FERNANDO CEZAR DOS SANTOS

ATIVIDADE HEMOLÍTICA E HEMAGLUTINANTE DE CÉLULAS VIÁVEIS E ANTÍGENOS SOLÚVEIS DE *Paracoccidioides spp.* SOBRE ERITRÓCITOS HUMANOS

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

Orientadora: Prof.ª Dr.ª Eiko Nakagawa Itano.

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Londrina, 13 de abril de 2015.

Este trabalho foi realizado no Laboratório de Imunologia Aplicada do Departamento de Ciências Patológicas da Universidade Estadual de Londrina, sob a orientação da Profª. Drª. Eiko Nakagawa Itano e contou com o apoio da Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação Araucária, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e PROPPG/UEL.

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‘‘Respirei fundo e escutei o velho e orgulhoso som do meu coração. Eu sou, eu sou, eu sou.’’

Sylvia Plat

SANTOS, F. C. **Atividade hemolítica e hemaglutinante de células viáveis e antígenos solúveis de *Paracoccidioides spp.* sobre eritrócitos humanos**. 2015. 52 f. Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2015.

RESUMO

Paracoccidioidomicose (PCM), uma micose sistêmica endêmica na América Latina, é causada pelos fungos termodimórficos *P. brasiliensis* e *P. lutzii*. A atividade hemolítica tem sido considerada um importante fator de virulência em diversos patógenos e tem sido detectada com células leveduriformes de *P. brasiliensis* e *P. lutzii.* O objetivo deste estudo foi avaliar a atividade hemolítica e hemaglutinante de células leveduriformes de *P. brasiliensis* e *P. lutzii* e seus antígenos solúveis. Inicialmente, células viáveis de *P. brasiliensis* (Pb339) e *P. lutzii* (LDR2) e seus antígenos livres de células (CFA) nativos ou aquecidos, em diferentes concentrações (101, 2, 3, 4, 5, 6,7 células/ml e 15.0; 7.5; 3.7 e 1.8 mg proteina/ml, respectivamente) foram submetidos ao teste de hemólise. Adicionalmente, os componentes solúveis de cultura dos fungos em meios sólidos, CFA (Saboraud com ou sem BHI) e sobrenadantes de cultura dos fungos (ExoAgs) em meios líquidos (RPMI, BHI), coletados a cada três dias por um período de 30 dias também foram avaliados. O teste de hemólise foi realizado em placas de 96 poços com eritrócitos humanos (1%) à 37ºC (3 hrs e 5 hrs) e o grau de hemólise foi avaliado por espectrofotometria no comprimento de onda de 550 nm. A atividade hemolítica foi observada em ambos os fungos na concentração de 107 células/poço e foi maior com LDR2 quando comparado ao Pb339 ($p<0,05$). Em contraposição, CFA de Pb339 apresentou maior atividade hemolítica que CFA de LDR2 ($p<0,05$). Em ambos, a atividade hemolítica do CFA foi maior em 5 hrs do que em 3 hrs de incubação e também quando a maior concentração foi usada. Surpreendentemente, foi também detectada atividade hemaglutinante com ambos os fungos em concentrações menores (106,105,104). Essa hemaglutinação foi detectada em CFA diluído e aquecido de Pb339 ou CFA de LDR2 aquecido, mas não diluído. O perfil hemolítico foi similar com antígenos solúveis de Pb339 obtidos de meios sólidos e líquidos, com atividade máxima começando em 9-12 dias de cultura, que foi mantida até a avaliação final. No entanto, somente antígenos solúveis de LDR2 crescidos em meio Saboraud apresentaram alta atividade hemolítica nos dias 15-18, que então decresceu. Todas as outras fontes de LDR2 apresentaram atividade diminuída durante todos os períodos de cultura avaliados. Em conclusão, células leveduriformes de *P. brasiliensis e P. lutzii* possuem a habilidade de lisar e aglutinar eritrócitos humanos, mas possivelmente Pb339 é mais eficaz em liberar esse fator em forma solúvel e tanto tempo quanto condições de cultura afetam a produção de fatores hemolíticos.

Palavras-chaves: Fungos. Meio de cultura. Paracoccidioidomicose. *P. brasiliensis. P. lutzii*. Fator de virulência.

SANTOS, F. C. **Hemolytic and hemagglutinating activity of *Paracoccidioides* sp. viable cells and soluble antigens on human erythrocytes.** 2015. 52 p. Dissertation (Master's Degree in Experimental Pathology) – Londrina State University, Londrina, 2015.

ABSTRACT

Paracoccidioidomycosis (PCM), an endemic systemic mycosis in Latin America, is caused by thermodimorfic fungi *P. brasiliensis* and *P. lutzii*. The hemolytic activity has been considered an important virulence factor in many pathogens and the hemolytic activity has been detected in yeast cells of *P. brasiliensis* and *P. lutzii.* This study was designed to evaluate the hemolytic and hemagglutinating activity of *P. brasiliensis* and *P. lutzii* yeast cells and theirs soluble antigens. Initially, *P. brasiliensis* (Pb339) and *P. lutzii* (LDR2) viable yeast cells and its native or heated cell free antigens (CFA), in different concentrations (101, 2, 3, 4, 5, 6,7 cells/ml and 15.0; 7.5; 3.7 and 1.8 mg protein/ml, respectively), were submitted to hemolysis test. Additionally, the soluble components from fungi grown on solid medium, CFA (Saboraud with or without BHI) and supernatants from fungi (ExoAgs) grown in liquid media (RPMI, BHI), collected every three days for a period of 30 days were also evaluated. The hemolysis test was performed in 96-well plates with human erythrocytes (1%), at 37ºC (3 hrs and 5 hrs) and the degree of hemolysis evaluated by spectrophotometry at a wavelength of 550 nm. The hemolytic activity was observed in both fungi at 107 cells and was stronger with LDR2 than Pb339 ($p<0.05$). However, the CFA from Pb339 presented stronger hemolysis than LDR2 ($p<0.05$). In both, CFAs activity was stronger in 5 than 3 hrs incubation as well as in higher concentration. Surprisingly, it was detected hemagglutinating activity with both yeast cells in lower concentrations (106,105,104). This hemagglutination was also detected in heated and diluted CFA from Pb339 or in heated and not diluted CFA from LDR2. The hemolytic activity profile was similar with soluble antigens from Pb339 grown on both solid or liquid media, with maximum activity starting in 9-12 days of culture remaining until the final evaluation. Nonetheless, only soluble antigens from LDR2 grown in Sabouraud medium presented stronger activity at 15-18 days followed by decrease. All other LDR2 sources presented low activity throughout the evaluated period of culture. In conclusion, *P. brasiliensis* and *P. lutzii* yeast cells present the ability to lyse and agglutinate human erythrocytes, but possibly Pb339 is more effective in releasing this factor in soluble form and both time and growth culture conditions affect the production of hemolytic factors.

Keywords: Fungi. Culture medium. Paracoccidioidomycosis. *P. brasiliensis. P. lutzii*. Virulence factor.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

°C	Graus Celsius
α	Alfa
β	Beta
BHI	Ágar Infusão de Cérebro de Coração
C1q	*C1 q Complex of Complement*
C3b	*Complement Protein 3*
C4b	*Complement Protein 4*
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CD35	*Cluster of Diferenciation 35*
CFA	*Cell-free Antigens*
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CR1	*Complement Receptor type 1*
DARC	*Duffy Antigen Receptor for Chemokines*
DNA	*Deoxyribonucleic acid*
E-CR1	*Human Erythrocyte Complement Receptor type 1*
ExoAgs	*Exoantigens*
GlcNAc	*N-acetylglucosamine*
Gp43	Glicoproteína de 43 kDa
Gp70	Glicoproteína de 70 kDa
GPI	Glicosilfosfatidilinositol
HIV-1	*Human Immunodeficiency Virus type 1*
IA	*Immune Adherence*
IC	I*mmune Complexes*
kDa	Quilodalton
LDR2	*P. lutzii* cepa LDR2
MBL	*Mannose Binding Lectin*
mg	Miligramas
ml	Mililitros
nm	Nanômetro
Pb339	*P.brasiliensis* cepa 339
PBS	Tampão Fosfato Salina 14

PCM	Paracoccidioidomicose/*Paracoccidioidomycosis*
PMSF	*Phenylmethanesulfonyl Fluoride*
TNF- α	Fator de Necrose Tumoral Alfa
UEL	Universidade Estadual de Londrina

SUMÁRIO

1 INTRODUÇÃO

Os fungos termodimórficos *Paracoccidioides brasiliensis* (espécies S1, PS2 e PS3) e *Paracoccidioides lutzii* são os agentes etiológicos da Paracoccidioidomicose (PCM), uma micose sistêmica que se distribui em vários países da América Latina (RESTREPO, 1985; TEIXEIRA *et al.*, 2009).

A primeira descrição da PCM ocorreu em 1908, pelo médico brasileiro Adolfo Lutz, em dois pacientes que apresentavam lesões orais e linfadenopatia cervical, identificando o material retirado das lesões e efetuando o primeiro isolamento do fungo em cultura (LUTZ, 1908).

O Brasil apresenta o maior número de casos de PCM, onde a prevalência, aspectos clínicos e epidemiológicos variam conforme a região (PANIAGO *et al.*, 2003).

Na região Centro-oeste há maior prevalência de infecções por *P. lutzii*, porém há casos descritos na região Norte, enquanto que nas regiões Sul e Sudeste, *P. brasiliensis* é a espécie predominante (GEGEMBAUER *et al.*, 2014).

A PCM constitui a oitava causa de morte no Brasil considerando as doenças infecciosas e parasitárias predominantemente crônicas (FORNAJEIRO *et al.*, 2005). A maior taxa de mortalidade pela PCM ocorre nas regiões Sul e Sudeste e é diagnosticada em quase todo o estado do Paraná, sendo a quinta causa de morte por doença infecciosa no estado (FORNAJEIRO *et al.*, 2005). Por não ser uma doença de notificação compulsória, estima-se sua incidência através de inquéritos epidemiológicos e relatos de casos (SHIKANAI-YASUDA *et al.*, 2006).

Esta patologia acomete preferencialmente agricultores do sexo masculino, com idade entre 30 a 50 anos (BRASIL, 2010). Mulheres possuem menor contato com áreas de risco infeccioso e possuem a proteção que lhes é conferida pelos estrógenos (SANO; NISHIMURA; MIYAJI, 1999).

A PCM é causada pela inalação de conídeos de *Paracoccidioides* que facilmente alcançam os alvéolos pulmonares e podem disseminar-se pela via linfohematogênica, sendo caracterizada por inflamação granulomatosa e altos níveis de anticorpos específicos (SAN-BLAS, 1993). Diversas outras manifestações clínicas e imunológicas são observadas, variando desde infecção assintomática à forma disseminada e severa. De acordo com a classificação atual, existem duas formas principais: PCM infecção, observada em indivíduos saudáveis e assintomáticos que vivem em áreas endêmicas e são reativos à intradermorreação com paracoccidioidina; PCM doença, forma sintomática que

divide-se em forma crônica/adulta e forma aguda/juvenil. A forma crônica apresenta alta frequência de comprometimento pulmonar, cutâneo e de mucosas e afeta indivíduos adultos, enquanto que a forma aguda é caracterizada por envolvimento sistêmico de linfonodos, hepatoesplenomegalia e lesões na medula óssea, afetando jovens de ambos os sexos (SHIKANAI-YASUDA *et al.*, 2006).

As colônias de *Paracoccidioides* apresentam-se em cor creme, cremosas e de aspecto cerebriforme na fase leveduriforme, sendo vistas em meio de cultura entre 10 e 15 dias de incubação (SIDRIM; ROCHA, 2004). Observam-se nas colônias leveduras de tamanhos diversos (4-30 μm), frequentemente ovais, dividindo-se por brotamento simples ou múltiplo da parede das células mães, originando uma forma típica conhecida como ‘’roda de leme’’(SIDRIM; ROCHA, 2004).

O principal componente que confere virulência ao fungo *P. brasiliensis* é a glicoproteína de 43 kDa (gp43), antígeno exocelular e imunodominante presente na superfície celular que atua como um receptor para a laminina presente na matrix extracelular (MEC), mecanismo pelo qual o fungo invade habilmente os tecidos (VICENTINI *et al.*, 1994).

A gp70, antígeno reconhecido em 96% dos soros de pacientes com PCM (BLOTTA; CAMARGO, 1993), pode induzir respostas linfoproliferativas quando testadas com linfócitos de pacientes com PCM (BENARD *et al.*, 1997) e diminuir a função de macrófagos peritoneais (fagocitose, NO, e produção de H_2O_2) (MATTOS GROSSO *et al.*, 2003).

Os fungos do complexo *Paracoccidioides* sintetizam diversos outros fatores associados à sua patogenicidade: paracoccina, uma lectina ligante de laminina, que estimula a liberação de TNF-α e óxido nítrico por macrófagos (COLTRI *et al.*, 2006); a serino-tiol protease exocelular, que pode clivar a laminina e outros componentes da matrix extracelular (MEC) (PUCCIA *et al.*, 1998); sialoglicoconjugados de superfície podem atuar como adesinas e aumentar o poder invasivo de *Paracoccidioides* (HAMILTON *et al.*, 1999); β-1,3-glucana, que desencadeia uma potente resposta inflamatória em células pulmonares e α-1,3-glucana, mais abundante na fase leveduriforme e induz resistência à fagocitose (SILVA *et al.*, 1994); glicolipídeos contendo β-galactofuranose que são altamente reativos à anticorpos de pacientes com PCM (TOLEDO *et al.*, 1995); fosfolipase B, que possui atividade hidrolítica sobre fosfolipídeos de membranas celulares do hospedeiro (TAVARES *et al.*, 2005); fosfolipase C, que causa a liberação de glicoproteínas antigênicas ancoradas ao glicosilfosfatidilinositol (GPI) de membrana do fungo e pode causar a degradação de GPIs presentes nas células do hospedeiro (HEISE; TRAVASSOS; DE ALMEIDA, 1995);

melanina, que protege o fungo da fagocitose e aumenta sua resistência à drogas antifúngicas (SILVA *et al.*, 2006); vesículas extracelulares contendo epítopos de α-galactosil que são altamente imunogênicos (VALLEJO *et al.*, 2011).

A capacidade de lisar eritrócitos tem sido descrita em diversos fungos patogênicos endêmicos, *Histoplasma capsulatum* e *Blastomyces dermatidis*, bem como patógenos oportunistas, incluindo *Candida albicans* e *Cryptococcus neoformans* (SALVIN, 1951) e isolados clínicos de várias espécies de fungos dermatófitos (AKTAS; YIGIT, 2014).

Estudos preliminares realizados em nosso laboratório demonstraram que *P. brasiliensis* e *P. lutzii* são hemolíticos quando inoculados em ágar sangue humano e que preparações antigênicas feitas a partir de culturas de *P. brasiliensis* são hábeis em causar lise de eritrócitos humanos (GONÇALVES; ONO; ITANO, 2001; GONÇALVES, 1999). Além disso, Schneider (2000) verificou que frações obtidas por cromatrografia presentes no CFA (cell-free antigen) de *P. brasiliensis* que possuem massa molecular entre 30 e 50 kDa podem lisar eritrócitos de carneiro.

Hemolisinas pertencem a uma classe de proteínas produzidas por bactérias e fungos e possuem funções pleiotrópicas. São tipicamente encontradas em conformação β-folha pregueadas e possuem efeitos citotóxicos em membranas de eritrócitos e fagócitos, podendo formar poros e destruir outras células eucarióticas e estruturas celulares (NAYAK; GREEN; BEEZHOLD, 2013; SCHAUFUSS; STELLER, 2003).

Outro fator de virulência descrito em micro-organismos patogênicos são as hemaglutininas, componentes que conferem a esses patógenos a potente capacidade de aderirem às células do hospedeiro (ROZDZINSKI; TUOMANEN, 1995; MENG *et al*., 1994). Taylor e colaboradores (2004) demonstraram que *Histoplasma capsulatum* possui a capacidade de aglutinar células vermelhas e que a secreção de hemaglutininas pode mediar a formação de coágulos sanguíneos e cooperar na disseminação da doença.

Haja vista que nosso grupo tem verificado que antígenos solúveis obtidos dos fungos que também causam infecção sistêmica *Arthrographis kalrae* (NAGASHIMA *et al.*, 2014) e *Candida parapsilosis* (ÁLVARES-E-SILVA, 2014) possuem atividade hemolítica sobre eritrócitos de camundongo e que antígenos solúveis de *Histoplasma capsulatum* possuem atividade hemaglutinina (VIVAN *et al*., 2010), este trabalho objetivou estudar o possível potencial de antígenos solúveis de *P. brasiliensis* e *P. lutzii* em lisar eritrócitos humanos e causar hemaglutinação e se a utilização de diferentes meios de cultura e a obtenção de antígenos solúveis destes meios em diferentes dias podem influenciar na produção destes fatores de virulência.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a atividade hemolítica e hemaglutinante de células viáveis de *P. brasiliensis* e *P. lutzii* e de seus antígenos solúveis sobre eritrócitos humanos.

2.2 OBJETIVOS ESPECÍFICOS

- Obter células leveduriformes e antígenos solúveis *P. brasiliensis* e *P. lutzii* em cultura;
- Avaliar a atividade hemolítica e hemaglutinante:

    - de células viáveis de *P. brasiliensis* e *P. lutzii*, em diferentes concentrações celulares sobre eritrócitos humanos 1%*;*

    - de antígenos solúveis nativos e aquecidos de *P. brasiliensis* e *P. lutzii* em 3 horas e 5 horas de incubação e em diferentes concentrações proteicas sobre eritrócitos humanos 1%;

    - de antígenos solúveis de *P. brasiliensis* e *P. lutzii* crescidos em meios sólidos Saboraud e Saboraud mais BHI no decorrer de cultivo de 30 dias.

    - de exoantígenos obtidos de sobrenadantes de cultivo de *P. brasiliensis* e *P. lutzii* crescidos em meios líquidos RPMI e caldo BHI no decorrer de cultivo de 30 dias.

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4 ARTIGO: *Paracoccidioides* sp. VIABLE CELLS AND SOLUBLE ANTIGENS PRESENT HEMOLYTIC AND HEMAGGLUTINATING ACTIVITIES ON HUMAN ERYTHROCYTES *IN VITRO*

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4.1 Abstract

Paracoccidioidomycosis (PCM), a systemic mycosis, is caused by thermodimorfics fungi of the *Paracoccidioides* genus. This study investigated the hemolytic and hemagglutinating activity of *P. brasiliensis* (Pb339) and *P. lutzii* (LDR2). Pb339 and LDR2 viable cells and its native or heated cell-free antigens (CFA) in different concentrations were incubated with human erythrocytes. Yeast cells of both species caused hemolysis, but LDR2 was more hemolytic. However, CFA of Pb339 was more hemolytic than LDR2. Yeast cells of both species demonstrated hemaglutination, but only heated CFA showed hemagglutinating activity. Additionally, CFA and Exoantigens (ExoAgs) of Pb339 and LDR2 obtained on alternate days in Saboraud, Saboraud plus BHI, RPMI media and BHI broth exhibited different hemolytic profiles. We report for the first time that *P. brasiliensis* and *P. lutzii* yeast

cells present the ability to lyse and agglutinate human erythrocytes and that both time and growth culture conditions affect the production of hemolytic factors.

Keywords: Fungi, culture medium, Paracoccidioidomycosis, cell-free antigens, virulence factor.

4.2 Introduction

Paracoccidioides genus comprises two species of thermodimorfics fungi, *P. brasiliensis* and *P. lutzii*, which are described as the etiological agents of paracoccidioidomycosis (PCM), a systemic granulomatous mycosis prevalent in Latin America [1, 2]. The infection of the individuals occurs by the inhalation of conidia present in nature, which can easily reach the alveoli and disseminate themselves by lymphohematogenic route [2]. Clinical manifestations and prognosis of the PCM depends on the balance between the fungus and host factors [3].

Several characteristics of *Paracoccidoides* sp. have been proposed as virulence factors that allow this organism to cause disseminated infection in susceptible hosts. For example, the ability to recognize and adhere to host tissues [4], resistance to phagocytosis and to antifungal drugs [5], as well as the production of hydrolytic enzymes including proteases and phospholipases are all important to determine virulence [6, 7, 8].

The ability to lyse erythrocytes has been described in many endemic pathogenic fungi such as *Histoplasma capsulatum* and *Blastomyces dermatidis*, as well as opportunistic pathogens including *Candida albicans* and *Cryptococcus neoformans* [9]. Clinical isolates of various species of dermatophyte fungi also exhibit hemolytic activity [10].

Bailão and coworkers [11] demonstrated that yeast cells of *P. brasiliensis* and *P.lutzii* have the ability to lyse sheep erythrocytes. Based on this, hemolysins, exotoxins that are able to destroy red and nucleated cells [9], are others putative virulence factors thought to

contribute to the pathogenesis of PCM and enable the uptake of the iron ion, a transient element described as cofactor in many biological processes in fungi, such as respiration, metabolism of amino acids, sterol and DNA biosynthesis [12].

A strong capability to adhere to host cells by hemagglutinins secretion is known in many other pathogenic micro-organisms [13, 14]. It has been described the capability to agglutinate erythrocytes in *Histoplasma capsulatum*, mechanism by which the fungus can form blood clots and cooperate with the pathogenesis of the disease [15].

Cell-free antigens (CFA) and Exoantigens (ExoAgs) corresponds to molecules that are secreted by fungus across the cell wall, adhere to surface of the fungus and then are released in soluble form during antigenic preparations or culture in liquid media. CFA of *P. brasiliensis* were first used by Camargo et al. [16] in serological tests for immunodiagnosis of PCM.

Since *Paracoccidoides* sp. have the ability to lyse sheep erythrocytes and hemagglutinating activity has been described in other micro-organisms, we hypothesize that soluble antigens of *P. brasiliensis* and *P. lutzii* are able to cause hemolysis and hemagglutination on human erythocytes and that *Paracoccidioides* culture in different days and in different culture media can influence the production of these virulence factors.

4.3 Material and Methods

4.3.1 Fungal isolates

P. brasiliensis (Pb339), gently donated by Dr. Zoilo Pires de Camargo of the Federal University of São Paulo (UNIFESP); and *P. lutzii* (LDR-2), isolated of a patient with PCM by Dr. Mário Augusto Ono of the State University of Londrina (UEL), were cultured on

Saboraud Dextrose Agar (SDA) (Acumedia, Michigan, USA) and maintained by subculture to 35ºC at intervals of 5 days.

4.3.2 Human erythrocytes

Human blood was obtained from a healthy individual by venipuncture without anticoagulant and immediately diluted 1/1 in Alsever's solution. Erythrocytes were washed by centrifugation (3 times with PBS, 175 *g* at 4°C for 10 min) (5804R, Eppendorf 82 Centrifuge, Hamburg, Germany) and adjusted to 1% in PBS. The current study was approved by the Internal Scientific Commission and the Research Bioethics Committee of the State University of Londrina.

4.3.3 Hemolysis test using *Paracoccidioides* sp. viable cells

Pb339 and LDR2 yeast cells cultured on SDA (Acumedia, Michigan, USA) for 5 days at 35ºC were collected, washed three times in PBS, counted in hemocytometer and cell viability assessed by using trypan blue staining. It was used cells with >95% viability. Yeast cells (10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1) were incubated 1:1 in a total volume of 200 µL, with a 1% erythrocyte suspension in bottom plates " V " for 3 hrs at 37°C. PBS was used as a negative control, and *Arthrographis kalrae* [17] was used as a positive control for hemolysis. Plates were centrifuged (155 *g* at 4 °C for 10 min), and collected supernatants were analyzed at 550 nm (Multiskan EX, Uniscience – Labsystems, Helsinki, Finland). The reading by spectrophotometry of the amount of hemoglobin released from red blood cells to the extracellular milieu reflects the hemolysis degree evaluated in this test.

4.3.4 Cell-free antigens (CFAs) preparations

Cell-free antigens (CFA) of Pb339 and LDR-2 were obtained according to the method described by Camargo et al. [16], with some modifications. Briefly, yeast mass were diluted in PBS (0.15 M, pH 7.4) with phenylmethylsulfonyl fluoride 2.5mM (PMSF) protease inhibitor (P7626 Sigma, St. Louis, MO, USA) and 0.02% thimerosal, shaken 60s by vortex-mixer and immediately centrifuged at 1005g at 4°C for 15 min (5804R74 Eppendorf Centrifuge, Hamburg, Germany). Supernatants (CFAs) were collected, the protein concentration was determined at 280 nm using NanoDrop Spectrophotometer Lite (Termo Fisher Scientific, Shangai, China) and then immediately frozen to - 80°C.

4.3.5 Hemolysis test using CFAs in different concentrations of protein

CFAs of Pb339 and LDR2 with protein concentration adjusted to 15 mg protein/ml and heated CFAs of both strains (56°C, 30 min and 100°C, 30 min) were serially diluted (ratio 1:2, 1:4 and 1:8, therefore 7.5, 3.7 and 1.8 mg protein/ml, respectively), and then incubated 1:1 in a total volume of 100 µl, with a suspension of 1% human erythrocytes in bottom plates "V" at for 3 hrs and 5 hrs at 37° C. PBS was used as a negative control, and distilled water was used as a positive control for hemolysis. Hemolytic activity was performed according to item 4.3.3.

4.3.6 Hemolysis test with CFAs and ExoAgs from different days and different culture media

Pb339 and LDR-2 were cultured in individual tubes with SDA (Acumedia, Michigan, USA) and SDA plus Brain Heart Infusion agar (S-BHI) (Merck, Darmstadt, Germany) for 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 days. The fungal mass of two tubes of each medium randomly

selected was collected (~200 g) and CFA was obtained according to the method described in item 4.3.4. For ExoAgs, Pb339 and LDR-2 were cultured in shaking at 37°C in RPMI 1640 incomplete medium (Invitrogen, Grand Island, NY, USA) and BHI broth (Acumedia, Michigan, USA) at a final concentration of 2 x 10^7 cells/ml. Aliquots were collected on days 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30, and the culture supernatants (ExoAgs) were obtained by centrifugation at 1005 g x at 4°C for 15 min (5804R74 Eppendorf Centrifuge, Hamburg, Germany) and immediately frozen at - 80°C. For hemolysis test, CFA or ExoAgs samples were incubated 1:1 in a total volume of 100 µl with a suspension of 1% human erythrocytes at 37°C for 5 hrs. PBS was used as negative control and distilled water was used as a positive control for hemolysis. Hemolytic activity was performed according to item 4.3.3.

4.3.7 Analysis of hemagglutinating activity of *Paracoccidioides* sp.

The visualization of erythrocyte agglutination was performed in all samples before the centrifugation for assessing hemolytic activity. A small pellet of erythrocytes at the bottom of the well was considered negative, and those containing even that one sheet of erythrocytes across the well were considered positive for hemagglutination.

4.3.8 Statistical Analysis

The data were analyzed by One Way-ANOVA, Two Way-ANOVA, Tukey test and t-test using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). We considered $p < 0.05$ to be statistically significant.

4.4 Results

4.4.1 Hemolysis test with *Paracoccidioides* sp. viable cells

In this test, significant hemolysis was observed when human erythrocytes were incubated with 10^7 viable cells/well of *P. brasiliensis* (Pb339) or *P. lutzii* (LDR2) (Fig. 1) and stronger hemolytic activity with LDR2 than Pb339 was observed ($p < 0.05$) (Fig. 2). Trace hemolysis was observed in the concentrations of 10^6 and 10^5 cells/well. Hemolysis was not observed at concentrations of 10^4, 10^3, 10^2 and 10^1 cells/well.

4.4.2 Hemolysis test using CFAs in different concentrations of protein

The hemolytic profile derived from hemolysis test with CFA in different concentrations of protein is shown in Figure 3 (A-D). Erythrocytes incubated with CFA from Pb339 and LDR2 at a concentration of 15 mg/ml showed stronger hemolysis and decreased when diluted 1/2 (7.5 mg protein/ml), 1/4 (3.7 mg protein/ml) and no hemolysis was observed at 1/8 (1.8 mg protein/ml). In this methodology, the hemolysis became significant at 3.7 mg protein/ml. The hemolytic activity was maintained in pure (15 mg/ml) Pb339 CFA even after heating at 56°C and 100°C, but in diluted samples it was detected decreased activity. Decreased hemolytic activity also was detected in CFA from LDR2. Although there was a decrease, the heating did not totally abolished the hemolytic activity. Pb339 CFA showed stronger hemolytic activity when compared to LDR2 CFA ($p<0,05$) (Fig. 3 E-F).

4.4.3 Hemolysis test with CFA from different days and different culture media

The hemolytic profile derived from hemolysis test with CFA from different days and different culture media are shown in Figure 4. CFA of Pb339 presented low hemolytic

activity on days 3, 6 and 9 of culture in SDA medium, increasing considerably in the 12th day of culture. From day 15, 100% hemolysis was detected. CFA of Pb339 from S-BHI medium had similar behavior, but reached 100% hemolysis from the 12th day of culture (Fig. 4A). When using CFA of LDR2, the hemolytic activity increased from the 3rd day culture in SDA, reaching a peak of 100% hemolysis on 15th and 18th days and then decreased. With CFA of S-BHI medium, hemolysis was increased, reaching a peak of 44% on the 30th day of culture (Fig. 4B).

4.4.4 Hemolysis test with ExoAgs from different days and different culture media

The hemolytic profile derived from hemolysis test with ExoAgs from different days and different culture media are shown in Figure 5. ExoAgs of Pb339 (Fig. 5A) and LDR2 (Fig. 5B) obtained from RPMI and BHI broth media were able to induce hemolysis in a time-dependent manner, but the hemolytic activity of Pb339 was stronger.

4.4.5 Analysis of hemagglutinating activity of *Paracoccidioides* sp.

Interestingly, during the hemolysis assays it was detected agglutination. This hemaglutination occurred when red cells were incubated with Pb339 viable yeast cells in all concentrations analyzed (10^7 before 3 hrs (Fig. 6) and 10^6, 10^5 and 10^4 cells/well, in 3 hrs (Fig. 1). The hemaglutination also occurred with *P. lutzii* (LDR2) in concentrations: 10^7 before 3 hrs (Fig. 6) and partial hemaglutination in 10^6, 10^5 and 10^4 cells/well in 3 hrs (Figure 1). Hemagglutination was not observed at concentrations of 10^3, 10^2 and 10^1 cells/well. Hemaglutination was also observed in the heated CFA from Pb339 (56° C and 100°C) in 1/2 to 1/8 dilutions and LDR2 only with CFA heated (100º C) and 1h incubation (Fig. 7).

4.5 Discussion

Hemolysins have been proposed as virulence factors for several fungal due to iron release by red cells. Iron is an important growth factor for pathogenic fungi especially during infection [9]. The hemolysin-like protein in the mycelium to yeast transition was showed by *P. brasiliensis* transcriptome investigation and suggested that *Paracoccidioides* can gain access to the intracellular hemoglobin by production of a hemolytic factor [18]. Bailão et al. [11] detected hemolytic activity in *P. brasiliensis* and *P. lutzi* yeast cells on sheep erythrocytes.

In concordance with this, our study detected hemolytic activity in *P. brasiliensis* and *P. lutzi* yeast cells on human erythrocytes. However, in our study *P. lutzi* (LDR2) presented stronger hemolytic activity than *P. brasiliensis* B339 (S1). This difference may be due to differences between fungal strains within the same species or by difference in growing condition used in our investigation. In addition, we verify at preliminary studies that ABO antigens do not interfere in hemolysis process (data not shown).

This study evidenced for the first time the release of hemolytic factor by fungi *P. brasiliensis* B339 (S1) and *P. lutzi* (LDR2), *in vitro*. This release possibly occurs *in vivo*, contributing as virulence factor in more distant infection site.

In a different way to cells, the soluble components from PbB339 showed greater activity than from *P. lutzi* (LDR2). A possible reason is that although *P. lutzi* (LDR2) presents in its cell body more hemolytic factor, PbB339 may release more to the outside.

The heating process does not eliminate totally the hemolytic activity of CFAs from both fungi. Probably the hemolytic factor has non-protein nature or the active site is not altered by protein conformation changing. Another possibility is that the hemolytic factor is constituted by more than one component with thermo-labile and thermo-resistant

components. Luo, Samaranayake and Yau [19] described the idea of existence of more than one component for the full hemolytic activity in *C. parapsilosis*. Nagashima et al. [17] detected more than one soluble component with hemolytic activity in *A. kalrae*.

Bailão et al. [11] demonstrated that *Paracoccidioides* has high growth in culture medium rich in hemoglobin and low growth in iron deficient medium. Furthermore, they reported that *Paracoccidioides* has a receptor anchored to the cell membrane, *rbt5*, orthologue of *Candida albicans*, which internalize the entire hemoglobin molecule, not only the iron, suggesting that the heme moiety is also used for regular metabolic pathways.

The role of hemolytic activity is to facilitate the growth of fungus and allow the uptake of iron, but also may participate in the PCM pathogenesis by other ways. For example, the moderate normochromic and normocytic anemia, with presence of microcytic and hypochromic erythrocytes, a discrete increase in reticulocyte counts, low hematocrit, and low levels of hemoglobin and iron can be seen in some PCM patients cases. This anemia may be more severe in cases of disseminated PCM [20]. The ability to promote the lysis of red cells by *Paracoccidioides* partially explain the reported anemia in patients with disseminated PCM, which may be a reflection of the increased fungal burden and hemolysins secretion. However, the antifungal therapy used should be taken into account, since treatment with sulfonamides, ketoconazole, and amphotericin B may induce an anemic state [21].

This work analyzed different media and different culture times for the purpose of optimizing the production of hemolytic factors and of investigating if there are differences between Pb339 and *P. lutzi* (LDR2). The results showed differences in hemolytic profile according to medium or time of LDR2 culture, suggesting that nutritional factors inherent to culture medium and the period of the culture may affect the production or release of these factors. However, stronger activity was reached from 9-12 days with Pb339 culture,

maintaining the level throughout the investigated period, regardless of culture medium. The hemolytic profile of soluble antigens originated from LDR2 showed a lower hemolytic activity than Pb339. However, we cannot rule out the hypothesis that LDR2 produces less hemolytic factor or that hemolytic factors production by Pb339 is unchanged according to media and period of culture. This is because we did not perform the analysis of yeast cells that originated its soluble antigens.

Surprisingly, in the present work it was detected hemaglutinating activity with both fungi (Pb339 and LDR2), which was evidenced in lower dilutions than hemolytic activity. But even in higher concentrations, the hemaglutinating activity was observed before the hemolysis occurrence. It is possible that initially occurs the adherence of the fungi on the surface of red blood cells by hemolytic factors or other components leading to agglutination and then to lytic effect. However, it was not performed lysis analysis in hemaglutinated samples in period greater than 3 hrs. The hemaglutination was also observed with preheated soluble antigens. It is possible that soluble antigens of *P. brasiliensis* and *P. lutzii* when heated can change the spatial molecular structure and expose the active sites that mediate the agglutination of erythrocytes. Vivan et al. [22] reported that cell-free antigens of *H. capsulatum* also have hemagglutinating activity, however this capability is lost when these antigens are heated.

Hemagglutinins were identified in various pathogenic micro-organisms as *Histoplasma capsulatum*, *Clostridium difficile*, *Staphylococcus saprophyticus*, *Vibrio cholerae* and showed critical roles in adhesion of these organisms to host tissues, although they perform other functions [15, 13, 23, 24]. The molecular mechanisms involved in hemagglutinating activity of *Paracoccidioides* are not understood, but we present some hypotheses. Extensive lipidomics analysis of the extracellular vesicles from pathogenic phase of *P. brasiliensis* showed the production of phosphatidylserine [25], a phospholipid

which has a strong ability to agglutinate human, rabbit, monkey, chicken and mouse erythrocytes [26]. Moreover, fungi have become a rich source of lectins, a class of non-immune proteins that recognize reversibly and specifically carbohydrates and have the ability to adhere to cells and host tissues and agglutinate human and animals erythrocytes [27]. Studies demonstrated that also ABO antigens that contain carbohydrates are targets of specific interactions of lectins of different micro-organisms, such as *Pseudomonas aeruginosa* and *Staphylococcus saprophyticus* [28]. Indeed, *Paracoccidioides* synthesizes a lectin of surface binding of N-acetylglucosamine (GlcNAc), the paracoccin, which binds to laminin in the extracellular matrix and is critical for the adhesion of pathogens to host tissues [4]. In this sense, GlcNAc is a carbohydrate constituent of the erythrocyte membrane that is facing to the extracellular fluid and is present in the chemical chain of the ABO antigens [29], which could be a target for attachment of paracoccin and subsequent agglutination of red cells. Further studies are required to determine whether this lectin is involved in this mechanism. Possibly the agglutination factors are distinct from hemolytic factors, but they may contribute to hemolysis by approaching more red blood cells with fungi.

The primary function of erythrocytes is the transport of the oxygen to the tissues. Furthermore, human erythrocytes have a complement receptor type 1, human erythrocyte CR1 (E-CR1, CD35), that binds to the immune complexes (IC) and transports them to the reticuloendothelial system, a phenomenon known as immune adherence (IA) [30], preventing inflammation induced by IC deposition in vasculature and tissues [31]. Considering our findings, we presume that *Paracoccidioides* can potentially cause hemolytic injury *in vivo* and compromise the clearance of IC. In fact, patients with PCM have high levels of circulating IC, which can aggravate the disease by the suppression of the cellular immunity [32, 33].

Nelson [34], more than 60 years ago, described by a series of refined experiments that bacteria become targeted for IA in the presence of specific antibodies and complement proteins. Nevertheless, opsonized promastigotes and amastigotes forms of *Leishmania* also bind to CR1 [35]. Thus, these studies show that erythrocytes are not able to depurate only IC, but also opsonized pathogens. IA mechanisms are mediated by the interaction of CD35 with the opsonic complement fragments C3b, C4b, C1q and mannose binding lectin (MBL) deposited directly on the microbial surface and also immune complexes [36]. Besides this, erythrocytes can transfer the pathogens linked to CR1 to human phagocytes [37]. Lachgar et al. [38] showed that the binding of the HIV-1 virus to erythrocytes through interaction with the Duffy antigen receptor for chemokines (DARC) and further, viral capsids of Herpes Simplex virus type 1 and the *Haemophilus influenzae* bacteria can bind to the erythrocyte without requirement for CR1 [39], suggesting that pathogens can also bind to erythrocytes by complement-independent mechanisms.

In line with this, we showed that *P. lutzii* has stronger hemolytic activity when this yeast is incubated directly with erythrocytes. Recently, Hahn et al. [40] reported a fatal case of fungemia due to infection by *P. lutzii*, in which the patient showed progressive worsening dyspnea, respiratory failure, severe hypoxemia and signs of muscle fatigue. Thereby, this finding reinforces the presupposed that the hemolytic injury caused by *Paracoccidioides* may impair these important immunological functions of pathogen intravascular clearance mediated by erythrocytes, allowing the survival of the fungus within the host, enabling increased fungemia due to decreased immune adherence and impairing the oxygen delivery to tissues, leading to the PCM progression.

In conclusion, we report for the first time that *P. brasiliensis* and *P. lutzii* yeast cells and their soluble antigens present the ability to lyse and agglutinate human erythrocytes. We suggest that Pb339 is more efficacious in releasing these factors in soluble form and that

both time and growth culture conditions affect the hemolytic factors production. Future studies in order to characterize these virulence factors will contribute with a better understanding of this complex host-pathogen interface and with the design of therapeutic interventions.

Conflict of interest statement:

The authors have no financial conflicts of interest.

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4.6 References

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4.7 Figures

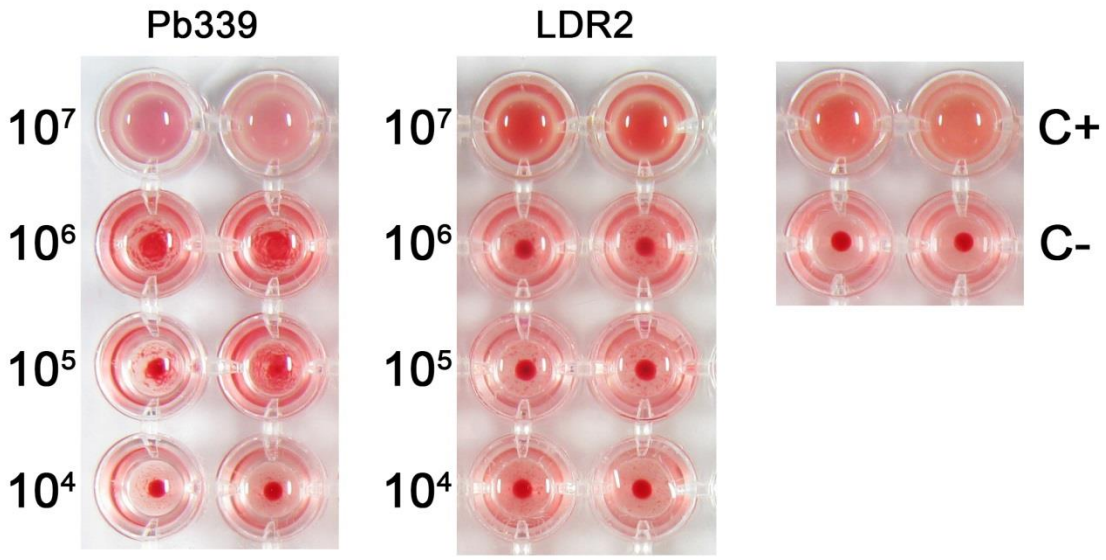

Fig.1 Hemolysis test with viable cells of *Paracoccidioides* sp.

Images depicting hemolysis of 1% human erythrocytes incubated with 10^7, 10^6, 10^5 and 10^4 yeast cells of *P. brasiliensis* (Pb339) and *P. lutzii* (LDR2. The experiment was performed in duplicate, and the average optical density of each condition was used to calculate the relative hemolysis of the experimental conditions or of the negative control (PBS) against the positive control (stronger hemolytic strain of *A. kalrae*).

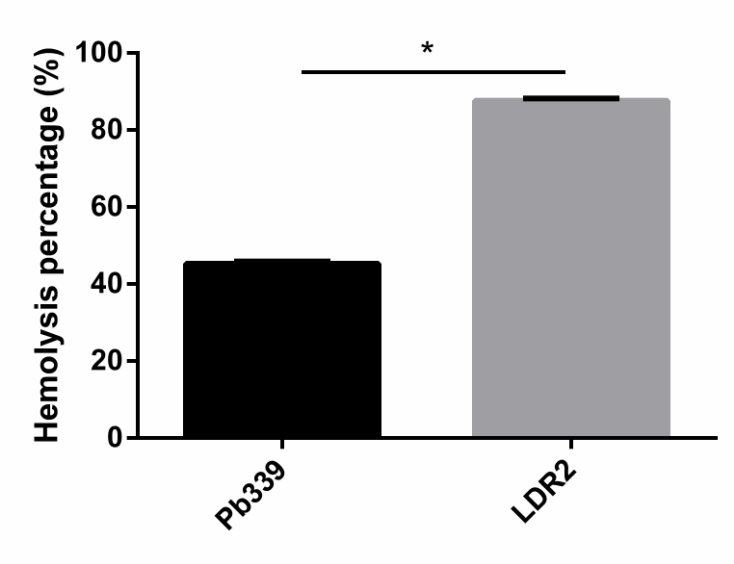

Fig. 2 Hemolysis percentage of LDR2 versus Pb339

Images depicting hemolysis of 1% human erythrocytes incubated with 10^7 yeast cells of *P. brasiliensis* (Pb339) and *P. lutzii* (LDR2). Data are plotted as the mean ± standart deviation. *p<0,05.

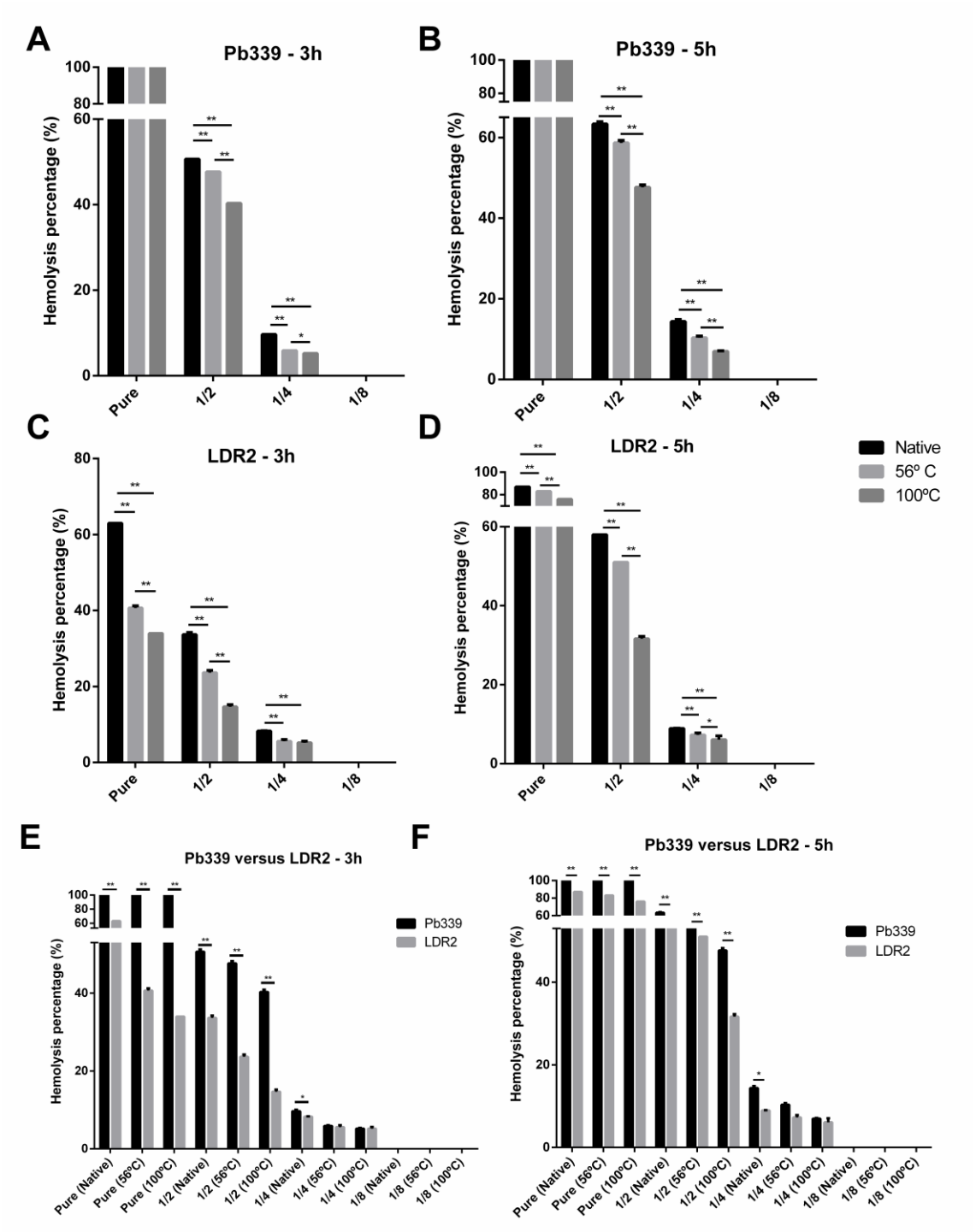

Fig.3 Hemolysis test using CFAs in different concentrations of protein

Native and heated CFAs (56°C, 30 min and 100°C, 30 min) of Pb339 pure (15 mg protein/ml) and diluted 1:2, 1:4 and 1:8, therefore 7.5, 3.7 and 1.8 mg protein/ml, respectively, were incubated in duplicate v/v with 1% human erythrocytes for 3 hrs (**A**) and 5

hrs (**B**) at 37° C. Native and heated CFAs (56 °C, 30 min and 100°C, 30 min) of LDR2 pure and diluted 1:2, 1:4 and 1:8, were incubated in duplicate v/v with 1% human erythrocytes for 3 hrs (**C**) and 5 hrs (**D**) at 37°C. Comparison of percentage of hemolysis of Pb339 versus LDR2 with 3h (**E**) and 5h (**F**) incubation was performed. Data are plotted as the mean ± standart deviation. * $p<0,05$; ** $p<0,01$.

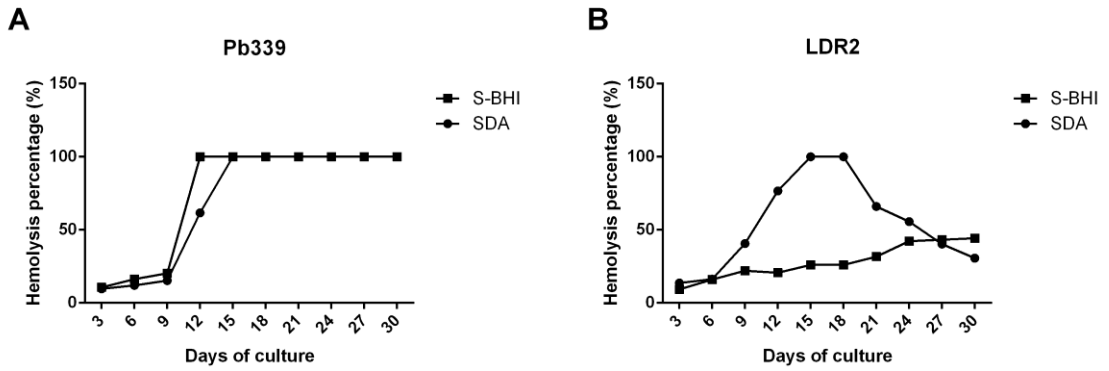

Fig. 4 Hemolysis test with CFA from different days and different culture media

CFAs of Pb339 (**A**) and LDR2 (**B**) obtained every 3 days of culture in Saboraud ( ) and S-BHI ( ) media were incubated v/v with 1% human erythrocytes to 37°C for 5h. The experiment was performed in duplicate, and the average optical density of each condition was used to calculate the relative hemolysis of the experimental conditions or the negative control (PBS) against the positive control (distilled water).

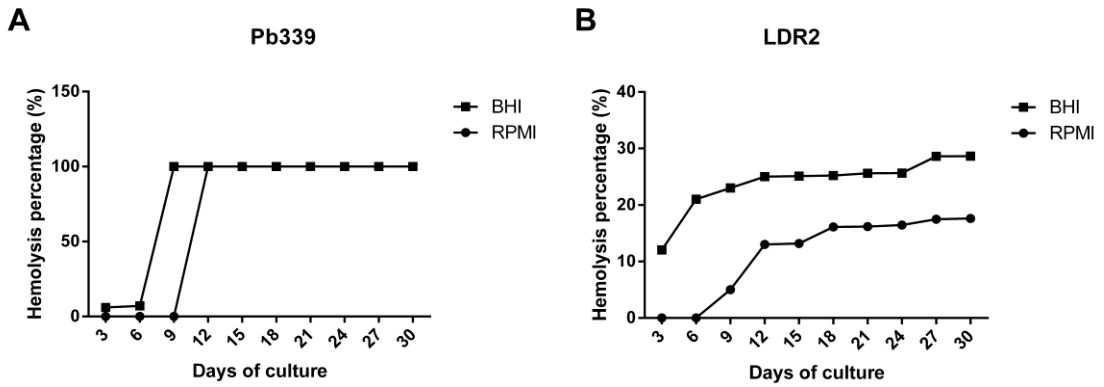

Fig. 5 Hemolysis test with ExoAgs from different days and different culture media

Culture supernatants of Pb339 (**A**) and LDR2 (**B**) collected from RPMI medium (-●-) and BHI broth (-■-) containing yeast cells of Pb339 and LDR2 were incubated v/v with 1% human erythrocytes to 37° C for 5 hrs. The experiment was performed in duplicate, and the average optical density of each condition was used to calculate the relative hemolysis of the experimental conditions or the negative control (PBS) against the positive control (distilled water).

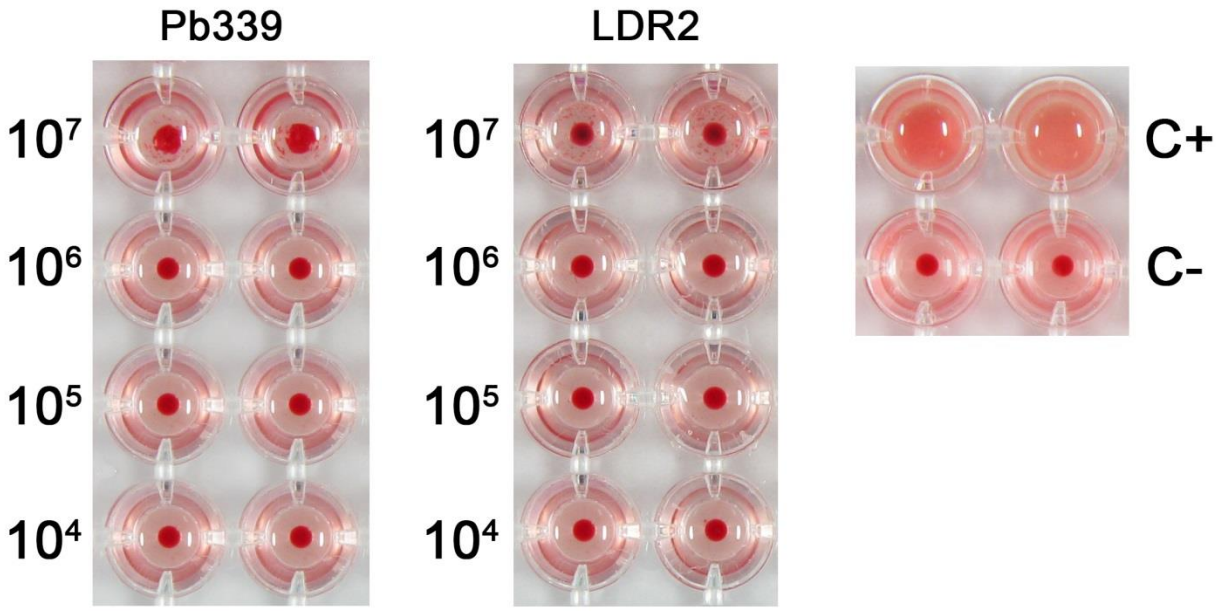

Fig. 6 Analysis of hemagglutinanting activity of *Paracoccidioides* sp. viable cells

Images depicting experimental conditions that present human blood agglutination. 10^7, 10^6, 10^5 and 10^4 yeast cells of *P. brasiliensis* (Pb339) e *P. lutzii* (LDR2) incubated v/v with a 1% human erythrocytes suspension for 1 hr at 37°C. As a negative (C-) or positive (C+) control, erythrocytes were incubated with PBS or stronger hemolytic strain of *A. kalrae*, respectively.

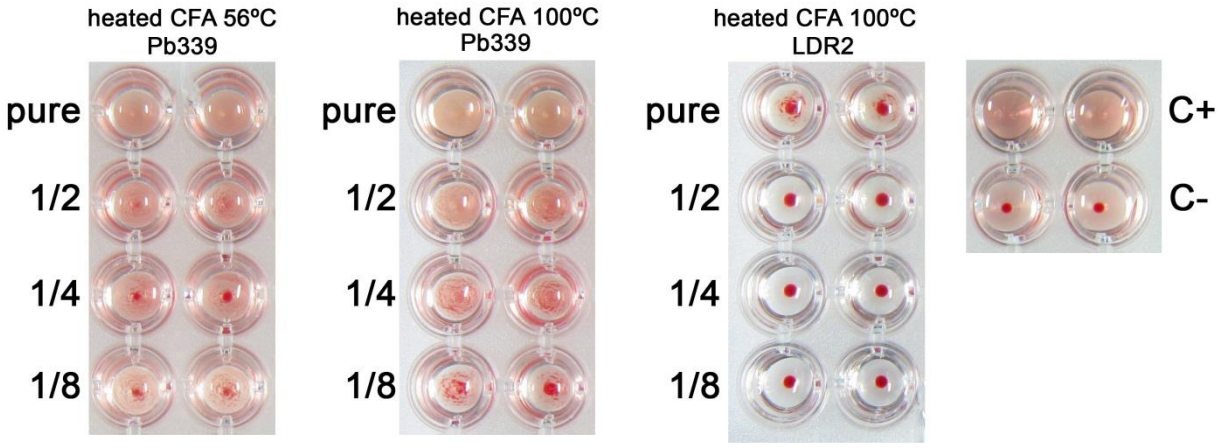

Fig. 7 **Analysis of hemagglutinanting activity of *Paracoccidioides* sp. CFAs**

Heated CFAs (56 °C, 30 min and 100°C, 30 min) of Pb339 and heated CFA (100°C, 30 min) of LDR2, pure (15 mg protein/ml) and diluted 1:2, 1:4 and 1:8, therefore 7.5, 3.7 and 1.8 mg protein/ml, respectively, were incubated v/v with 1% human erythrocytes for 3 hrs at 37° C. As a negative (C-) or positive (C+) control, erythrocytes were incubated with PBS or distilled water, respectively.

5 CONCLUSÕES

Células leveduriformes de *P. brasiliensis* e *P. lutzii* induzem hemólise em eritrócitos humanos.

Antígenos solúveis de *P. brasiliensis* e *P. lutzii* também possuem atividade hemolítica.

Antígenos solúveis de *Paracoccidioides spp.* são parcialmente termolábeis quando aquecidos.

Tempo e condições de cultura influenciam na produção de hemolisinas por *Paracoccidioides spp*. Tanto células viáveis quanto antígenos são mais hemolíticos quando incubados por 5 horas com eritrócitos humanos em relação à 3 horas de incubação.

Células leveduriformes e antígenos solúveis aquecidos aglutinam eritrócitos humanos.

Estudos futuros com o uso de frações proteicas de diferentes pesos moleculares contribuirão no intuito de melhor caracterizar os fatores hemolíticos de *Paracoccidioides spp*.

Pesquisas a fim de investigar o potencial hemaglutinante de fosfolipídeos e lectinas extraídas de *Paracoccidioides* sp. ajudarão a elucidar os mecanismos moleculares envolvidos no processo de hemaglutinação.

A partir de dados obtidos, conclui-se que: as leveduras de *P. brasiliensis* e de *P. lutzii,* como os seus antígenos solúveis, apresentam a capacidade de lisar e aglutinar eritrócitos humanos e, tempo e condições de cultura influenciam na produção de hemolisinas por *Paracoccidioides spp.*