¹⁷⁾ Costa et al. (1995)⁽¹⁸⁾ realizaram teste intradérmico utilizando a paracoccidioidina como antígeno em animais domésticos e selvagens e obtiveram taxas de positividade de 64% em equinos, 41% em ovelhas e 40% em bovinos; em animais selvagens uma positividade de 95% em quatis e 45,4% em felinos. Em outro estudo demonstraram maiores taxas de positividade em animais terrestre com 83%, do que em animais arbóreos com 22,45%.⁽¹⁹⁾ Em animais selvagens já ocorreram relatos da doença em morcegos frugívoros (*Artibeus lituratus*), preguiças (*Choloepus didactylus*) e primatas.^(20,21,22) Em 1986, Naiff et al.⁽²³⁾ relataram o isolamento de *P. brasiliensis* de tatus (*Dasypus novemcinctus*) capturados em Tucuruí (PA), área considerada não-endêmica para esta doença. O fungo já foi isolado de outra espécie de tatu, *Cabassous centralis*, sendo este animal indicado como um provável reservatório do fungo.⁽²⁴⁾ Estudos têm demonstrado que os fenômenos ambientais como o El Niño determinam o aumento do crescimento fúngico, devido ao elevado índice pluviométrico e como consequência, da umidade do solo, que favorecia a esporulação dos *Paracoccidioides* spp.⁽²⁵⁾

Admite-se que a infecção seja adquirida quando propágulos da fase micelial do fungo são inalados, instalando-se nos alvéolos pulmonares. A seguir, o fungo passa à fase leveduriforme após 12-18h, transformação considerada fundamental para que se

estabeleça a infecção.^(26,27,28) O fungo pode, então, se disseminar por via hematogênica e, ou, linfática para qualquer parte do organismo.⁽²⁶⁾ A penetração cutânea direta do fungo é muito rara, apesar de ter sido documentada em alguns casos, em acidentes com espinhos e em laboratório.

Ao entrar no organismo, *P. brasiliensis* pode ser destruído imediatamente ou multiplicar-se, produzindo uma lesão de inoculação e se disseminando para linfonodos regionais. A infecção paracoccidióidica pode regredir ou progredir, dependendo de fatores ligados ao fungo e ao hospedeiro. A regressão pode ser acompanhada de destruição de todos os fungos, formando-se cicatriz local estéril ou se acompanhar da persistência de focos quiescentes, com fungos viáveis. A progressão da infecção determina o aparecimento de sinais e sintomas, o que caracteriza a doença ativa. Os focos latentes podem apresentar reativação posterior, denominada reinfecção endógena, e levar à doença ativa.⁽²⁹⁾ Outros fatores influenciam no progresso da infecção, forma clínica e gravidade, como estado nutricional, tabagismo e alcoolismo.⁽³⁰⁾

As manifestações clínicas da PCM se relacionam, em geral, ao comprometimento de pulmões, pele, mucosa das vias aero digestivas superiores, adrenais e de órgãos ricos no sistema fagocítico mononuclear, tais como fígado, baço linfonodos e medula óssea.⁽²⁶⁾ No entanto, deve-se registrar que a PCM pode comprometer qualquer órgão, aparelho ou sistema.

A PCM se apresenta sob três formas clínicas principais: forma aguda ou subaguda (moderada e grave), forma crônica (leve, moderada e grave) e forma residual. A forma aguda ou subaguda, também chamada forma juvenil, é responsável por 20 a 25% dos casos, acomete em geral crianças, adolescentes e adultos jovens, caracteriza-se por apresentar instalação mais rápida, de algumas semanas a poucos meses e apresentar envolvimento predominante do sistema reticulo endotelial, isto é, baço, fígado, nódulos

linfáticos e medula óssea. Nessa forma clínica as manifestações pulmonares são raras e a presença de lesões de mucosa das vias aero digestivas superiores é pouco freqüente. Nos tecidos são encontrados muitos fungos em multiplicação e granulomas em geral frouxos. A forma crônica ou do adulto ocorre em 75% a 80% dos casos, em geral acomete indivíduos com idade superior a 30 anos e que apresentam história clínica de longa duração, com freqüência acima de seis meses. As manifestações pulmonares são muito freqüentes e, em geral, associadas ao comprometimento de outros órgãos, tais como mucosa das vias aero digestivas superiores, pele e, por vezes, adrenais. As formas residuais, também denominadas seqüelas, são observadas após tratamento e se caracterizam pelas manifestações clínicas ligadas à fibrose de lesões prévias, entre as quais se destacam as pulmonares, em geral acompanhadas de enfisema, e as adrenais, como síndrome de Addison.⁽³¹⁾ Como não é doença de notificação compulsória no Brasil, a real prevalência da PCM não pode ser calculada. A PCM é autóctone da América Latina e endêmica no Brasil e em outros países, tais como México, Argentina, Equador, Venezuela e Colômbia, com uma estimativa anual de 1 a 3 casos/ 100.000 habitantes e, no Brasil, se coloca como a oitava causa de mortalidade entre as doenças infecciosas crônicas,⁽³²⁾ com 3.360 novos casos por ano no Brasil⁽³³⁾. Estudos de casos atendidos em hospitais universitários e de atestados de óbito sugerem que este país apresenta a maior incidência da doença. O Estado de São Paulo registra a maior média de óbitos por ano (59,38), com taxa anual de 2,02.⁽³⁴⁾ A cidade de Botucatu - SP apresenta taxa de óbitos em torno 8,73/1.000.000 de habitantes.⁽³⁵⁾ O Serviço de Doenças Infeciosas e Parasitárias da Faculdade de Medicina de Botucatu recebe em média 15 casos novos por ano e mantém cuidadoso seguimento de seus pacientes.⁽³⁶⁾ A PCM é observada em pacientes que tiveram, ou se encontram, em contato direto e prolongado com o solo, como os trabalhadores rurais.^(37,38,39) Predomina em indivíduos

do sexo masculino, com razão de masculinidade de 22.0:1.0 na forma crônica e 1.7:1.0 na forma aguda/subaguda.⁽⁴⁰⁾ O estrogênio pode explicar a menor prevalência de PCM em pacientes do sexo feminino, pois retarda ou impede as transições micélio-levedura e conídio-levedura, necessárias para que o fungo atinja a fase patogênica, o que pode explicar o reduzido número de mulheres afetadas pela doença na idade adulta e a observação da mesma prevalência em ambos os sexos em pacientes com idade inferior a 13 anos.^(39,41)

À semelhança de outras doenças infecciosas granulomatosas, a PCM induz deficiência imunológica antígeno-específica.⁽⁴²⁾ Falhas na apresentação do antígeno, excesso de antígenos e de imunocomplexos circulantes e deficiência de receptores para interleucina-2 (IL-2) parecem estar ligados à resposta insatisfatória das células T. O comprometimento da resposta das células T se faz acompanhada de diminuição da atividade fungicida dos macrófagos. Portanto, os pacientes apresentam comprometimento do braço Th1 da resposta imune celular, com baixos níveis de TNF- α , IFN- γ e IL-2, associado à manutenção ou à elevação da produção de IL-5, IL-10 e TGF- β , que caracteriza a exacerbação do braço Th2.⁽⁴³⁾ A resistência ao fungo é caracterizada pela atuação eficaz do braço Th1 da resposta imune celular, caracterizada pela formação de granulomas compactos e produção de citocinas produzidas por linfócitos T CD4+, que ativam macrófagos. Os pacientes com a forma grave apresentam deficiência acentuada dessa resposta, induzindo maior ativação de linfócitos tipo B e produção de anticorpos.⁽⁴⁴⁾ No entanto, além de facilitar a opsonização de células fúngicas, não se conhece outra ação dos anticorpos na defesa do hospedeiro. Além disso, deve-se registrar que a gravidade da doença guarda relação inversa com a resposta imune celular e direta com os níveis séricos de anticorpos específicos.^(45,46) A fibrose é ligada à evolução progressiva dos granulomas em direção à cicatrização e, em

menor grau, provavelmente à indução direta pelos fungos.⁽⁴⁷⁾

O diagnóstico de PCM é estabelecido a partir da demonstração do *Paracoccidioides* spp. em materiais biológicos. O exame direto, realizado ao microscópio óptico, com escarro homogeneizado⁽⁴⁸⁾, permite a identificação do fungo devido à sua morfologia característica, isto é, à presença de exo-esporulações. Resultados falsos positivos podem ocorrer nas formas diminutas, que são confundidas com *Histoplasma capsulatum* var. *capsulatum* e *Cryptococcus* spp. pouco capsuladas. Outros materiais clínicos, como o raspado de lesões tegumentares, ou mucosas, e os espirados de linfonodos comprometidos, também devem ser analisados por exame direto, apresentando elevada sensibilidade, devido à grande quantidade de fungos neles presentes.

A citoinclusão do escarro em parafina também é utilizada com coloração pela hematoxilina-eosina (HE) e pela prata (Gomori-Grocott), com elevada sensibilidade.⁽⁴⁹⁾

Fungos do gênero *Paracoccidioides* também podem ser identificados através do seu cultivo em meios como o Mycosel (BBL) ou Mycobiotic ágar (Difco), BHI (Difco), ágar-Sabouraud ou ágar extrato de levedura.

Além disso, o exame histopatológico de tecidos biopsiados permite o reconhecimento do fungo empregando-se as colorações pela HE e prata, entre outras.

Métodos sorológicos também são utilizados para o diagnóstico da PCM. O teste padronizado é a reação de imunodifusão dupla em gel de ágar (IDD), uma prova semi-quantitativa.⁽⁵⁰⁾ Apresenta sensibilidade de 90% e especificidade de 100%, empregando-se um preparado antigênico rico em gp43, como o obtido da cepa Pb339. Poucos são os casos de reação cruzada, que predominam com soros de pacientes com histoplasmose clássica. Este método também permite avaliar a gravidade dos casos, pois títulos elevados são observados em pacientes mais graves. A IDD também é muito útil no

seguimento dos pacientes com PCM, verificando-se diminuição gradual dos títulos de anticorpos durante a terapia antifúngica, até sua negatificação. A contra-imunoeletoforese é outro método sorológico empregado, também baseado em precipitação em gel, utilizando-se os mesmos preparados antigênicos que a IDD; é mais sensível que a IDD, mas apresenta a mesma especificidade. Pode ser realizado de maneira semi-quantitativa. Outros métodos como a reação de fixação do complemento, reação de precipitação em tubos, reação de imunofluorescência indireta e os métodos imunoenzimáticos também podem ser utilizados. Um estudo sorológico demonstrou que os soros de pacientes infectados por *P. lutzii* não reagem com antígenos produzidos a partir de isolados de *P. brasiliensis*.⁽⁵¹⁾ Outros métodos para o diagnóstico da PCM são as técnicas de biologia molecular, como a reação em cadeia da polimerase (PCR).⁽⁵²⁾

O tratamento da PCM contempla duas fases – tratamento inicial ou de ataque e tratamento complementar. O tratamento inicial deve ser mantido até que se observe cura clínica e normalização da velocidade de hemossedimentação. O tratamento de consolidação deve ser feito até um ano após a negatificação da sorologia (medida pela IDD), que caracteriza a cura sorológica.⁽⁵³⁾ Diversos fármacos demonstraram ser eficazes no tratamento da PCM, incluindo os derivados sulfamídicos, a associação sulfametoxazol-trimetoprim, também denominada cotrimoxazol (CMX), anfotericina B (AmB), e derivados azólicos, tais como cetoconazol (CTC) e itraconazol (ITC). Atualmente, os fármacos mais utilizados no tratamento são o itraconazol e o cotrimoxazol.⁽⁵⁴⁻⁵⁶⁾

1.1. Paracoccidioidomicose experimental

Os modelos experimentais utilizados na PCM têm trazido grande contribuição para o conhecimento da imunopatologia desta micose sistêmica. O modelo animal permite avaliar o estudo da resposta imune celular e humoral, a interação parasita-

hospedeiro, a morfologia do fungo nos tecidos, a progressão da doença desde o momento da infecção, até sua disseminação, a ação de medicamentos e o dimorfismo do fungo.⁽⁵⁷⁾

Montenegro (1927)⁽⁵⁸⁾ inicialmente utilizou um modelo experimental para o estudo da doença, infectando hamsters por via intratesticular demonstrando que esse animal é suscetível à doença e trazendo grandes contribuições ao conhecimento da interação entre *Paracoccidioides* e o hospedeiro.

Lacaz reproduziu a PCM em camundongos por via intravenosa e intraperitoneal, causando infecção disseminada em pulmões, fígado, baço, linfonodos, cavidade peritoneal e coração. Observou-se que animais infectados pela via intravenosa apresentavam lesões mais extensas. Vários fatores podem interferir nos resultados das pesquisas com animais tais como sexo, idade, local de origem, linhagem, tamanho do inóculo e via de inoculação.^(57,59,60)

O camundongo é um bom modelo experimental para o estudo da PCM, pois apresenta lesões disseminadas com quadro histopatológico muito semelhante ao do homem. Tanto a via intravenosa como a intraperitoneal e a intratraqueal são adequadas para obtenção de infecção disseminada.^(61,62)

Calich et al. (1985)⁽⁶²⁾ pesquisaram a susceptibilidade das linhagens BALB/c, B10.A e A/Sn, infectados com isolados muito e pouco virulentos, trazendo grande contribuição ao conhecimento de aspectos imunogenéticos da PCM. As linhagens apresentaram padrões variados de susceptibilidade à infecção paracoccidióidica, sendo que a linhagem BALB/c apresenta susceptibilidade intermediária, B10.A grande susceptibilidade e A/Sn resistência à doença.

Xidieh et al. (1999)⁽⁶³⁾ através da análise histopatológica demonstraram a influencia genética em lesões desenvolvidas por camundongos resistentes A/Sn e

suscetíveis B10.A.

Outros modelos vêm se constituindo em alternativa para o uso de experimentação animal, com destaque para o uso de *Galleria mellonella* e testes *in vitro*, como uso de cultura de células utilizando as células mononucleares do sangue periférico (PBMCs, na sigla inglesa) de pacientes com PCM para estudos imunológicos.^(64,65)

1.2. Revisão da literatura

A avaliação da patogenicidade e imunogenicidade de seis isolados de pacientes com formas clínicas distintas e bem definidas de PCM foi feita por Singer-Vermes et al. (1994)⁽⁶⁶⁾, utilizando estudos *in vitro* e em infecção murina. Os autores procuraram correlacionar gravidade do quadro clínico com patogenicidade e imunogenicidade dos isolados.

Os isolados foram comparados quanto à sua virulência, tropismo a diferentes órgãos e capacidade de induzir uma resposta específica imune celular e humoral em camundongos B10.A suscetíveis a *P. brasiliensis*.

A patogenicidade foi avaliada pelo cálculo da dose letal 50% (DL 50), não se revelando associação entre a DL 50% de cada isolado e a gravidade da doença do paciente-fonte. Não se observou correlação entre o tropismo fúngico observado em pacientes e em camundongos B10.A. Os camundongos isolados com as amostras Pb56 e Pb57, que foram isoladas de pacientes com PCM leve, apresentaram valores maiores de DL 50%. E os isolados de Pb50 e Pb57 apresentaram maior recuperação de fungos nos pulmões. Os resultados observados sugerem que a associação entre a patogenicidade e a imunogenicidade na doença em humanos e em camundongos só era perceptível em amostras isoladas de casos leves de PCM.

Zacharias et al. (1986)⁽⁶⁷⁾ realizaram estudo comparativo da infecção causada por três isolados de *P. brasiliensis* (Pb 18, Pb 192 e Pb 265). Foram comparados os achados histopatológicos de pulmões, fígado, baço, rins e adrenais, a resposta imune humoral pela avaliação dos níveis séricos de anticorpos específicos determinados pela reação de imunodifusão dupla em gel de ágar - IDD e a imune celular, pelo teste do coxim plantar. O isolado avirulento 265 não provocou lesões histopatológicas específicas, não induziu a produção de anticorpos específicos detectáveis pela IDD e apresentaram resposta imune celular inferior à dos outros dois isolados. As lesões extrapulmonares foram mais freqüentes em camundongos infectados com o isolado Pb18. Os isolados Pb 18 e Pb 192 apresentaram respostas imune humoral e celular anti-*P. brasiliensis* semelhantes. Os isolados não apresentaram diferenças relevantes na parede celular.

Em outro estudo, realizado por Singer-Vermes et al. (1989)⁽⁶⁸⁾, foram utilizados isolados pouco virulentos (Pb 265 e IVIC Pb 267), intermediários (Pb 192, IVIC Pb 9 e Pb SN), virulento (Pb 2052) e muito virulento (Pb 18). Com os resultados obtidos neste estudo foi possível associar a virulência dos isolados com o resultado da DL50%. Os isolados pouco virulentos não foram letais, não sendo possível calcular a DL50%. Os granulomas induzidos pelos isolados individuais foram semelhantes embora a evolução da inflamação e os órgãos afetados variassem de acordo com o isolado. A imunogenicidade de cada isolado de *P. brasiliensis* foi avaliada pela dosagem de títulos de IgG pelo método ELISA. Os isolados pouco virulentos e intermediários induziram baixa produção de anticorpos, que foi elevada com fungos virulentos e muito virulentos. Assim, observaram-se diferenças na cinética de produção de anticorpos provocada pelos diferentes isolados.

Como se pode observar, poucos trabalhos avaliaram patogenicidade e imunogenicidade de isolados do gênero *Paracoccidioides*, correlacionando-as com o

quadro clínico dos pacientes, objeto do presente estudo.

Tabela 1. Estudos em patogenicidade e imunogenicidade de isolados do gênero *Paracoccidioides*.

Patogenicidade	Imunogenicidade	Linhagem	Local de inoculação	Inóculo	Semanas sacrifício	Órgãos / UFC	Autores/ Ano
Histopatológico	IDD	Swiss	Intravenoso	$2,5 \times 10^5$	2, 4 e 8	Baço, fígado, rim e adrenais	Zacharias et al. 1986
DL50%, UFC e Histopatológico	ELISA	B10.A	Intraperitoneal	1×10^6 , 8×10^4 – 20×10^6	4, 8, 12, 16 e 24	Baço, omento, fígado, pulmões, linfonodos, rins e coração	Singer-Vermes et al. 1989
DL50%, UFC e Histopatológico	DTH, ELISA	B10.A	Intraperitoneal	8×10^4 , 5×10^6 e 18×10^6	2, 4, 12 e 16	Baço, omento, fígado, pulmões, linfonodos e diafragma	Singer-Vermes et al. 1994

DL50% - dose letal 50%; UFC - unidades formadoras de colônias; IDD – reação de imunodifusão dupla em gel de ágar; DTH - reação de hipersensibilidade tardia, na sigla inglesa.

2. Objetivos

O presente estudo tem como objetivos geral e específicos os que se seguem.

2.1. Objetivo geral

Identificar, por métodos moleculares, isolados do complexo *Paracoccidioides brasiliensis* e determinar sua imunogenicidade e a patogenicidade.

2.2. Objetivos específicos

1. Determinar a espécie críptica de cada isolado clínico.
2. Determinar a patogenicidade e a imunogenicidade de cada isolado de *P. brasiliensis*.
3. Correlacionar a imunogenicidade e a patogenicidade de cada isolado com a gravidade de seu paciente-fonte.

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Capítulo II - Artigo

Pathogenicity and immunogenicity of clinical *Paracoccidioides* spp. isolates and their correlation with disease severity

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Abstract. We have compared the pathogenicity and immunogenicity of four clinical isolates from different and well-defined clinical forms of paracoccidioidomycosis and three collection isolates evaluating their virulence and ability to induce specific cellular and humoral immune responses in BALB / c mice. Pb234 and Pb417 were isolated from patients with the chronic moderate form, Pb326 from an acute severe form and Pb531 from a chronic severe form. The strains Pb417 and Pb326 joined the strains identified as *P. brasiliensis* S1a, Pb531 as *P. brasiliensis* S1b, and Pb234 and Pb192 as *P. restrepiensis* (PS3). Pathogenicity was evaluated by the determination of the LD50% (LD₅₀) estimation and by colony-forming unit (CFU) counts in the lungs and liver. Immunogenicity was evaluated by determining the specific antibodies serum levels, and interleukins and VEGF pulmonary concentrations. It was possible to observe a direct correlation between pathogenicity and disease severity. Pb531 and Pb326, isolated from PCM severe cases, showed the lowest values of LD₅₀. Distribution of the isolates regarding virulence showed Pb531 strain as high, Pb326 as intermediate and the other - Pb192, Pb234, Pb417, Pb01, and Pb8334 as low. Specific serum levels of antibodies

were detected only in animals infected with the Pb326 strain, at the sixth post-infection week. In the first two stages of the infection a balanced concentration of cytokines and VEGF was observed, but the third stage showed a predominant induction of IL-10; very little differences among strains were noted. Taken together, our results indicate a direct correlation between pathogenicity and disease severity.

Keywords. Paracoccidioidomycosis, systemic mycoses, virulence, severity, murine model and sequencing.

Introduction

Paracoccidioidomycosis (PCM) is a granulomatous systemic mycosis caused by thermally dimorphic fungi of the *Paracoccidioides brasiliensis* complex and *Paracoccidioides lutzii*^(1,2) which is confined to Latin America and is endemic in an area that stretches from Mexico to Argentina.⁽³⁾

PCM has two main clinical forms: a) acute / subacute (juvenile form) - AF, which compromises 15 to 25% of cases, reveals equal distribution as to sex, has a faster installation, and generally reveals generalized lymphadenomegaly, digestive manifestations, hepatosplenomegaly, and bone and cutaneous lesions, being rare the pulmonary involvement; its clinical presentations are moderate and severe; b) chronic form (of the adult), which is the most frequent - compromises 75 to 85% of the patients, predominates in the male sex, compromises the lungs in the vast majority of cases and reveals cutaneous and mucosal lesions of the upper aerodigestive routes frequently ; may present mild, moderate and severe clinical forms. This clinical form installs more slowly, and its main clinical manifestations are related to pulmonary damage.⁽⁴⁾

The relationship between the severity of the clinical conditions and the host's immune response was well documented – direct with humoral response and indirect with cell mediated immunity.⁽⁵⁾

However, few studies have attempted to correlate severity of PCM patients and characteristics of isolates of such patients, such as pathogenicity and immunogenicity, as in the studies by Singer-Vermes et al. (1994)⁽⁰⁶⁾ and Zacharias et al. (1986).⁽⁷⁾ Using *in vitro* studies and in murine infection, the authors sought to correlate severity of the clinical picture with pathogenicity and immunogenicity of the corresponding isolates.

Thus, the present study aimed to correlate the pathogenicity and immunogenicity of isolates from clinical samples of fungi of the *Paracoccidioides* genus with the severity of the disease caused by them.

MATERIALS AND METHODS

Patients and case definition, inclusion and exclusion criteria

Four patients were studied at admission in the University Hospital of the Faculdade de Medicina de Botucatu - São Paulo State University (UNESP), with active PCM confirmed by the isolation and identification of the etiological agent in culture. We also studied three collection isolates - one of the *Paracoccidioides brasiliensis* complex (Pb192) and two *Paracoccidioides lutzii* (Pb01 and 8334). Patients with any comorbidity of infectious, noninfectious inflammatory or neoplastic origin, except smoking and alcoholism, were excluded from the study.

Data on sex, site of isolation, affected organs/tissues, clinical form according to the specifications of Mendes (1994)^(4,2) and serum level of specific antibodies, determined on admission by double agar gel immunodiffusion test, as described in Table 1.

Table 1. Identification, clinical characteristics, serum specific antibody levels at the time of fungal isolation of patients with paracoccidioidomycosis (PCM).

Strains <i>P. brasiliensis</i> / <i>lutzii</i>		Sex	Site of isolation	Affected organs/tissues	DID	Clinical form of PCM	
Pb234	M	Tongue	Lungs and mucous membrane of the upper air-digestive tract	1/4	chronic	moderate	
Pb326	F	Oral mucosa	Lymph nodes and superior aerodigestive pathways.	1/16	acute	severe	
Pb417	M	Sputum	Lungs (mild injury), and larynx	Neg.	chronic	- moderate	
Pb531	M	Testicle	Lungs (mild injury), superior aerodigestive pathway mucosa, intestine, and testis.	1/16	chronic	- severe	
Pb192	...	...	...	...	...	...	...
Pb01	...	...	...	...	...	chronic	-
8334	...	...	...	...	...	...	...

DID - double immunodiffusion reaction on agar gel; **M** - male; **F** - female; ... - no information.

Fungus isolates

Patient and collection isolates were stored on Sabouraud agar and maintained by successive tests, every 15 days and stored in a greenhouse at 37 ° C at the Tropical Diseases Research Laboratory - Medical Mycology, Faculty of Medicine of Botucatu - UNESP.

Identification of isolates

The strains were identified by sequencing the Exon 2 region of the gp43 glycoprotein, which allows species differentiation as a highly variable genetic region.⁽⁸⁾

DNA extraction was done using the "Quick CTAB" protocol⁽⁹⁾ and later the exon 2 of gp43 was amplified by PCR in a Veriti Thermocycler thermocycler (AppliedBiosystems, Foster City, CA, USA) using primers gp43-E2F (CCAGGAGGCGTGCAGGTGTCCC-3') and gp43-E2R (GCCCCCTCCGTCTTCCATGTCC-3').⁽¹⁰⁾ To perform the amplification, the GoTaq®Green Master Mix kit (Promega, Madison, WI, USA) was used with 25 µL reaction volume containing 3 µL genomic DNA (300 ng / µL) and 0.5 µM of every primer. The cycling was as follows, an initial DNA denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min; finally, there was a final extension at 72°C or 10 min.

After confirming the amplification by 1.5% agarose gel visualization, the PCR product was purified using ExoProStar (GE Healthcare, Milwaukee, WI, USA) and sequenced on the ABI 3500 DNA analyzer (AppliedBiosystem, Foster City, CA, USA) according to the manufacturer's instructions. The quality of the sequences was verified by Sequencing Analysis software (Applied Biosystems), and we only included nucleotides with Phred ≥ 20 . Sequencing editing and phylogenetic analysis were performed using the MEGA v 7.0 software.⁽¹¹⁾ Molecular identification was performed by the Basic Local Alignment Search Tool (BLAST) of the National Center Biotechnology Information (NCBI). Sequence alignments were performed using the Clustal W algorithm. Phylogenetic construction was performed by the Maximum Likelihood (ML) method with 1000 replicates of Bootstrap and using *P. lutzii* as an outgroup.

Finally, we deposited the sequences in the GenBank database: *Paracoccidioides brasiliensis* strain 192 immunodominant antigen Gp43 (GP43) gene, partial cds, GenBank access key: MH367527, *Paracoccidioides brasiliensis* strain 234

immunodominant antigen Gp43 (GP43) gene, partial cds , GenBank access key: MH367528, *Paracoccidioides brasiliensis* strain 326 immunodominant antigen Gp43 (GP43) gene, partial cds, GenBank access key: MH367529, *Paracoccidioides brasiliensis* strain 417 immunodominant antigen Gp43 (GP43) gene, partial cds, GenBank access key: MH367530, *Paracoccidioides brasiliensis* strain 531 immunodominant antigen Gp43 (GP43) gene, partial cds, GenBank access key: MH367531.

Mice

A total of 294 male BALB/c mice, aged 8 to 12 weeks, from the Experimental Research Unit (Unipex) of Faculdade de Medicina de Botucatu - São Paulo State University (UNESP) were used. They were kept in collective boxes with 30x20x13cm containing on average five animals, with bed of autoclaved wood, in an environment at a temperature ranging from 23 to 25°C, with luminosity controlled by electromechanical time switch, that is, 12h in the clear and 12h in the dark, receiving filtered water ad-libitum and balanced commercial mouse ration, autoclaved.

Cultivation of clinical isolates

Isolates of the *Paracoccidioides* genus were incubated at 35°C in the yeast phase, with weekly subcultures, in GPY culture medium containing 2% glucose, 1% peptone and 0.5% yeast extract. We monitored the viability of the fungus by Trypan blue staining (Sigma). At the time of animal inoculation, these isolates were maintained in this condition for no longer than 2 weeks. For the live cell assays, we used suspensions with more than 90% viable fungi.⁽¹²⁾

Experimental design

The study was delineated in two experiments, with four groups of mice in experiment 1 and two groups in experiment 2. These animals were infected with seven

isolates of patients, prioritizing the constitution of at least one isolate per clinical form.

Experiment 1- Consisting of four groups of six animals infected with each isolate of the *Paracoccidioides* genus. For analysis of the 50% lethal dose (LD₅₀) and a control group, consisting of animals that received a saline solution.

The three infected groups (G1, G2, G3) consisted of 42 mice which were inoculated by the intraperitoneally route; six animals were inoculated with each of the seven clinical isolates, with daily mortality observed for 120 days. They differed as to the inoculum.

1. Group 1 (G1): inoculum of 8×10^4 cells/mL;
2. Group 2 (G2): inoculum of 5×10^6 cells/mL;
3. Group 3 (G3): inoculum of 18×10^6 cells/mL;
4. Group 4 (G4): consisting of 28 mice, which received a 0.9% physiological solution intraperitoneally, constituting the healthy control group, with daily mortality observed for 120 days.

Experiment 2 - Groups of 16 animals infected with each isolate of *Paracoccidioides* sp. for viable fungi recovery, serological evaluation, and dosage of cytokines, and a control group, consisting of animals that received a saline solution.

1. Group 5 (G5): consisting of 112 mice, which were infected by the intraperitoneally route with a concentration of 5×10^6 cells/mL, inoculating 16 animals with each of the seven clinical isolates, with daily mortality observation, until the sacrifice, which was performed in the 2th, 4th and 6th week post-infection, with organ collection and microbiological evaluation of fungal load,

cytokines and vascular endothelial growth factor (VEGF) dosage and determination of antibodies serum levels.

2. Group 6 (G6): consisting of 28 mice, which received intraperitoneally 0.9% physiological solution, with daily mortality count until sacrifice, which was performed at the 2nd, 4th and 6th week after administration of physiological solution, with the collection of organs for microbiological assessment of fungal load, cytokine and VEGF dosage and determination of antibodies serum levels, constituting the healthy control group.

The selection of animals from groups 5 and 6 for sacrifice was made by drawing lots.

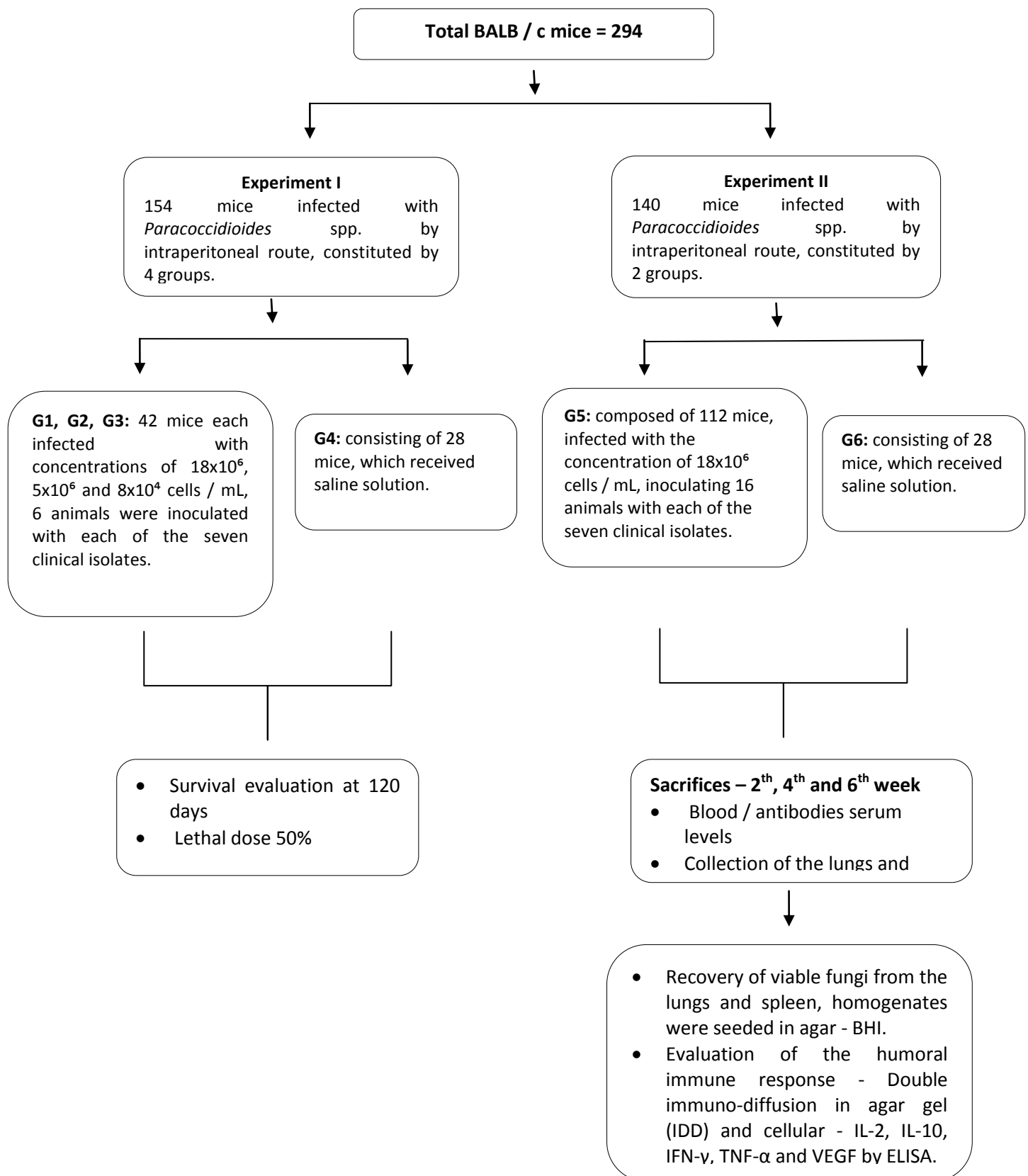


Figure 1. Flowchart of the experimental design performed with isogenic BALB / c mice infected with different strains of fungi of the *Paracoccidioides* genus, for evaluation of pathogenicity and immunogenicity.

Virulence recovery

Virulence recovery was performed by infection and reisolation in male BALB/c mice, according to Kashino et al. (1990)⁽¹³⁾, with modifications. They were infected intraperitoneally with 0.25 mL of the suspension of *Paracoccidioides* sp. containing 5×10^6 viable yeast cells with the aid of a disposable syringe with a volume of up to 1.0 mL. After two weeks, an autopsy was performed to collect the spleen, which was macerated with 1.0 mL of sterile PBS until we obtain the homogenate. We selected 100 μ L aliquots of the homogenates over the entire surface of the plates with enriched brain heart infusion-BHI medium (Difco) containing 0.5% gentamicin, 4.0% horse serum and culture filtrate for fungi of the *Paracoccidioides* genus.⁽¹⁴⁾

Preparation of the inoculum and infection of the mice

The yeast phase cells suspended from the five-day old culture were washed in saline and counted in the Neubauer chamber. Mice were infected intraperitoneally with 0.25 mL of the suspension of *Paracoccidioides* sp. containing different concentrations, 18×10^6 , 5×10^6 and 8×10^4 of viable yeast cells, using a disposable syringe with a volume calibrated up to 1.0 mL.

Collection of biological material

To sacrifice mice we initially anesthetized them with a solution of 24 μ L ketamine, 32 μ L xylose and 24 μ L distilled water administered intraperitoneally with a disposable syringe millimeter up to 1.0 mL. After sedation, blood was collected by cardiac puncture using a 1.0 mL syringe and a 25 x 7 gauge needle. Blood was transferred to a sterile tube, with anticoagulant, and identified with the number of that mouse.

Anti - *P. brasiliensis* serum levels were determined by the double agar gel immunodiffusion (DID) test. We centrifuged the blood at 3,500 rpm in Revan Cycle C I centrifuge. The obtained serum was transferred to a 2.0 mL screw-capped tubes and then stored in a freezer at -80°C for later dosage of the specific antibodies. An autopsy was then performed to collect spleen and lungs. All procedures were performed with sterile materials and in an aseptic environment, in a vertical laminar flow chamber. The left and right lobes of the lungs were weighed and macerated with 1.0 mL of sterile PBS until we obtain the homogenate. The volume of 100 µL was reserved for recovery of viable fungi, and the remainder was centrifuged for 10 min at 10,000 rpm at 4°C (Eppendorf Centrifuge mod 5804 R). After collecting, the supernatant was filtered on 0.22 µm nylon disk, aliquoted and frozen at -80°C for subsequent cytokine and VEGF dosage.

Pathogenicity evaluation

Each isolate of *Paracoccidioides* sp. was evaluated for its pathogenicity by calculating the 50% lethal dose (LD₅₀) and counting the number of colonies forming units (CFU).

Calculation of lethal dose 50%

We calculated the 50% lethal dose by the probit method⁽¹⁵⁾. Three different concentrations of *Paracoccidioides* sp isolates, i.e., 8×10^4 , 5×10^6 , and 18×10^6 cells/mL, were inoculated in 18 mice per isolate. Mortality was observed every day for 120 days.⁽⁶⁾

The virulence score ranged from 1 to 7, with 1 being the one that presented the highest LD₅₀, that is, it was less virulent and, therefore, progressively. The isolate that killed all the mice, in all concentrations used, received score 7. In the case of ties, each isolate received the average of the scores that they would receive.

Calculation of the fungal load

Recovery of viable fungi

Recovery of viable fungi was evaluated in groups of eight animals infected with 5×10^6 cells/mL of each isolate of *Paracoccidioides* sp. and sacrificed at the 6th post-infection week. Fragments of spleen and lungs of the mice were submitted to microbiological evaluation to determine the fungal load. Thereby, we weighed and macerated the fragments in 1.0 ml of sterile buffered saline (PBS). We seeded one hundred μ L aliquots of the homogenates over the entire surface of the plates with enriched brain heart infusion-BHI medium (Difco) containing 0.5% gentamicin, 4.0% horse serum and culture filtrate for fungi of the genus *Paracoccidioides*⁽¹⁴⁾ in duplicate with the help of L. glass rods. The plates were then sealed and incubated at 35°C. The plates were read after 30 days of culture, with the aid of a colony counter. Finally, the count of the number of colony forming units (CFU) was adjusted to a gram of tissue, using the formula:

$$\text{Count (CFU per gram of tissue)} = \frac{\text{average of the CFU counts} \times 10}{\text{weight of the fragment (grams)}}$$

The score of the virulence, based on the fungal load, ranged from 1 to 7, in the direct relation ship between the fungal load, that is, 1 for the lowest fungal load and 7 for the highest.

Evaluation of the specific humoral immune response

The humoral immunity was evaluated by the semi-quantitative dosage of the serum anti-*Paracoccidioides* antibodies, using the double agar gel immunodiffusion (DID) test, according to Restrepo specifications⁽¹⁶⁾ and stained by Schuvarz 10B starch (Merck). Serum was considered to be reactant when observed precipitation line (s); the

serum level was defined by the highest dilution presenting precipitation lines. Culture filtrate prepared in the Laboratory of Clinical Mycology of the Faculdade de Ciências Farmacêuticas de Araraquara – UNESP was used as antigen. Briefly, *P. brasiliensis* grown in Fava Netto medium at 35°C for five days was inoculated in modified Negróni medium according to Siqueira⁽¹⁷⁾ and incubated in a shaker incubator at 35°C for 15 days. Thimerosal was added at the final concentration of 0.2 g / mL and incubated under the same conditions for another four days. The antigen was then filtered, concentrated 10-fold with polyethylene glycol (Sigma P-2263) and dialyzed with PBS on dialysis membrane (Sigma D-9377). The protein content of the concentrated extract was analyzed by SDS-PAGE, separated into small aliquots and frozen at -70°C. The Schuvarz starch solution, used as a dye, consisted of 4.0 g of Schuvarz 10B starch (Merck), 100 mL of glacial acetic acid and distilled water in an amount sufficient to complete 1,000 mL and stored in an amber flask protected from light solar.

Cell free antigen from Paracoccidioides lutzii

This cell-free antigen was prepared in the Laboratory of Tropical Diseases of the Faculdade de Medicina de Botucatu – UNESP, using the strain Pb01 in the yeast phase, according to Camargo et al. (1991).⁽¹⁸⁾ Pb01 was cultivated in Fava Netto medium at 35°C for five days, to which 1.0 mL of sterile PBS, pH 7.4 and 0.01M, was added. This colonies-PBS mixture was vortexed for 30 seconds and centrifuged at 400 x g for 1 minute. The supernatant (cell-free antigen) was removed, aliquoted and stored at -20°C.

Evaluation of the cellular immune response

The cytokines interleukin-2 (IL-2), tumor necrosis factor- α (TNF- α), interleukin-10 (IL-10), and the vascular endothelial growth factor (VEGF) were quantified by ELISA using Cytokine DuoSet kit (R & D Systems, Minneapolis, USA). We also

quantified the interferon- γ cytokine (IFN- γ) by ELISA, however using the commercial mouse kit (BD Biosciences and R & D Systems) BDTM Cytometric Bead Array (BD, Becton Dickinson, USA). All dosages were carried out according to the specifications of the respective manufacturers.

The cut-off line for the evaluation of cytokines and VEGF was determined by the results of the control group, consisting of 28 uninfected BALB/c mice, kept in the same laboratory conditions during the same time as the infected animals and receiving physiological solution at 0.9%, at the same volume as that of the infecting solution.

To compare the concentrations of each cytokine and VEGF determined in lung homogenate, the rate of each substance, defined as the ratio of the cytokine concentration of lung homogenate of each infected animal to the mean of the concentrations observed in the mice of the control group.

$$\text{Rate} = \frac{\text{concentration in the infected animal}}{\text{average of the concentrations of the controls}}$$

Ethical aspects

This study was approved by the Research Ethics Committee of the Faculdade de Medicina de Botucatu - UNESP, under the evaluation report number 79373417.5.0000.5411 and by the Ethics Committee on Animal Use (CEUA), Faculdade de Medicina de Botucatu - UNESP, with protocol number 1224/2017.

Statistical analysis

All analyses were carried out blind to the corresponding clinical form and degree of severity of the patients. LD₅₀ was calculated using the probit method. Survival curves were compared using the Kaplan-Meier-log-rank method and comparisons of survival

time between strains and concentrations were performed by factorial analysis of variance, followed by multiple comparisons performed by the Tukey test. The recovery of fungi, measured in colony forming units per gram of tissue, was compared according to the group using the Kruskal-Wallis test and in the comparison of the fungal recovery in each group, the Wilcoxon test was used. We compared the concentrations of cytokines by analysis of variance in a factorial scheme, and the results were presented by the mean and standard error, followed by the multiple comparisons made by the Tukey test. Correlations between fungal recovery scores of different organs and humoral and cellular immune responses were made using the Spearman correlation. The comparison of more than two related samples, for continuous variables, was done by the Friedman test. All statistical tests were performed using the SPSS 19.0 program (IBM SPSS Statistics, IBM Corporation, Chicago, IL, USA), assuming an α -type error equal to or less than 0.05 for rejection of the null hypothesis.

Results

Figure 2 shows the phylogeny obtained from the phylogenetic analysis of the Gp43 Exon 2 region. Strains 417 and 326 were grouped with strains deposited as *P. brasiliensis* S1a; the strain 531 as *P. brasiliensis* S1b; strains 234 and 192 as PS3, nowadays called *P. restrepiensis*.

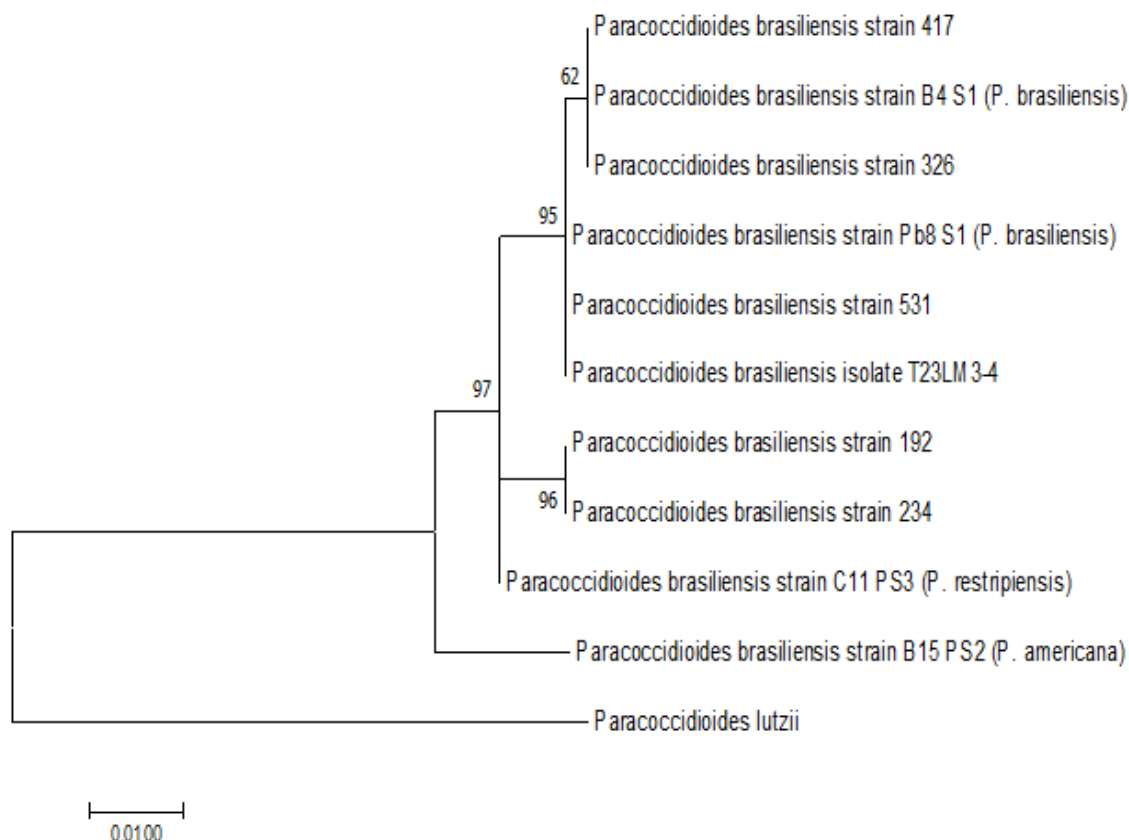


Figure 2. Analysis of molecular phylogeny by Maximum Likelihood method using the exon two regions of gp43. The evolutionary history was inferred using the Maximum Likelihood method based on Kimura parameters (1980).⁽¹⁹⁾ The tree with the highest probability of log is evident. The percentage of trees in which the associated rate is grouped became visible next to the branches. The tree design was in scale, with lengths of branches measured by the number of substitutions per site. The analysis involved 11 nucleotide sequences. We performed the evolutionary analyses in the MEGA7.⁽¹¹⁾

Pathogenicity

All strains were lethal to BALB/c mice; the LD₅₀ and the virulence score of the isolates are in table 2. All mice infected with strain Pb531 (S1b) died at the doses used, that is the reason why it was not possible to calculate its LD₅₀ [table 2].

Table 2. Lethal dose 50% (LD₅₀) and virulence score of seven isolates of the *Paracoccidioides* genus. Evaluation of BALB/c mice after 120 days of infection with three different concentrations - 8x10⁴, 5x10⁶ and 18x10⁶ of viable fungal cells/inoculum.

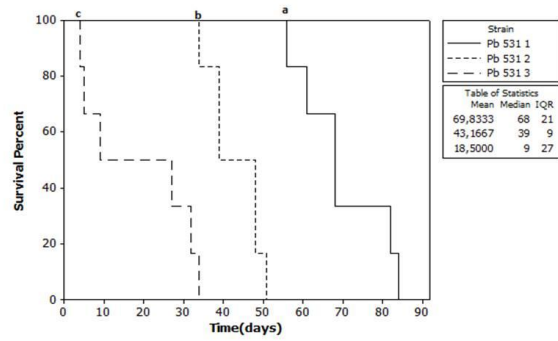
Strains	LD ₅₀ (x10 ⁶ viable yeasts)	Virulence Score
<i>P. brasiliensis</i> complex		
Pb234 (PS3)	39.3	1.0
Pb326 (S1a)	7.2	6.0
Pb417 (S1a)	27.5	2.5
Pb531 (S1b)*	...	7.0
Pb192 (PS3)	21.4	4.5
<i>P. lutzii</i>		
Pb01	27.5	2.5
8334	21.4	4.5

* All mice died at the doses used.

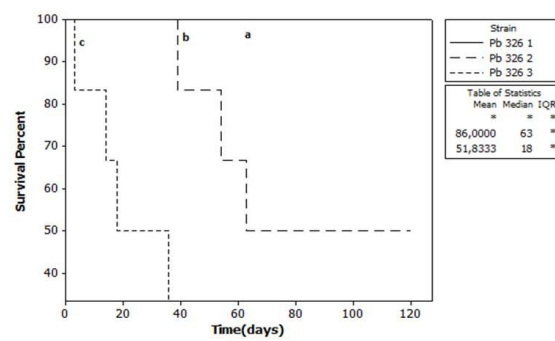
Comparison of the survival time among strains revealed a wide variation, but the mice infected with Pb531 had the shortest survival time, with a mean of 43.8 days ($p<0.001$) [figure 3 and table 3]. The evaluation of the survival time regarding concentration of the infecting inoculum showed differences only with (in) strains Pb531 and Pb326. Survival time decreased with the increase of the Pb531 inoculum. However, with strain Pb326, the survival time was higher with the lowest inoculum, but it did not differ when the two highest inoculum concentrations were used [figure 3 and table 4].

Comparison among concentrations showed that mice infected with the highest fungal concentration (18x10⁶ cells/mL) presented the shortest survival time, with a mean of 79.5 days ($p<0.001$) [table 4].

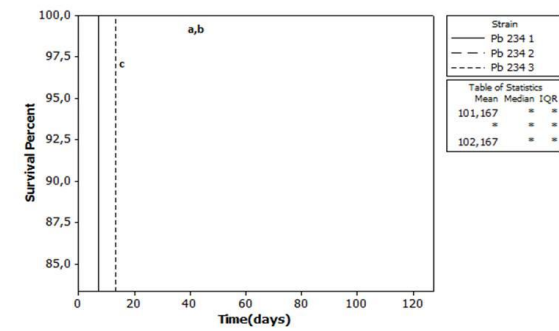
Pb531



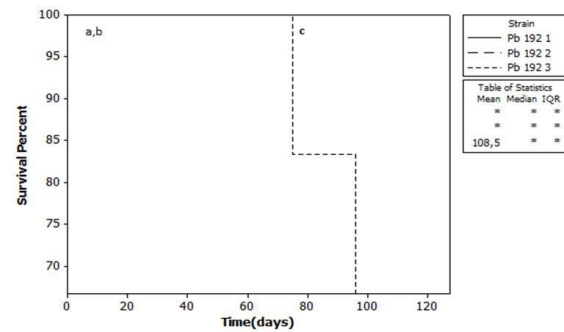
Pb326



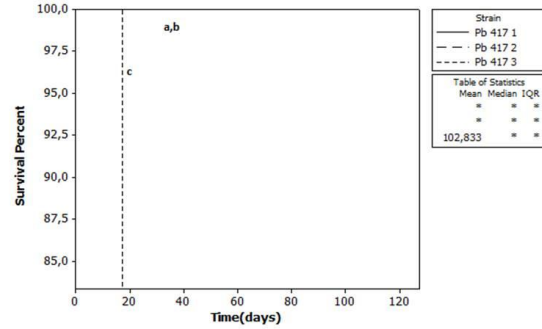
Pb234



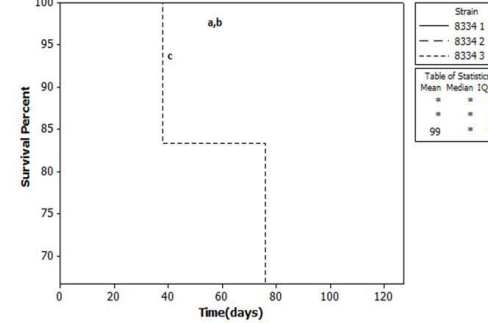
Pb192



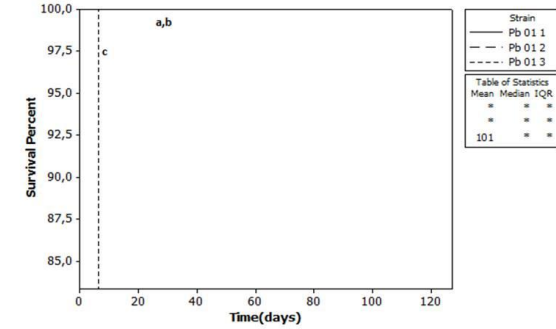
Pb417



83347



Pb01



Strain	8x10 ⁴ (a)	5x10 ⁶ (b)	18x10 ⁶ (c)	p value
Pb 531	69.8	43.2	18.5	< 0.0001 (a>b>c)
Pb 326	-	86.0	51.8	
Pb 234	101.2	-	102.2	0.35
Pb192	-	-	108,5	0.11
Pb417	-	-	102.8	0.37
8334	-	-	99.0	0.11
Pb01	-	-	101.0	0.37

survival time: mean [interquartile] -: no deaths (120 days)

Figure 3. Survival curves of BALB / c mice infected with seven different strains of the genus *Paracoccidioides* in three inocula - 8×10^4 (a), 5×10^6 (b) and 18×10^6 (c). Estimates by Kaplan-Meier log-rank (time in days versus survival in percentage).

Table 3. Mean and standard deviation of survival time, evaluated in days, of BALB/c mice infected with seven strains of the *Paracoccidioides* genus.

Strains	Mean	Standard deviation
Pb192 (PS3)	114.3 A	24.3
Pb234 (PS3)	107.8 AB	35.6
Pb417 (S1a)	114.3A	24.3
Pb326 (S1a)	85.9 B	45.8
Pb531 (S1b)	43.8 C	23.9
Pb01-like	113.7 A	26.9
8334 (<i>P. lutzii</i>)	113.0 AB	21.4

Capital letters compare means among lines. Means followed by the same letter do not differ from each other ($p > 0.05$), while means followed by different letters differ from each other ($p \leq 0.05$).

Table 4. Mean and standard deviation of survival time, in days, of BALB/c mice infected with three concentrations of seven fungal strains of the *Paracoccidioides* - Pb531, Pb326, Pb234, Pb192, Pb417, Pb01-like and 8334, analyzed in their set.

Concentrations	Mean	Standard deviation
8×10^4	108.5 A	26.0
5×10^6	101.5 A	32.7
18×10^6	79.5 B	50.0

Capital letters compare means among lines. Means followed by the same letter do not differ from each other ($p > 0.05$), while means followed by different letters differ from each other ($p \leq 0.05$).

Evaluation of fungal load

The strain Pb531 (S1b) was more virulent than all others except Pb326 (S1a), which revealed intermediate virulence. Pb531 presented a special tropism for lungs, where the fungal load was higher than that of all the other strains, except Pb326. The tropism for lung and spleen was similar for five of the seven strains; spleen was more affected ($p=0.04$) than the lung only by strain Pb234 (PS3) and tended ($p=0.06$) to be more affected than the lung by strain Pb326 (S1a), as shown in table 5 and 6.

Table 5. Fungal lung and spleen load, evaluated by the number of colonies forming units, measured by medians and interquartile ranges, observed in BALB/c mice infected with different strains of fungi of the *Paracoccidioides* genus.

Grup	Organ		P
	Lung	Spleen	
Pb01(P. lutzii)	0[0; 0] a,B	17[0; 6] a,B	0.18
Pb326 (S1a)	12[0; 54] a,AB	291[77; 853] a,AB	0.06
Pb531 (S1b)	58240[45760;76960] a,A	37818[36418;45258] a,A	0.20
8334 (P. lutzii)	0[0; 0] a,B	0[0; 185] a,B	0.44
Pb234 (PS3)	0[0; 0] b,B	32[7; 88] a,AB	0.04
Pb417 (S1a)	0[0; 0] a,B	0[0; 0] a,B	1.00
Pb192 (PS3)	0[0; 15] a,B	0[0; 2523] a,AB	0.46
p	0.001	0.03	

Lower case letters compare medians in the same line by the Wilcoxon test. Capital letters compare medians in the same column by the Kruskal-Wallis test. Medians followed by the same letter do not differ from each other ($p>0.05$), while medians followed by different letters differ ($p\leq0.05$).

Correlation between severity and variables that evaluate the pathogenicity

We found correlation by analyzing the newly isolated isolates of patients, the severity of the cases and the experimental variables that evaluated virulence (pathogenicity).

Table 6 - Correlation between the severity scores of four patients with paracoccidioidomycosis and the scores of the variables that evaluate the fungus virulence isolated from these patients, in BALB/c mice. Spearman correlation coefficient.

Strain	Severity (score)	Lethal dose 50% (score)	Fungal load Lung (score)	Fungal load Spleen (score)
Pb234 (PS3)	1.5	1.0	1.5	2.5
Pb326 (S1a)	3.5	3.0	3.0	2.5
Pb417 (S1a)	1.5	2.0	1.5	1.0
Pb531 (S1b)	3.5	4.0	4.0	4.0

Severity vs LD₅₀: $r_s = 0,90$ $p < 0,05$

r_s critical _{4;0,05} = 0,80

Severity vs lung fungal load: $r_s = 0,95$ $p < 0,05$

Severity vs splenic fungal load: $r_s = 0,75$ $p > 0,05$ [tendency]

LD₅₀ vs lung fungal load: $r_s = 0,95$ $p < 0,05$

LD₅₀ vs splenic fungal load: $r_s = 0,65$ $p > 0,05$

Pulmonary fungal load vs splenic fungal load: $r_s = 0,85$ $p < 0,05$

Specific humoral response

Serum levels of specific antibodies induced by infection with the five isolates of the *P. brasiliensis* complex and the two *P. lutzii* isolates were not detectable at the second and fourth week of infection. At the sixth week, only the Pb326 (S1a)-infected mice had detectable antibodies serum levels, with a median title of 1/32, while animals from all other groups - Pb192 (PS3), Pb234 (PS3), Pb417 (S1a), Pb531 (S1b), Pb01-like and 8334 (*P. lutzii*) remained unreactors.

Specific cellular immune response

The cell-mediated response was evaluated by determining the concentrations of five cytokines and the vascular endothelial growth factor (VEGF) in lung homogenate and will be presented, for each mediator, regarding the infecting isolate and the time of infection.

Interleukin-2 (IL-2)

Concentrations of IL-2 only varied by time, i.e., stage of infection, in mice inoculated with Pb417 (S1a), which was higher at week 6. The comparison between isolates showed the same behavior in the first two stages, in which they did not differ. At the sixth week, we observed the lowest concentration in animals infected with Pb531 (S1b). The others occupied intermediate positions [table 7].

Table 7. Influence of strain and stage of infection on the concentration (pg/mL) of interleukin-2 (IL-2) in lung homogenate of BALB/c mice infected with different isolates of the *Paracoccidioides* genus and healthy controls receiving saline solution 0.9%.

IL-2		Stage of infection		
Strain	2 th week	4 th week	6 th week	
Pb234 (PS3)	41.2±18.2 a,B	27.1±18.2 a,B	72.0±13.7 a,BC	
Pb326 (S1a)	34.1± 18.2 a,B	56.3±18.2 a,AB	63.9±14.8 a,BC	
Pb417 (S1a)	34.9±18.2 b,B	85.0±18.2 b,AB	194.3±18.2 a,A	
Pb531 (S1b)	40.1±18.2 a,B	47.6±18.2 a,B	30.3±21.0 a,C	
Pb192 (PS3)	49.1±21.0 a,B	51.7±21.0 a,AB	57.0±13.7 a,BC	
Pb01-like	95.0±18.2 a,AB	67.9±18.2 a,AB	121.4±16.2 a,AB	
8334 (<i>P. lutzii</i>)	101.8±18.2 a,AB	60.3±18.2 a,AB	108.1±18.8 a,AB	
Control	151.7±18.2 a,A	136.4±18.2 a,A	144.2±18.2 a,AB	

Analysis of variance, followed by the Tukey test ($p < 0.001$). Lowercase letters compare medians as a function of stage, for each strain (lines). Capital letters compare medians in the same column, regarding strain. Medians followed by the same letter do not differ from each other ($p > 0.05$), while medians followed by different letters differ from each other ($p \leq 0.05$).

Interleukin-10 (IL-10)

Concentrations of IL-10 only varied by time, i.e., stage of infection, in mice inoculated with Pb234 (PS3), which was higher at week 6. The comparison among strains showed the same behavior in the first two stages, in which they did not differ. At the sixth week, we observed the highest concentration in animals infected with Pb234 (PS3), much higher than controls. The others occupied intermediate positions [table 8].

Table 8. Influence of strain and stage of infection on the concentration (pg/mL) of interleukin-10 (IL-10) in lung homogenate of BALB/c mice infected with different isolates of the *Paracoccidioides* genus and in healthy controls receiving saline solution 0.9 %.

IL-10		Stage of infection		
Strain	2 th week	4 th week	6 th week	
Pb234 (PS3)	142.8±45.5 b,B	202.0±45.5 b,B	346.7±34.4 a,A	
Pb326 (S1a)	194.1±45.5 a,AB	212.6±45.5 a,B	221.4±37.2 a,AB	
Pb417 (S1a)	91.0±45.5 a,B	194.8±45.5 a,B	161.7±45.5 a,BC	
Pb531 (S1b)	104.4±45.5 a,B	147.5±45.5 a,B	108.7±52.6 a,BC	
Pb192 (PS3)	173.4±52.6 a,AB	133.6±52.6 a,B	190.3±34.4 a,BC	
Pb01-like	276.6±45.5 a,AB	231.2±45.5 a,AB	215.1±37.2 a,AB	
8334 (<i>P. lutzii</i>)	191.4±45.5 a,AB	200.0±45.5 a,B	157.1±32.2 a,BC	
Control	374.0±45.5 a,A	413.9±45.5 a,A	23.1±45.5 b,C	

Analysis variance, followed by the Tukey test ($p < 0.001$). Lowercase letters compare cytokine concentrations, as a function of time, for each strain (lines). Capital letters compare cytokine concentrations, depending on the strains, at each moment (columns). Concentrations followed by the same letter do not differ from each other ($p > 0.05$), while concentrations followed by different letters differ from each other ($p \leq 0.05$).

Interferon-gamma (INF- γ)

The concentration of IFN- γ varied as to the stage of the infection and to the strain. Pb234 and Pb417 increased from the second to the sixty week of infection, Pb192, Pb236 and Pb531 decreased, and Pb01-like and 8334 did no altered [table 9]. In addition, analysing the three stages, at week 2 Pb326, Pb01-like and 8334 showed similar concentrations, but higher than the others; at week4, Pb326 presented higher concentration than Pb234, Pb417 and Pb192, which were similar; finally, at week 6 Pb234 and Pb326 showed higher concentrations than Pb531, Pb01-like and 8334 [table 9].

Table 9. Influence of strain and stage of infection on interferon-gamma (INF- γ) concentration (pg/mL) in lung homogenate of BALB/c mice infected with different isolates of the *Paracoccidioides* genus and healthy controls receiving 0.9% saline solution.

Strain	INF- γ Stage of infection		
	2 th week	4 th week	6 th week
Pb234 (PS3)	37.5 $\pm$ 10.4 b,BC	52.3 $\pm$ 10.4 b,B	89.6 $\pm$ 7.8 a,A
Pb326 (S1a)	112.0 $\pm$ 10.4 a,A	115.4 $\pm$ 10.4 a,A	103.4 $\pm$ 8.5 a,A
Pb417 (S1a)	15.8 $\pm$ 14.7 b,C	53.9 $\pm$ 10.4 ab,B	80.5 $\pm$ 10.4 a,AB
Pb531 (S1b)	57.5 $\pm$ 10.4 a,BC	53.9 $\pm$ 10.4 a,B	37.9 $\pm$ 12.0 a,B
Pb192 (PS3)	53.3 $\pm$ 12.0 ab,BC	33.0 $\pm$ 12.0 b,B	67.6 $\pm$ 7.8 a,AB
Pb01 -like	106.5 $\pm$ 10.4 a,A	77.4 $\pm$ 10.4 ab,AB	48.2 $\pm$ 8.5 b,B
8334 (<i>P. lutzii</i>)	78.8 $\pm$ 10.4 a,A	64.9 $\pm$ 10.4 ab,AB	44.6 $\pm$ 7.3 b,B
Control	76.9 $\pm$ 10.4 a,AB	81.0 $\pm$ 10.4 a,AB	76.7 $\pm$ 10.4 a,AB

Analysis variance, followed by the Tukey test ($p < 0.001$). Lowercase letters compare cytokine concentrations, as a function of time, for each strain (lines). Capital letters compare concentrations of cytokines, depending on the strains, at each moment (columns). Concentrations followed by the same letter do not differ from each other ($p > 0.05$), while concentrations followed by different letters differ from each other ($p \leq 0.05$).

Tumor necrosis factor-alpha - (TNF- α)

No effect of interaction between moments and isolates was observed. The overall mean TNF- α concentration of each isolate was lower than that of the controls and did not differ from each other, while the overall mean of the sixth week was higher than the second and fourth weeks, which did not differ each other [Table 10].

Table 10. Influence of strain and stage of infection on tumor necrosis factor-alpha (TNF- α) concentration (pg/mL) in lung homogenate of BALB/c mice infected with different isolates of the *Paracoccidioides* genus and in healthy controls receiving 0.9% saline solution.

TNF- α		Stage of the infection		
Strain	2 th week	4 th week	6 th week	The overall mean of isolates
Pb234 (PS3)	82.2 $\pm$ 90.5	82.2 $\pm$ 90.5	209.5 $\pm$ 68.4	124.7 $\pm$ 48.4 B
Pb326 (S1a)	138.0 $\pm$ 90.5	180.3 $\pm$ 90.5	366.7 $\pm$ 73.9	228.3 $\pm$ 49.3 B
Pb417 (S1a)	91.2 $\pm$ 90.5	155.3 $\pm$ 90.5	281.3 $\pm$ 90.5	175.9 $\pm$ 52.2 B
Pb531 (S1b)	140.6 $\pm$ 90.5	355.8 $\pm$ 90.5	474.1 $\pm$ 104.5	323.5 $\pm$ 55.1 B
Pb192 (PS3)	112.6 $\pm$ 104.5	89.5 $\pm$ 104.5	164.3 $\pm$ 68.4	122.1 $\pm$ 54.3 B
Pb01 –like	251.5 $\pm$ 90.5	151.6 $\pm$ 90.5	150.1 $\pm$ 73.9	184.4 $\pm$ 49.3 B
8334 (<i>P. lutzii</i>)	206.0 $\pm$ 90.5	139.6 $\pm$ 90.5	163.8 $\pm$ 64.0	169.8 $\pm$ 47.7 B
Control	738.2 $\pm$ 90.5	655.1 $\pm$ 90.5	719.0 $\pm$ 90.5	704.1 $\pm$ 52.2 A
The general average of moments	220.0 $\pm$ 32.7 b	226.2 $\pm$ 32.7 B	316.1 $\pm$ 28.4 a	

Analysis variance, followed by the Tukey test ($p < 0.001$). Lowercase letters compare concentrations of cytokines as a function of time, for each strain (lines). Capital letters compare cytokine concentrations as a function of strains, at each time point (columns). Averages followed by the same letter or averages not followed by letters do not differ from each other ($p > 0.05$), while means followed by different letters differ from each other ($p < 0.05$).

Vascular endothelial growth factor - VEGF

VEGF lung concentrations varied regarding the stage of infection only in mice infected with Pb417 (S1a), which were higher at week 6. The comparison among strains showed no difference in the first two stages. However, at the sixth week the concentrations were quite variable, but catching the attention the lowest VEGF concentration in mice infected with Pb531 (S1b) [table 11].

Table 11. Influence of strain and stage of infection on endothelial cell growth factor (VEGF) concentrations (pg/mL) in lung homogenate of BALB/c mice infected with different isolates of the *Paracoccidioides* genus and in healthy controls receiving saline solution at 0.9 %.

Strain	Stage of infection		
	2 th week	4 th week	6 th week
Pb234 (PS3)	231.0±40.9 a,A	253.2±40.9 a,AB	301.9±30.9 a,BC
Pb326 (S1a)	304.5±40.9 a,A	189.8±40.9 a,AB	271.1±33.4 a,BCD
Pb417 (S1a)	197.7±40.9 b,A	244.1±40.9 b,AB	432.2±40.9 a,AB
Pb531 (S1b)	142.6±40.9 a,A	189.5±40.9 a,AB	110.8±47.2 a,D
Pb192 (PS3)	161.9±47.2 a,A	104.5±47.2 a,B	238.0±30.9 a,CD
Pb01-like	217.8±40.9 a,A	202.0±40.9 a,AB	276.5±36.6 a,BCD
8334 (<i>P. lutzii</i>)	212.6±40.9 a,A	214.6±40.9 a,AB	240.0±28.9 a,CD
Control	304.9±40.9 b,A	337.2±40.9 b,A	533.4±40.9 a,A

Analysis of variance, followed by the Tukey test ($p < 0.001$). Lowercase letters compare cytokine concentrations, as a function of time, for each strain (lines). Capital letters compare concentrations of cytokines, depending on the strains, at each moment (columns). Concentrations followed by the same letter do not differ from each other ($p > 0.05$), while concentrations followed by different letters differ from each other ($p \leq 0.05$).

Comparison of immunogenicity of isolates

The concentration of cytokines and VEGF, measured in pg/mL, in the lung homogenate of control mice receiving 0.9% saline revealed very different means from one mediator to another: IL-2 = 144, 1; IL-10 = 270.3; IFN- γ = 78.2; TNF- α = 704.1 and VEGF = 391.8. As TNF- α concentrations were almost 10-fold greater than that of IFN- γ , the inducing capacity of cytokines and VEGF production by the isolates was evaluated by rates, i.e, the ratio between the observed concentrations and the mean of the controls at each time of infection [tables 12 to 14, and figure 4].

Evaluation of the rates at weeks 2 and 4 of infection showed a predominance of higher values for IFN- γ and lower values for TNF- α , in almost all the strains. However, at week 6 there was a predominance of higher rates for IL-10 and lower rates for TNF- α in six out of the seven strains studied, with a tendency to this finding in the last one ($p=0.07$).

Table 12 - Evaluation of the cytokines and vascular endothelial growth factor in lung homogenate of BALB/c mice infected with different strains of the *Paracoccidioides* genus, presented as median, 10 and 30 quartiles, in brackets, according to strain, after two weeks of infection (stage 1).

Strain	Cytokine and VEGF					<i>p</i>
	IL-2	TNF- α	IFN- γ	IL-10	VEGF	
Pb234 (PS3)	0.28ab [0.23;0.32]	0.11b [0.08;0.15]	0.51ab [0.40;0.58]	0.37ab [0.32;0.44]	0.74a [0.70;0.82]	0.004
Pb326 (S1a)	0.19b [0.17;0.28]	0.19b [0.15;0.23]	1.46a [1.29;1.62]	0.56ab [0.42;0.62]	0.99ab [0.83;1.17]	0.004
Pb417 (S1a)	0.18ab [0.11;0.35]	0.12ab [0.08;0.17]	0.03b [0.00;0.21]	0.17ab [0.16;0.33]	0.68a [0.45;0.84]	0.04
Pb531 (S1b)	0.28ab [0.20;0.33]	0.20b [0.15;0.23]	0.72a [0.71;0.79]	0.27ab [0.22;0.34]	0.51ab [0.40;0.53]	0.009
PB192 (PS3)	0.39 [0.15;0.48]	0.18 [0.10;0.20]	0.66 [0.65;0.74]	0.45 [0.45;0.48]	0.45 [0.39;0.69]	0.08
Pb01 – like	0.74ab [0.50;0.76]	0.33b [0.26;0.42]	1.35a [1.17;1.60]	0.75ab [0.66;0.82]	0.68ab [0.58;0.85]	0.012
8334 (<i>P. lutzii</i>)	0.68ab [0.35;1.00]	0.30b [0.18;0.38]	1.00a [0.90;1.15]	0.51ab [0.43;0.60]	0.71ab [0.61;0.79]	0.009

Friedman's test. Lowercase letters compare cytokines for each strain. Medias followed by hair ($p > 0.05$), while medians followed by different letters differed from each other ($p \leq 0.05$). Concentration in pg/mL.

Table 13. Concentration of cytokines and vascular endothelial growth factor in lung homogenate (VEGF) of BALB/c mice infected with different strains of the *Paracoccidioides* genus, presented as median, 10 and 30 quartiles, in brackets, regarding strain, after four weeks of infection (stage 2).

Strain	Cytokines and VEGF					<i>p</i>
	IL-2	TNF- α	IFN- γ	IL-10	VEGF	
Pb234 (PS3)	0.21ab [0.18;0.22]	0.11b [0.09;0.17]	0.69ab [0.48;0.81]	0.44ab [0.32;0.65]	0.76a [0.69;0.82]	0.006
Pb326 (S1a)	0.33ab [0.25;0.58]	0.27b [0.20;0.35]	1.18a [1.04;1.81]	0.46ab [0.43;0.60]	0.55ab [0.43;0.69]	0.015
Pb417 (S1a)	0.51 [0.45;0.79]	0.22 [0.15;0.32]	0.64 [0.46;0.87]	0.42 [0.30;0.64]	0.70 [0.63;0.82]	0.06
Pb531 (S1b)	0.32 [0.27;0.43]	0.32 [0.20;0.88]	0.68 [0.54;0.79]	0.32 [0.25;0.47]	0.58 [0.45;0.68]	0.17
PB192 (PS3)	0.41ab [0.33;0.42]	0.13b [0.12;0.15]	0.40a [0.39;0.43]	0.33ab [0.31;0.33]	0.29ab [0.26;0.36]	0.04
Pb01 – like	0.50ab [0.38;0.62]	0.24b [0.18;0.29]	1.03a [0.84;1.07]	0.55ab [0.53;0.58]	0.58ab [0.48;0.72]	0.01
8334 (<i>P. lutzii</i>)	0.45ab [0.38;0.50]	0.21b [0.17;0.26]	0.83a [0.70;0.90]	0.49ab [0.42;0.55]	0.64ab [0.57;0.70]	0.004

Friedman's test. Lowercase letters compare cytokines for each strain. Medians followed by at least one letter in common do not differ from each other ($p > 0.05$), while medians followed by different letters differ from each other ($p \leq 0.05$). Concentration in pg/mL.

Table 14. Concentration of cytokines and vascular endothelial growth factor (VEGF) in lung homogenate of BALB/c mice infected with different strains of the *Paracoccidioides* genus, presented as median, 10 and 30 quartiles, in brackets, regarding strain, after six weeks of infection (stage 3).

Strain	Cytokines and VEGF					<i>p</i>
	IL-2	TNF- α	IFN- γ	IL-10	VEGF	
Pb234 (PS3)	0.55bc [0.36;0.60]	0.20c [0.18;0.41]	1.19ab [0.91;1.43]	13.90a [12.25;15.84]	0.53bc [0.47;0.74]	<0.001
Pb326 (S1a)	0.44b [0.36;0.54]	0.47b [0.21;0.53]	1.24ab [1.16;1.41]	10.06a [6.51;11.04]	0.54b [0.32;0.57]	<0.001
Pb417 (S1a)	1.45ab [1.18;1.51]	0.40b [0.30;0.49]	1.09ab [0.95;1.15]	6.91a [4.63;9.36]	0.85ab [0.60;1.02]	0.003
Pb531 (S1b)	0.24 [0.14;0.27]	0.60 [0.37;0.96]	0.64 [0.25;0.71]	3.41 [3.13;6.59]	0.16 [0.15;0.28]	0.07
PB192 (PS3)	0.44bc [0.29;0.53]	0.18c [0.16;0.28]	0.82ab [0.76;1.05]	7.54a [6.93;9.68]	0.43bc [0.36;0.53]	<0.001
Pb01 – like	0.78ab [0.65;0.83]	0.21b [0.19;0.25]	0.65ab [0.53;0.74]	6.98a [6.36;7.81]	0.50b [0.33;0.53]	<0.001
8334 (<i>P. lutzii</i>)	0.79ab [0.47;0.97]	0.25c [0.14;0.29]	0.57abc [0.49;0.64]	6.80a [5.98;7.86]	0.39bc [0.26;0.64]	<0.001

Friedman's test. Lowercase letters compare cytokines for each strain. Medians followed by at least one letter in common do not differ from each other ($p>0.05$), while medians followed by different letters differ from each other ($p\leq 0.05$). Concentration in pg/mL.

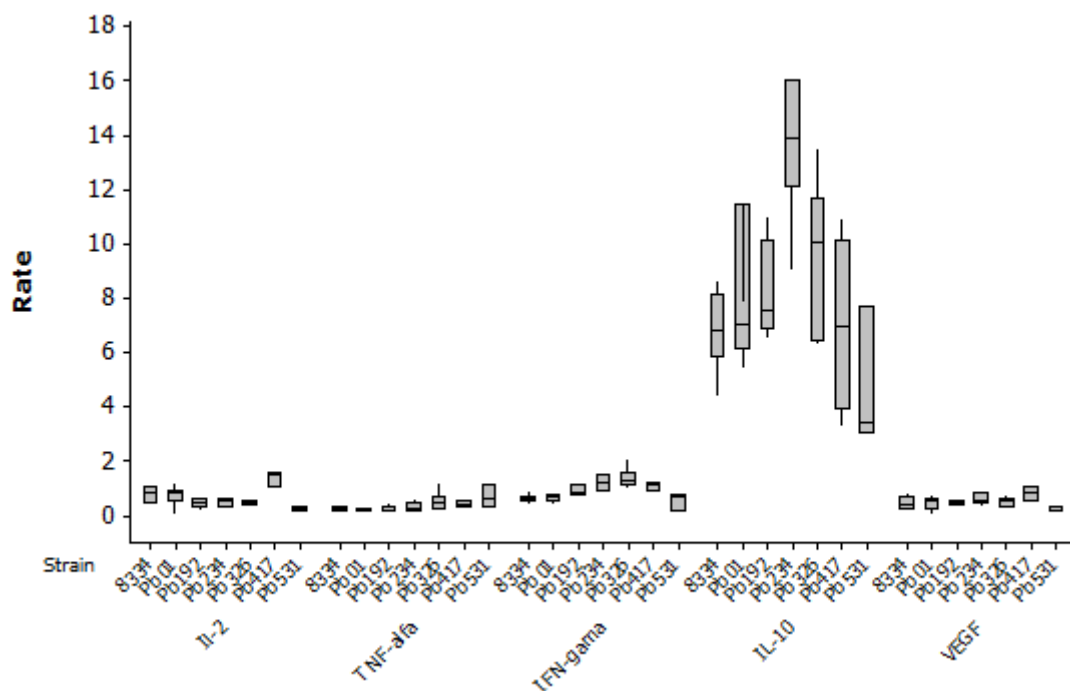


Figure 4. Box plot for concentrations of cytokines and vascular endothelial growth factor (VEGF) in lung homogenate of BALB/c mice, regarding strain, after six weeks of infection (stage 3). IL-2-interleukin-2; TNF- α - tumor necrosis factor- α ; IFN- γ - interferon- γ ; IL-10-interleukin-10; VEGF - vascular endothelial growth factor. Concentration in pg/mL.

Analysis of the correlation between variables

The correlation between the severity of the clinical condition of the patients and the pathogenicity of the isolates determined by the 50% lethal dose was significant and direct, i.e, the higher the pathogenicity in BALB/c mice (the lower the LD₅₀), the greater the severity presented ($r_s = 0.90$, $p < 0.05$), as well as between clinical severity and pulmonary fungal load ($r_s = 0.95$, $p < 0.05$).

Among the other correlations analyzed, considering the variables studied in BALB/c mice, three were significant: a) LD₅₀ and VEGF, inverse; b) IL-2 and fungal load of the spleen, inverse; c) INF- γ and IL-10, direct, as shown in Table 15.

Table 15. Correlation between variables indicating pathogenicity and those indicating immunogenicity in BALB/c mice infected with different strains of the *Paracoccidioides* genus.

	LD50%	Fungal load of the lung	Fungal load of the spleen	IL-2	IL-10	IFN- γ	TNF- α	VEGF
LD50%								
Fungal load of the lung	0,607 $p>0,05$							
Fungal load of the spleen	0,500 $p>0,05$	0,563 $p>0,05$						
IL-2	-0,428 $p>0,05$	-0,607 $p>0,05$	-0,857 $p<0,05$					
IL-10	-0,428 $p>0,05$	-0,678 $p>0,05$	0,071 $p>0,05$	0,142 $p>0,05$				
IFN-γ	-0,053 $p>0,05$	-0,0428 $p>0,05$	0,071 $p>0,05$	0,178 $p>0,05$	0,821 $p<0,05$			
TNF-α	0,607 $p>0,05$	0,383 $p>0,05$	0,571 $p>0,05$	-0,214 $p>0,05$	-0,178 $p>0,05$	0,035 $p>0,05$		
VEGF	-0,714 $p=0,05$	-0,321 $p>0,05$	-0,357 $p>0,05$	0,428 $p>0,05$	0,357 $p>0,05$	0,25 $p>0,05$	-0,142 $p>0,05$	

LD50% - lethal dose 50%; IL-2-interleukin-2; IL-10 interleukin-10; IFN- γ -interferon-gamma; tumor necrosis factor - alpha - TNF- α ; endothelial cell growth factor - VEGF.

Discussion

PCM is a systemic mycosis caused by thermally dimorphic fungi of the *Paracoccidioides* genus which develops a mycelial form at room temperature and a multiple-budding yeast form in the infected host or in culture at 37°C.⁽²⁾ Studies carried out with randomly amplified polymorphic DNA (RAPD) analysis⁽²⁰⁾, gene genealogy⁽⁸⁾, genealogic concordance method of phylogenetic species recognition (GCPSR)⁽¹⁾, and nuclear gene genealogy demonstrated that fungi of the *Paracoccidioides* genus can be distributed in five phylogenetic species, four of which constitute the *Paracoccidioides brasiliensis* species complex⁽²¹⁾. *Paracoccidioides restrepiensis*, formerly known as phylogenetic species 3 (PS3), was initially constituted of 21 Colombian isolates. Although considered restrict to this country, this specie has also been found in Venezuela⁽²²⁾ and possibly in Brazil - there is a Brazilian isolate whose identification has been difficult and appears as the outgroup of the dyad PS3/PS4⁽²¹⁾. It is worth mentioning that a sample characterized as *P. restrepiensis* (PS3) was isolated from one of our patients, who was born in the Nova Fátima municipality (Paraná state) and had never travelled or moved to other countries, confirming that this specie is not restricted to Colombia.

PCM clinical presentation ranges from mild forms to severe disease.⁽²⁾ It presents two main clinical forms of active disease in patients without any cause of immunosuppression – the acute/subacute form (AF) and the chronic form (CF). Age is the main dividing line of these clinical forms – the AF predominates in children and young adults while the CF in adults. However, some adults can present the AF, specially in some Brazilian regions. In addition, a wide spectrum of severity has been observed among patients with the CF.

The difference among PCM clinical forms could be ascribed to characteristics of the host such as age, gender and genetic patterns and/or pathogenicity and immunogenicity of the fungal isolate.^(23,6)

The great development of the knowledge on the immune system and its importance on the host-parasite relationship, asymptomatic infection or disease, and on the PCM clinical manifestations has dominated the scenario of the different degrees of dissemination and severity of the disease. The degree of severity of PCM patients presents a direct correlation with the humoral immunity, and an indirect one with the cell mediated immunity^(24,25) PCM patients with the severe clinical forms usually presents high serum levels of specific antibodies and intensively impaired cellular immune response, while those with the mild forms in general show low antibody serum levels and cell mediated immunity not much compromised.

The existence of distinct *Paracoccidioides* strains that display different degrees of virulence was very well demonstrated.^(23,7,26,27,28,29,30) However, contradictory results have been observed, which could be due to the type of experimental models used. In addition, the lack of well characterized virulent and avirulent strains could be an obstacle to the recognition and identification of mycological virulence factors.^(23,31)

The present investigation was carried out in BALB/c mice intraperitoneally infected with different isolates of the *Paracoccidioides* genus, identified by genotyping methodology. The LD₅₀ and the pulmonary and spleen fungal load were used to evaluate the virulence of the isolates, and the immunogenicity was analysed by determining the specific antibody serum levels (humoral immunity) and by the concentration of cytokines and VEGF in pulmonary homogenates (cellular immune response). These data were evaluated regarding the clinical forms and the degree of severity presented by four patients from whom these fungi were isolated. Furthermore,

correlation between clinical and experimental findings and among different experimental results were also carried out.

A direct correlation was observed between severity of the clinical forms of four patients and virulence determined by LD₅₀ of the corresponding isolates in BALB/c mice. This type of correlation was only partially observed by Singer-Vermes et al. (1994)⁽⁶⁾ and it was not noted in a previous study.⁽²³⁾ However, it should be considered that both studies were performed in B10.A mice, previously recognized to be highly sensitive to intraperitoneal infection with *P. brasiliensis*.⁽³²⁾

Vascular endothelial growth factor (VEGF) is a signaling protein that stimulates the vascularization and the angiogenesis, besides participating in the system responsible for recovering the oxygen supply to the tissue when the blood circulation is not appropriate. These activities are related to the increase of the eNOS expression and NO production.⁽³³⁾ Hypoxia and some cytokines are potent stimulators of the VEGF production.^(34,35) In our study, the VEGF rates and cytokines rates were similar in the stages 1, 2 and 3; however, VEGF rates were much lower in stage 3 than in stages 1 and 2. This decreased intensity was also observed in a recent study performed in a murine paracoccidoidal infection⁽³⁶⁾ and can explain the progress of the disease severity over time.

The evaluation of cytokines concentration and VEGF in pulmonary homogenates showed few differences among isolates in the first and second stages of the infection. Thus, in the third stage, with six weeks of infection, the isolates show different results. Moreover, the rates of the different cytokines and VEGF reveal similar results in all the stages of infection, independently of the isolate. However, it is evident the higher rates of IL-10 concentration, independently of the isolate. The sequence of events, presented by Defavere (1999)⁽³⁷⁾ and summarized as follows, could explain our

findings.

In experimental model using BALB/c mice, Biagioni et al. (1987)⁽³⁸⁾ and Defaveri (1999)⁽³⁷⁾ demonstrated the deposition of IgG and C3 in the fungal wall, into the granulomas, and increase of the IgG+ cells in the lymphoid halo perigranuloma, a very active process since the fourth week of infection. Thus, the specific antibodies could play a role in the defense against the *Paracoccidioides* cells in the early phase of the infection, by attracting neutrophils to phagocytize and to lyse fungal cells or to facilitate the release of lytic factors from cells of the granuloma, such as proteases and free radicals. Nevertheless, a predominant Th2 response follows this phase, with great production of IL-4 by T CD4+ cells, basophils and mast cells.⁽³⁹⁾ This increased production of IL-4 in the granuloma could play a role in the progress of the infection by eliciting the decrease of the IFN- γ production by T lymphocytes, the decrease of TNF- α production by macrophages and natural killer (NK) cells, and the increase of the IL-10 production. IL-10 is a potent inhibitor of several protective responses against fungal cells of the *Paracoccidioides* genus: a) production of IFN- γ b) production of TNF- α by macrophages and NK cells; c) delayed-type hypersensitivity response; d) local production of NO, which presents great activity on *Paracoccidioides* spp in the murine infection. Furthermore, IL-10 stimulates the antibodies production and increases the mast cells proliferation.⁽⁴⁰⁾ This sequence of events could explain our findings in the first two stages, and mainly those of the sixth week of infection, characterized by low concentrations of IL-2, IFN- γ and TNF- α , and very high IL-10 concentrations.

VEGF regulates angiogenesis by inducing proliferation, migration and permeability of endothelial cells.⁽³⁴⁾ Besides hypoxia, other factors regulate the VEGF expression, such as the cytokines TNF- α and TGF- β . The decreased concentrations of the pulmonary cytokines, TNF- α among them, could explain the low VEGF

concentrations, which could contribute to the progress of the paracoccidioidic lesions.

The recent progress of the molecular biology techniques made it possible to investigate the diversity of pathogen strains based on genomic characteristics⁽²⁰⁾, including the fungi of the *Paracoccidioides* genus.⁽²¹⁾ Thus, genotypic analysis was used to investigate possible relationship among *Paracoccidioides* clusters and geographical origin, virulence and/or clinical manifestations. Association between genetic clusters and geographical origin was demonstrated, but not with virulence evaluated in experimental infection or severity of clinical manifestations, in the human disease.^(20,41)

The pathogenicity has been evaluated by LD₅₀ and fungal load measured by plating different tissues, specially the lungs. The immunogenicity has been evaluated by the dosage of specific serum antibodies and, mainly, by the quantification of cytokines, including in tissue homogenates. However, the cytokines play an important role in the response of the host to the parasite. It could be suggested that LD₅₀ and the tissue fungal load are expressing the pathogenicity-immunogenicity interaction.

In addition, it is well documented the difference of response to the same isolate when it is evaluated in distinct experimental conditions / models. Thus, a standardization of the experimental model might be proposed to this type of evaluation. As there was a correlation between patients' severity and LD₅₀, and between patients' severity and pulmonary fungal load in BALB/c mice, in the sixth week of infection, this could be a model to take into account for this evaluation. In addition, this species of mice is more easily available. Moreover, it is necessary to stimulate the isolation of *Paracoccidioides* fungal cells from clinical specimens for further genotyping and evaluation of its virulence. In the future, when virulence genes could be easily identified, this information should be investigated and shared with physicians.

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III. Conclusões

A avaliação da patogenicidade e da imunogenicidade de isolados clínicos do gênero *Paracoccidioides*, nas condições dos experimentos desenvolvidos, permitiu concluir o que se segue.

1. A identificação molecular dos cinco isolados clínicos, pelo seqüenciamento da região Exon 2 da gp43, revelou que dois pertencem à espécie críptica S1a, um à S1b e dois à PS3, demonstrando a variabilidade de espécies que comprometem pacientes da Região de Botucatu.

2. A identificação da cepa Pb234, caracterizada como *Paracoccidioides restrepiensis* (PS3), demonstra que esta espécie não está restrita à Colômbia.

3. Os parâmetros dose letal 50%, recuperação fúngica de tecidos de camundongos infectados e tempo de sobrevivência demonstram que a virulência da cepa Pb531 é alta, da Pb326 é intermediária e a das demais - Pb192, Pb234, Pb417, Pb01 e 8334 é baixa.

4. Somente os animais infectados com a cepa Pb326, de virulência intermediária, apresentaram níveis séricos de anticorpos detectáveis na sexta semana pós-infecção.

5. A produção das diferentes citocinas e fator de crescimento endotelial vascular - VEGF, determinadas em tecido pulmonar, foi em geral uniforme nos dois primeiros estágios da infecção, mas revelou nítido predomínio da IL-10 no terceiro.

6. Observou-se correlação direta entre gravidade do quadro clínico apresentado pelos pacientes e patogenicidade de seus isolados no modelo experimental estudado – camundongos BALB/c, concentrações utilizadas do inóculo, via intra-peritoneal de infecção e variáveis estudadas – dose letal 50%, curva de sobrevivência e intensidade da infecção pulmonar.

IV. Apêndice



Comissão de Ética no Uso de Animais

Criada através da Portaria DFM nº 611 de 13/12/2012



UNIVERSIDADE ESTADUAL PAULISTA
CAMPUS DE BOTUCATU
FACULDADE DE MEDICINA

unesp

CERTIFICADO Nº 1224/2017-CEUA

Certificamos que a proposta intitulada "Patogenicidade de isolados clínicos do complexo *paracoccidioides brasiliensis*", registrada com o nº 1224/2017, sob a responsabilidade de Beatriz Aparecida Soares Pereira – Orientador: Prof. Dr. Rinaldo Poncio Mendes Coorientador: Prof. Dr. James Venturini – que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei n. 11.794, de 8 de outubro de 2008, do Decreto n. 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais da Faculdade de Medicina de Botucatu, em reunião de 31 de agosto de 2017.

Finalidade () Ensino (X) Pesquisa Científica	
Vigência da autorização	23/02/2019
Espécie/Linhagem/Raça	Camundongo Isogênico/BALB-C
Nº de animais	320
Peso/Idade	25 gramas/ 8 a 12 semanas
Sexo	Madro
Origem	Biotério Central da UNESP - FMB

Prof. Dr. Guilherme Antônio Moreira de Barros
Presidente da CEUA

Alberto Santos Capelluppi
Secretário da CEUA



UNESP -FACULDADE DE
MEDICINA DE BOTUCATU



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: "Patogenicidade e imunogenicidade de isolados clínicos do complexo Paracoccidioides brasiliensis"

Pesquisador: Beatriz Aparecida Soares Pereira

Área Temática:

Versão: 2

CAAE: 71276817.4.0000.5411

Instituição Proponente: Departamento de Doenças Tropicais e Diagnóstico por Imagem

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.258.227

Apresentação do Projeto:

Trata-se os autos de resposta do pesquisador referente ao questionamento do parecerista constante no Parecer nº 2.208.807 de 09 de agosto de 2017 sobre o Projeto intitulado " Patogenicidade e imunogenicidade de isolados clínicos do complexo Paracoccidioides brasiliensis". As pendências relatadas foram em relação a:

1. Esclarecer quanto ao período de seleção dos pacientes que fornecerão a amostra de ambos centros participantes. Se ainda estiveram em acompanhamento é necessária a aplicação do TCLE (inserir). Esclarecer quanto ao período de seleção dos pacientes que fornecerão a amostra de ambos centros participantes.

Resposta: Não haverá seleção de pacientes pois o projeto utilizará amostras já estocadas em estufa 35°C obtidas em um primeiro atendimento na rotina do ambulatório de Micologia Clínica da Faculdade de Medicina de Botucatu – UNESP e da Faculdade de Medicina da Universidade Federal do Mato Grosso do Sul – UFMS. Dessa forma pede-se a dispensa do TCLE, já que os pacientes não fazem mais acompanhamento ambulatorial nesses Centros.

2) Quanto aos riscos da pesquisa, este só se refere ao técnico?

Resposta: Sim, pois as amostras já foram coletadas sendo armazenada no Laboratório de Pesquisa de Doenças Tropicais – Micologia Médica da Faculdade de Medicina de Botucatu – UNESP. Somente

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PARECER CONSUBSTANCIADO DO CEP

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Continuação do Parecer: 2.258.227

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_P ROJETO_922005.pdf	24/08/2017 12:52:44		Aceito
Outros	OFICIO.pdf	24/08/2017 12:52:11	Beatriz Aparecida Soares Pereira	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	BIORREPOSITORIO.pdf	23/08/2017 13:18:18	Beatriz Aparecida Soares Pereira	Aceito
Outros	UFMS.pdf	23/08/2017 13:17:46	Beatriz Aparecida Soares Pereira	Aceito
Projeto Detalhado / Brochura Investigador	PCMBeatrizprojeto.doc	15/05/2017 21:06:03	Beatriz Aparecida Soares Pereira	Aceito
Outros	AnuenciaUnipex.pdf	15/05/2017 21:03:55	Beatriz Aparecida Soares Pereira	Aceito
Outros	AnuenciaFMB.pdf	15/05/2017 21:03:15	Beatriz Aparecida Soares Pereira	Aceito
Folha de Rosto	Folhaderosto.pdf	15/05/2017 21:02:13	Beatriz Aparecida Soares Pereira	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

BOTUCATU, 04 de Setembro de 2017

Assinado por:

SILVANA ANDREA MOLINA LIMA
(Coordenador)

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