PAULA LEONELLO ÁLVARES E SILVA

ALTERAÇÃO NEUROLÓGICA NA INFECÇÃO EXPERIMENTAL MURINA POR *Candida Parapsilosis* E CARACTERIZAÇÃO DE ANTÍGENOS SOLÚVEIS

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

Orientadora: Prof.ª Dr.ª Eiko Nakagawa Itano

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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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LISTA DE ABREVIATURAS E SIGLAS

3HAA	Ácido 3-hidroxiantralinico
°C	Graus Celsius
Ags	Antígenos
AIDS	*Acquired Immunodeficiency Syndrome*
Anti-E-CFA	*Anti-isogenic erythrocytes sensitized with*
CFA	*Cell-free Antigens*
O.D.	Densidade Óptica
DC	Células dendriticas
ELISA	*Enzyme Linked Immunosorbent Assay*
GAPDH	*Glyceraldehyde 3-phosphate dehydrogenase*
HE	Hematoxilina-Eosina
i.v.	Intravenosa
IDO	Indoleamine 2,3-dioxigenase
IFN-γ	Interferon gama
IgG	Imunoglobulina G
IL-17	Interleucina 17
IL-4	Interleucina 4
kDa	Quilodalton
mA	Miliamperes
MM	Massa Molecular
nm	Nanômetro
OPD	Orto-Feniletilenodiamino Diidrocloreto
PBS	Tampão Fosfato Salina
PCR	Reação da Cadeia da Polimerase
pg	Picogramas
p.i	*Post infection*
PMSF	*Phenylmethanesulfonyl Fluoride*
QUIN	Ácido quinolinico
qRT-PCR	PCR em Tempo Real
RNA Ácido	Ribonucléico

SAPs	Proteases de aspartilo
SDS	Sodium Dodecyl Sulfate
SNC	Sistema Nervoso Central
TGF-β	Transforming Growth Factor beta
Th1	Linfócito T auxiliar ou helper 1
Th2	Linfócito T auxiliar ou helper 2
TMB	3;3;5;5-tetrametilbenzidina
TNF-α	Fator de Necrose Tumoral alfa
TRP	Triptofano
UEL	Universidade Estadual de Londrina
V	Volt
Mg	Micrograma
μL	Microlitro
μm	Micrômetro

ÁLVARES E SILVA, Paula Leonello. **Efeito imunopatológico na infecção experimental murina por *Candida parapsilosis* e caracterização parcial de fator hemolítico.** 2014. 64f. Tese (Doutorado em Patologia Experimental) - Universidade Estadual de Londrina, Londrina, 2014.

RESUMO

Candida parapsilosis é um importante patógeno associado a infecções sanguíneas mas estudos sobre virulência e patogenicidade são escassos. Considerando que estudo evidenciou alteração comportamental em camundongo infectado, avaliou-se efeito imunopatológico no decorrer de infecção sistêmica em camundongos, aliada a caracteriazação parcial de fator hemolítico de *C. parapsilosis*. Grupos de camundongo SWISS e BALB/c foram infectados por via endovenosa ($1x10^8$ leveduras/animal), sendo o primeiro por 56 dias e o segundo durante 7, 14, 28 e 56 dias, tendo como controle o PBS. Foram analisadas: alterações comportamentais determinando-se número de giros/5min, detecção de células de Purkinje por imunofluorescência (anti-calbindin), contagem de células Purkinje por histopatologia, análise de apoptose utilizando anti-ligante de Fas por imunohistoquímica, no cerebelo de camundongos infectados durante 56 dias. Determinou-se também antigenemia por CFU e ELISA CAPTURA, níveis de citocinas (INF-γ, IL- 4, IL-17 e IL-10), expressão e atividade de indoleamine 2,3 dioxigenase (IDO) no cerebelo de camundongos infectados durante 7, 14, 28 e 56 dias. No decorrer de infecção foi avaliada também respostas imune humoral (ELISA), celular (DTH) anti "*cell free antigen*" (CFA) de *C. parapsilosis* e citocinas circulantes (INF-γ, IL- 4, IL-17 e IL-10). Para a caracterização de fator hemolítico*,* foi avaliada a atividade lítica de eritrócitos de camundongos utilizando CFA na forma nativa e aquecida (56°C, 1 h) e as frações de CFA obtidas por gel filtração (Sephadex G-100/120) e por filtração em Amicon (100k e 50k). Os níveis de carboidratos totais, capacidade de ligação à ConA e de reatividade aos anticorpos específicos (anti-eritrocito autólogo sensibilizado com CFA) foram determinados em frações de gel filtração. Além disso, as frações foram analisadas por *western blotting*, investigada a capacidade de anticorpos neutralizarem a atividade hemolítica e foram determinados os níveis de IgG anti-fração hemolítica em camundongos infectados com *C. parapsilosis*. Os resultados demonstraram diminuição de células de Purkinje ($p<0,05$) e correlação significativa entre a diminuição dessas células e o aumento no número de giros ($r= 0.9876$) e reação com anti-ligante de Fas foi negativa, no cerebelo. Detectou-se aumento significativo em CFU (7 e 14 dias) ($p<0,05$), de todas as citocinas (7 dias) ($p<0,05$) e expressão de IDO (7>14>28 dias) ($p<0,05$) ou de atividade de IDO (7,14 dias) ($p<0,05$) no cerebelo, em relação ao controle. Além disso, níveis elevados de anticorpos IgG ($p <0,05$) ou DTH ($p <0,05$) a CFA ($p <0,05$) (28 e 56 dias) e de níveis séricos de citocinas INF-γ (7 e 14 dias) ($p<0,05$), IL-10 (56 dias) ($p<0,05$) e IL-17 (56 dias) ($p<0,05$) foram detectados no decorrer de infecção de camundongos. Concluímos pelos resultados que as respostas imunes especificas e modulação de citocinas devem participar na diminuição de carga fúngica no cérebro, mas por outro lado devem apresentar efeito imunopatológico, possivelmente com participação de IDO levando a diminuição quantitativa de células Purkinje no cerebelo e como consequência induzindo alteração comportamental dos camundongos infectados, o que requer estudos adicionais. Em relação ao fator hemolítico, concluímos que o fungo *C. parapsilosis* apresenta fator hemolítico de alta MM (~ 280kDa e ~ 300kDa), é termoestável e apresenta a capacidade de induzir resposta imune no decorrer de infecção e, se essa resposta específica participa na defesa, requer estudos adicionais

Palavras-chaves: Alteração comportamental. Cerebelo. Células de Purkinje. Citocinas. IDO. Fator de virulência. Micose.

ÁLVARES E SILVA, Paula Leonello. **Immunopathological effects in experimental murine infection with *Candida parapsilosis* and partial characterization of hemolytic factor**. 2014. 64p. Tese (Doutorado em Patologia Experimental) - Universidade Estadual de Londrina, Londrina, 2014.

ABSTRACT

Candida parapsilosis has emerged as an important pathogen of bloodstream infections and studies on the virulence and experimental pathogenicity concerning this species are few. In our previous study it was observed behavioral change in *C. parapsilosis* infected mice. The objective of this study was to evaluate immunopathological effect on the course of systemic mice infection and partially characterize *C. parapsilosis* hemolytic factor. Groups of SWISS and BALB/c mice were infected intravenously ($1x10^8$ yeast), the first for 56 days and second for 7, 14, 28 and 56 days and as a control only PBS in both groups. It were analyzed: behavioral changes by determining the number of turns/5min, Purkinje cells detection by immunofluorescence (anti-calbindin), quantitative Purkinje cells determination by histopathology, apoptosis with anti-Fas ligand by immunohistochemistry in the mice cerebellum infected for 56 days. Fungi CFU and CAPTURE ELISA, cytokines (IFN-γ, IL-4, IL-17 and IL-10), indoleamine 2,3-dioxygenase (IDO) expression (qRT-PCR) and activity (kynurenine) were evaluated in the cerebellum of mice infected for 7, 14, 28 and 56 days. Also humoral immune responses (ELISA), cellular (DTH) to "cell free antigen" (CFA) from *C. parapsilosis* and circulating cytokines (INF-γ, IL-4, IL-17 and IL-10) levels were assessed during the course of infection. For hemolytic factor characterization it was evaluated mice erythrocytes lysis by using native form and heated (56 ° C, 1 h) CFA and fractions CFA obtained by gel filtration (Sephadex G-100/120) and Amicon filtration (100k and 50k). The total carbohydrates levels, ConA binding capacity and reactivity with specific antibodies (immunization with autologous erythrocyte sensitized with anti-CFA) were determined in gel filtration fractions. In addition, components recognized by western blotting, the antibodies inhibitory ability and IgG anti-hemolytic fraction levels in infected were evaluated. The results showed a reduction in number of Purkinje cells ($p<0.05$), correlation between the decrease of these cells and the increase in the number of turns ($r = 0.9876$) and no reactivity with anti-Fas ligand in cerebellum. In the course of infection a significant increase in CFU (7 and 14 days) ($p <0.05$), all cytokines (7 days) ($p <0.05$) and expression (7> 14> 28 days) or activity of IDO (7,14 days) were resulted in the cerebellum of infected, in relation to control. Also higher levels of antibody IgG ($p <0.05$) or DTH($p <0.05$) to CFA and serum INF-γ (7 and 14 days) ($p <0.05$), IL-10 (56 days) ($p <0.05$) and IL-17 (56 days) ($p <0.05$) were detected in the course of mice infection. By the results of experimental immunopathological effect in a systemic *C. parapsilosis* mice infection study, we conclude that the specific immune responses and modulation of cytokines induced should participate in the reduction of fungal burden in the brain, but on the other hand must display pathological effect, with possible involvement of IDO in quantitative decrease of Purkinje cells in the cerebellum and consequently inducing behavioral changes of infected mice, which requires further study. In relation to hemolytic factor, we conclude that the fungus *C. parapsilosis* has hemolytic factor with high MM (~ 280kDa and ~ 300kDa) which is thermostable and has the ability to induce immune response during infection and if this specific response participates in the defense, requires additional studies.

Keywords: Behavioral changes. Cerebellum. Purkinje cells. Cytokines. IDO. Virulence factor. Mycosis.

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INTRODUÇÃO

Desde 1980, os fungos tem emergido como os principais causadores de doenças humanas, principalmente em pacientes imunocomprometidos e hospitalizados com doenças subjacentes graves (PFALLER & DIEKEMA, 2007).

Dentre essas infecções fúngicas, a mais frequente é a candidíase, causada pelo fungo *Candida ssp*. (ALVARES, 2007). Dentre as espécies consideradas emergentes destaca-se *Candida parapsilosis (*LEVY *et al*., 1998; LEVIN *et al*., 1998).

Na ultima década, a ocorrência de *C. parapsilosis* aumentou dramaticamente, sendo é a segunda espécie mais comumente isolada em culturas de sangue (TROFA *et al*., 2008).

C. parapsilosis é um comensal típico da pele humana, com patologia limitada ao tegumento intacto, sendo o aumento e frequência da infecção atribuída a uma variedade de fatores de risco.

Considerando que seja comensal normal da pele humana, *C. parapsilosis* representa uma ameaça aos pacientes em contato com profissionais de saúde colonizados, quando ocorre a violação do protocolo de lavagem de mão (TROFA *et al*., 2008).

Pacientes imunocomprometidos, recém nascidos em estado crítico, pacientes cirúrgicos sob uso prolongado de cateter venoso central ou dispositivo de habitação, estão expostos a maior risco de infecção por *C. parapsilosis*.

Estudos tem demonstrado a transmissão horizontal de fontes ambientais em locais de surto, com implicação frequente de mãos dos profissionais de saúde (VAN ASBECK *et al*., 2007; BLISS *et al*., 2008; HERNÁNDEZ-CASTRO *et al*., 2010).

Doenças invasivas por *C. albicans* e *C. tropcalis* são normalmente precedidas por uma colonização prévia e transmissão vertical, geralmente de mãe para filho na época do nascimento. Em contraste, a doença invasiva por *C. parapsilosis* pode ocorrer sem colonização prévia e é frequentemente transmitida horizontalmente através de fontes externas contaminadas (TROFA et al., 2008).

Comparação a outras espécies de Candida, *C. parapsilosis* apresenta uma ampla distribuição na natureza. Ao contrário da *C. albicans* e *C. tropicalis*, a *C. parapsilosis* não é um patógeno humano obrigatório, podendo ser isolada de animais domésticos, insetos, solo e ambiente marinho (FELL & MEYER, 1967; WEEMS, 1992).

C. parapsilosis foi isolada pela primeira vez em Porto Rico (1928) por Ashford, em fezes de paciente com diarreia.

Anteriormente a 2005, *C. parapsilosis* foi separada em três grupos de I a III: *C. parapsilosis, C. orthopsilosis* e *C. metapsilosis*. Estudos demostraram diferença suficiente no grupo, capaz de justificar a designação em três espécies distintas, mas estreitamente relacionadas e fenotipicamente indistinguíveis (TAVANTI *et al*., 2005).

C. parapsilosis apresenta morfologia oval, redonda ou cilíndrica e em ágar Sabouraud dextrose, apresenta colônia branca, cremosa, brilhante, lisa e/ou enrugada. Não forma hifas verdadeiras e pode ser encontrada na fase de levedura ou pseudohifa (TROFA *et al*., 2008).

Os fatores de virulência associados a *C. parapsilosis* incluem aderência a uma ampla variedade de superfícies biológicas e protéticos, formando biofilme em dispositivo médicos implantáveis e a secreção de enzimas hidrolíticas capazes de causar dano tecidual (CHOW *et al*., 2012).

C. parapsilosis tem a notória capacidade de crescer em nutrição parenteral, formar biofilmes em cateteres e outros dispositivos implantados por disseminação nosocomial, pelo transporte das mãos e pela persistência no ambiente hospitalar (CLARK *et al*., 2004).

Existe uma associação bem documentada entre infecção por *C. parapsilosis* e a presença de um dispositivo intravascular (WEEMS *et al*., 1992; GIRMENIA *et al*., 1996; LEVY *et al*., 1998).

Em 1940, *C. parapsilosis* foi diagnosticado como agente causador de um caso fatal de endocardite em usuário de droga por via intravenosa (JOACHIM & POLAYES, 1940). Inicialmente, os investigadores associaram a infecção com a introdução endógena de *C. parapsilosis*, que prenunciou a ligação de *C. parapsilosis* com instrumentação invasiva e soluções de hiperalimentação.

C. parapsilosis em meio contendo alta concentrações de glicose e lipídios, prontamente produzem biofilme indicando associação com aumento da prevalência de infeção vascular em pacientes sob nutrição parenteral (NOSEK *et al*., 2009).

Em contraste com *C. albicans*, biofilme de *C. parapsilosis* é mais fino, menos estruturado, não apresenta hifas verdadeiras e consistem exclusivamente de agregado de blastosporos (KUHN et al. 2002).

Espécies de *Candida* secretam enzimas hidrolíticas associadas à virulência, incluindo proteases de aspartilo (SAPs), fosfolipases, lipases e hemolisinas (CHOW et al., 2012).

O papel da SAP em infecções sistêmicas por *C. parapsilosis* permanece

desconhecida, embora produção de SAP pode ter um papel na evasão da resposta imune, tal como a supressão de SAP resultou em um aumento da susceptibilidade à fagocitose e morte pelos macrófagos (HORVÁTH *et al*., 2012).

Fosfolipases são importantes fatores de virulência em fungos patogênicos, contribuindo para dano tecidual (KUHN *et al*., 2002). O papel da fosfolipase na virulência de *C. parapsilosis* permanece incerto, embora o aumento de fosfolipase se associa ao aumento da adesão a células epiteliais (DAGDEVIREN *et al*., 2005).

Produção de lipase, é reconhecido desempenham um papel importante na infecção por *C. parapsilosis*, já que cepas de *C. parapsilosis* sem lipases Cplip1 e Cplip2, diminuiu virulência no modelo murino de infecção (GÁCSER *et al*., 2007).

Atividade hemolítica de *C. parapsilosis*, em geral, tem sido relatada ser ausente ou muito baixa (ISSA *et al*., 2011; SENEVIRATNE *et al*., 2011). Um estudo analisou a atividade hemolítica de 34 isolados de *C. parapsilosis*, todos (34) apresentaram atividade hemolítica fraca (FRANÇA et al., 2011). O papel da atividade hemolítica na patogênese de *C. parapsilosis* permanece mal compreendido.

Experimentalmente, a virulência de *C. parapsilosis* é menor que *C. albicans* e outras espécies de *C.* não-*albicans*, devido à sua menor aderência a células epiteliais (ABI SAID *et al*., 1998). Por outro lado, *C. parapsilosis* tem capacidade de aderir de forma mais eficiente em material estranho (WEEMS *et al*., 1992).

Esta característica explica a associação com cateteres e também a mortalidade significativamente menor quando comparado com outras espécies de *C. não-albicans* (GIRMENIA *et al*., 1996; LEVY et al., 1998; KRCMERY, 1998; VISCOLI et al., 1999; KRCMERY, 1999).

Embora *C. parapsilosis* seja frequentemente considerado menos virulento do que *C. albicans* e outras espécies de *C. não-albicans* (TROFA et *al*., 2008) existem casos de infecções de rins, pulmão, coração, articulações, ossos e sistema nervoso central (LACAZ *et al*., 2002), sendo que as formas clínicas severas têm sido associadas com pacientes imunocomprometidos, neutropênicos, transplantados e recém-nascidos em estado critico (COLOMBO & GUIMARÃES, 2003; BENJAMIN *et al*., 2006).

A interação patógeno hospedeiro é crucial na manifestação e gravidade da infeção fúngica, os mecanismos de defesa do hospedeiro influenciam as formas clinicas da doença. Uma resposta de defesa do hospedeiro frente a um patógeno, se for exacerbada, pode levar à lesão tecidual no hospedeiro, podendo gerar mais dano que o próprio patógeno.

Os objetivos deste trabalho incluem estudar efeito imunopatológico no decorrer de infecção experimental murina por *C. parapsilosis* e caracterizar melhor os fatores de virulência relacionados às atividades hemolítica de *C. parapsilosis*.

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OBJETIVOS

1. OBJETIVOS GERAIS

Avaliar efeito imunopatológico de infecção sistêmica com *C. parapsilosis* em camundongo e caracterizar parcialmente o fator hemolítico de *C. parapsilosis*.

2. OBJETIVOS ESPECÍFICOS

- Obter antígenos solúveis de *C. parapsilosis;*
- Obter anticorpos policlonais a antígenos solúveis totais de *C. parapsilosis;*
- Obter anticorpos policlonais específicos aos fatores hemolíticos de *C. parapsilosis;*
- Avaliar grau de infecção (CFU) no cérebro de camundongos infectados experimentalmente com *C. parapsilosis*
- Avaliar alterações comportamentais de camundongos infectados experimentalmente com *C. parapsilosis*
- Avaliar lesões em cérebro (cerebelo) de camundongos infectados com *C. parapsilosis* por meio de análise histopatológia, por imunoflurescencia e por imunohistoquímica.
- Avaliar expresssão e atividade de IDO em cerebelo de camundongos infectados com *C. parapsilosis;*
- Avaliar níveis de citocinas (IFN-γ, IL-4, IL-17 e IL-10) em cerebelo e soro no decorrer de infecção com *C. parapsilosis*
- Determinar nível de IgG anti-antígeno total no decorrer de infecção com *C. parapsilosis;*
- Determinar resposta de celular por meio de DTH no decorrer de infecção com *C. parapsilosis;*
- Analisar atividade hemolítica de *C. parapsilosis* e seus antígenos solúveis;
- Obter frações cromatográficas e analisar perfil proteico e de carboidrato de antígenos solúveis de *C. parapsilosis;*

Analisar perfil de reatividade a anticorpos específicos e a ConA em frações de antígenos solúveis de *C. parapsilosis;*

Determinar MM aproximada de fator hemolitico de *C. parapsilosis* (*Western Bloting* e filtração em amicon);

- Avaliar o grau de inibição de anticorpos na atividade hemolítica de *C. parapsilosis*
- Determinar nível de IgG anti-fração hemolítica no decorrer de infecção com *C. parapsilosis;*

1º ARTIGO: Experimental systemic *Candida parapsilosis* murine infection: Behavioral Changes, Decrease in Cerebellum Purkinje Cells Numbers and Immunomodulation

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1. Abstract

Candida parapsilosis has emerged as an important pathogen in bloodstream infections, and practically no knows the host immunopathological effects. This study evaluated immunopathological effect *C. parapsilosis* in systemic infection in mice. Swiss and Balb'c mice were intravenously infected with *C. parapsilosis* IFM 663581 ($1x10^8$ yeast/animal) and control with PBS. The number of turns/5min, brain histopathology and Purkinje cells was analyzed in Swiss mice infected during 56 days. Fungi CFU, cytokines (IFN-γ, IL-4, IL-17 and IL-10), indoleamine 2,3-dioxygenase (IDO) expression and activity were evaluated in the cerebellum of Balb/c mice infected for 7, 14, 28 and 56 days. Also humoral (ELISA) and cellular (DTH) immune responses to "cell free antigen" (CFA) from *C. parapsilosis* and circulating cytokines (INF-γ, IL-4, IL-17 and IL-10) levels were assessed during the course of Balb/c mice infection. The results showed a reduction in number of Purkinje cells, correlation between the decrease of these cells and the increase in the number of turns ($r = 0.9876$) ($p<0.05$). Increased number of CFU (7 and 14 days), increased levels in all cytokines evaluated (7 days) and also increased IDO expression (7> 14> 28 days) or IDO activity (7,14 days) were observed in the cerebellum of infected group ($p<0.05$). High levels of antibody IgG or DTH to CFA and serum INF-γ (7 and 14 days),

IL-10 (56 days) and IL-17 (56 days) were also detected in the course of mice infection (p <0.05). In conclusion, systemic *C. parapsilosis* mice infection induce specific immune responses and local and systemic cytokines modulation that should participate in the reduction of fungal burden in the brain, but on the other hand must display pathological effect, with possible involvement of IDO in decreasing in number of Purkinje cells in the cerebellum and consequently inducing behavioral changes, which requires further study.

2. Introduction

Candida parapsilosis has been revealed as an important pathogen associated with bloodstream infections, although often considered less virulent that *C. albicans* and others *non-C. albicans* species (TROFA *et al*., 2008). Host defense mechanisms affect manifestation and severity of fungal infections, and clinical forms of the disease depends on the immune response (ROMANI, 2004). To avoid pathologic consequences of excessive inflammatory cell-mediated reactions, the immune system apply various protection mechanisms (BOZZA *et al*., 2005).

The Th1 response is considered the main protective response against fungal infectious diseases, while Th2 have deleterious effects (WÜTHRICH et al., 2012). TAVARES *et al*., (2000) observed increasing resistance against systemic Candidiasis in IL-10 depleted mice. NETEA *et al*. (2004) related that resistance against *C. albicans* decreased in IL-10 production, which was associated with increase in IFN- production and decreased number of Treg cells in TLR2 deficient mice. The cytokine IL-17, produced in Th17 was been important in antifungal defense (ABBAS *et al*, 2012) but also has been described as relevant in autoimmunity (STUMMVOLL *et al.,* 2008). The enzyme indoleamine 2,3-dioxygenase (IDO) and tryptophan metabolites also contribute to immune homeostasis by inducing Tregs and taming overzealous or heightened inflammatory responses (ROMANI, 2008). IDO has an important role in regulating immune responses to infections (MOFFETT & NAMBOODIRI, 2003; MELLOR *et al*., 2004; POPOV *et al*., 2008), however, IDO induction in the central nervous system (CNS) is delicate, because many metabolites from the kynurenine pathway have well known neurotoxic effects (SCHWARCZ *et al*., 1983). Motor activity coordination is fundamentally influenced by the cerebellum, such that body movements and sustentation are disturbed when the cerebellum is damaged (PORRAS-GARCÍA, *et al*., 2013).

There are studies involving cytokines in candidiasis by *C. albicans*, but no concerning *C. parapsilosis* infection. This fungus has emerged as an important pathogen of bloodstream infections and currently is the second or third most commonly isolated Candida species from blood cultures worldwide (SAN MIGUEL *et al*., 2005; VAN ASBECK *et al*., 2009; MARRA *et al*., 2011) and infections by this fungus are a significant problem in the premature neonate and contribute significantly to neonatal mortality and morbidity (PAMMI *et al*., 2013).

In our previous study it was observed behavioral change in *C. parapsilosis* infected mice and this study investigated the immunopathological effect in brain from mice infected with C. parapsilosis by behavioral change, cytokines, histopathological and IDO activity and expression in brain (cerebellum). Also systemic immune responses and cytokines were evaluated on the course of systemic mice infection.

3. Materials and Methods

3.1. Fungal Isolates

Patient isolate (663581) with histoplasmosis, received hospital care at University Hospital of Londrina – HU/UEL and initially identified through selective medium, Chromagar Candida (CA221, Paris, France). The identification followed by confirmation by polymerase chain reaction (PCR), using as first primer ITS-5 e ITS-4 and second primer ITS-5, ITS-4, ITS-2 and ITS-3, two consecutive PCRs and analysis by sequencing (Applied Biosystes) at Chiba University, Chiba, Japan.

3.2. Cell Free Antigen (CFA)

CFA was obtained as described by Camargo *et al.* (1991) with modification and it was stored at -80ºC until use.

3.3. Mouse infection

First experience: SWISS mice (6- to 8-week-old female) were fed ad libitum and kept in a 12-h light-dark cycle at 25ºC. The animals were randomly divided into two groups with ten animals each for experiment: d) 56 days (infected and killed fifty six days) and (e) Control (inoculated with phosphate-buffered saline - PBS) (each group = 5). Second experience: BALB/c mice (6- to 8-week-old female) were fed ad libitum and kept in a 12-h light-dark cycle at 25ºC. The animals were randomly divided into five groups with ten animals each

for experiment: (a) 7 days (infected and killed with seven days), (b) 14 days (infected and killed with fourteen days), (c) 28 days (infected and killed with twenty eight days), (d) 56 days (infected and killed fifty six days) and (e) Control (inoculated with PBS). Infected groups were inoculated with 100 ul of *C. parapsilosis* isolated ($1x10^8$ viable yeast cells/ml in PBS) by intravenous route. The animals were sacrificed, cerebellum and bloods were aseptically removed (each group =5). All the procedures applied to animals in this study were approved by the Animal Care and Use Committee of the State University of Londrina (CEEA nº. 100/09).

3.4. Behavioral analysis in SWISS mice infected with *C. parapsilosis*

The day prior euthanasia, each as filmed 5 minutes and images were analyzed for number of spins.

3.5. Perfusion in SWISS mice infected with *C. parapsilosis*

The animals (infected and control) underwent the cardiac perfusion process at 56th days of infection. The animals were anesthetized with ketamine (80mg/kg) and xylazine (8mg/kg) intramuscularly. After full anesthesia, perfused through the ascending aorta with a 4% solution of paraformaldehyde in 0.12 M phosphate buffer, pH 7.4. After one day fixation in the same fixative, underwent dehydration in 70% alcohol for 30 min, 2 passes in 95% ethanol for 30 min, 3 passes in absolute ethanol for 30 min, a passage in absolute alcohol for 1 hour and 4 passages in xylene for 30 min. Then, they were subjected to 30 min 4 inclusions in paraffin. Sections of 5 mm were fixed on microscope slides for immunofluorescence, immunohistochemistry and staining with HE.

3.6. Cerebellum histopathological analysis

The cerebellum histopathological analysis, was carried out counting the number of Purkinje cells. The sections fixed on a microscope slide were hydrated in xylene, alcohol and xylene (v/v) absolute alcohol, 95 and 70% ethanol (10 min for each solution). Staining was done with hematoxylin (1 min) and eosin (1min) and wash in running water for 5 min. The analysis was carried by serial sections of each cerebellum and sections 10 with 20mm spacing between the cuts was analyzed. The images captured by optical microscopy at magnification 100, were analyzed by Motic Images Plus 2.0 software.

3.7. Immunofluorescence of Purkinje cells

The cerebellum sections with a thickness of 0.5 mm, were deparaffinized (xylene) and hydrated (70% to absolute alcohol), washed with distilled water then PBS (0.1 M, pH 7.4). Antigenic recovery by heat mediation in citrate buffer. Endogenous peroxidase was blocked by methanol and hydrogen peroxide followed by blocking with 2% BSA + 0.1% Triton X in PBS. After primary antibody - antibody anti-calbindin - ab82812 (1:50 in 1% BSA + 0.1 % Triton X in PBS) incubation for 18h at 4°C it was incubated with secondary antibody - anti-mouse IgG FITC - (F0257 - Sigma, St. Louis, MO, USA) (1:1000 in 1% BSA + 0.1 % Triton X in PBS) over 1h 30' at room temperature in the dark. For reading, were added to the slides, glycerol with PBS (v/v). The images were captured by fluorescence microscope (Leica DFC300 FX) with increased 200.

3.8. Immunohistochemistry apoptosis Purkinje cells

The cerebellum section was processed as 3.8. Antigenic recovery by heat mediation in citrate buffer, blocked and treated with primary antibody anti-Fas Ligand (15285 ab) as above. Then it was incubated with anti-rabbit IgG peroxidase conjugate 1:4000 (A1949, Sigma, St. Louis, MO, USA) for 1h 30' at room temperature and revelation by DAB and H_2O_2. Counter staining was performed with hematoxylin Herris (1min) and wash in running water for 5 min. The stained sections were then dehydrated in alcohol and xylene and coverslipped with fixed balm. The images captured by optical microscopy at magnification 200, were analyzed by Motic Images Plus 2.0 software.

3.9. IgG anti- *C. parapsilosis* serum levels by ELISA

Plates were coated with 100 mL/well containing 25 µg/mL of CFA *C. parapsilosis* diluted in carbonate-bicarbonate buffer and incubated for 1 hour at 37°C and overnight at 4°C, followed by blocking and washing step. The serum samples (1:10) was added (100 µL/well) and incubated for 2 hours at 37°C and followed by incubation with anti-mouse IgG peroxidase conjugate 1:4000 (A8924, Sigma, St. Louis, MO USA). The revelation was made with OPD and H_2O_2 (100 mL/well) and read at 492 ηm (Multiskan EX,Uniscience-Labsystems, Helsinki, Finland).

3.10. CAPTURE ELISA for Ag of *C. parapsilosis*

ELISA immunoplates were coated with IgG anti-CFA *C. parapsilosis* (30 μg/mL), in carbonate-bicarbonate buffer at pH 9.6 for 1 h at 35 ºC and overnight at 4 ºC. The plates were washed and blocked for 1 h at room temperature, followed by washing again. The plates were then incubated with homogenated brain (pure) samples for 2 h at 35 ºC. After the washes and incubated with IgG anti-E-CFA *C. parapsilosis* (30 μg/mL). Then it was incubated with sheep anti-E-CFA (1:50 for 2 h at 35 °C) followed by anti-sheep IgG (A3415, Sigma, St, Louis, MO, USA), diluted 1:4000 for 1.5 h at 35 °C and processed as above. The reaction was developed with a substrate solution and stopped with 4N H2SO4. The absorbance was read at 492 nm.

3.11. Delayed-Type Hypersensitivity Assay

Delayed-type hypersensitivity (DTH) reactions were evaluated by employing a footpad test previously described [26]. Briefly, mice were inoculated with 50ul of CFA, and footpad thickness was measured with a caliper (Mitutoyo Corporation, Tokyo, Japan) immediately before and 24 h after inoculation (7.5ug/ml). The increase in footpad thickness was calculated and expressed in millimeters.

3.12. Colony-Forming Units (CFU)

Cerebellum was aseptically removed, weighted and homogenized in PBS (0.2 g of tissue/mL). The homogenates (50 μL) were plated on sabouraud dextrose agar plates (Himedia, Mumbai, India), and the colonies were counted after 24 h of incubation at 37 ºC. The results were expressed as log10 CFU/g.

3.13. Cytokines Assay in serum e cerebellar tissue

IFN-γ, IL-4, IL-17 and IL-10 homogenized cerebellum levels and serum levels were determined by ELISA Kits from eBiocience according to the manufacturer's instructions.

3.14. IDO gene expression in cerebellar tissue by qRT-PCR

The cDNA were obtained according to the following thermal profile: 60 minutes at 37°C and 5 minutes at 95°C. The gene expression for the gene of interest IDO1 (indoleamine 2,3-dioxygenase1) and endogenous gene GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) has been characterized through the technique of polymerase chain reaction in real time (qPCR) (Applied Biosystems, Foster City, CA). The samples were placed in

microcentrifuge tubes for specific qPCR assay and put them 2μL of cDNA samples from + 10μL TaqMan PCR Master Mix (Applied Biosystems, Foster City, CA). Subsequently, the microtubes were placed in the apparatus Mx3500p Stratagene (Agilent Technologies) to read the samples made according to the following thermal profile: denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds and extension at 72°C for 15 seconds. The sequences of the primers IDO1 (Mm00524206_m1) and endogenous GAPDH gene (Mm99999915_g1) were obtained ready assays from Applied Biosystems. The ratio of gene of interest was calculated according to the formula: ratio = $2^{-\Delta\Delta Ct}$. GAPDH was used to normalize for IDO mRNA expression, respectively.

3.15. Kynurenine activity assay

To monitor IDO enzymatic activity, kynurenine was detected using a modified spectrophotometric assay (Braun *et al*., 2005). Cerebellum homogenates were washed and resuspended in Hanks Buffer (HBSS) containing 100μg/mL of L-tryptophan (Life Technologies, Grand Island, NY) and incubated for 5 hours. The amount of 50 μL of 30% trichloroacetic acid was added to 100 μL of cerebellum homogenates, vortexed, incubated for 30 min at 50° C to hydrolyze N-formylkynurenine to kynurenine and centrifuged at 8000 x g for 10 min. A volume of 75 μL of the supernatant was then added to an equal volume of Ehrlich reagent (0,2 g p-dimethylbenzaldehyde, 10 mL glacial acetic acid) in a 96 well microtiter plate. Optical density was measured at 492 nm after 30 min, using a Multiskan MS (Labsystems, Helsinki, Finland) microplate reader. A standard curve of defined L-Kynurenine concentrations (0-5000 μg/mL) was used to determine unknown kynurenine concentrations.

3.16. Western blotting for IDO

Western blotting was performed according to the method described by RIGOBELLO *et al.* (2013), with some modifications. We used mercaptoethanol sample-buffer (BIO-RAD, Bio-Rad Laboratories, Inc. USA) to treat homogeneized cerebelum samples, pre-stained molecular weight protein standards (Precision Plus Protein Kaleidoscope Standards, Catalog No.: 161-0375. BioRad), which was then submitted to 12,5% polyacrylamide gel electrophoresis in tris-glycine buffer, pH 8.2, at 100V. After nitrocellulose-membrane transfer was incubated with purified anti-IDO (Monoclonal anti-mouse, clone mIDO-48, produced in mouse IgG2b, Biolegend and clone mIDO-80, produced in rabbit, Santa Cruz Biotechnology) for 2h at room temperature. The detection using anti-mouse and anti-rabbit

IgG conjugated to peroxidase respectively (Sigma-Aldrich). Then, the membrane was revealed using a solution of hydrogen peroxide with Luminol Pierce ECL Western Blotting (Thermo Scientific) and exposed for 5 minutes in a fotodocumentador Gbox iChemi XR (Syngene International Ltd., Bangalore, India). The reuse of membranes for ressondagem with another antibody (Monoclonal Anti-β-Tubulin, clone TUB 2.1, produced in mouse, Sigma-Aldrich) was performed using stripping solution (Thermo Scientific). The membrane was incubated on shaker at 37 °C for 60 minutes. The membrane was washed and the protocol followed by hybridization with antibodies departing from the lock step. After the incubations with antibodies and washes, the membrane was again incubated with chemiluminescence reagents and exposed for 5 minutes in a fotodocumentador Gbox iChemi XR.

3.17. Statistical Analysis

The data were analyzed by GraphPad Prism 6.0 software using ANOVA and Tukey's test or t-test; we considered $p<0.05$ to be statistically significant.

4. Results

4.1. Behavioral analysis on mice infected with *C. parapsilosis*

Infected mice presented a behavioral change in the course of infection while the control mice did not show this change (figure 1). The number of turns on its axis was analyzed in 56 days post infected mice and resulted in higher turns number in 5 min (168.8 ± 16.84) while the control mice did not show turn (0.0 ± 0.0) n = 5 (figure 2).

4.2. Histopathology analysis of cerebellum

Histological analysis of cerebellum showed a significant ($p<0.05$) decrease in number of Purkinje cells of infected group, but this decrease was not all the cerebellum, happened in some regions of the cerebellum. The number of Purkinje cells counted in the cerebellum of 56 days infected group was lower (0.941 ± 0.031) than control group (1.087 ± 0.040) (Figure 3 and 4).

4.3. Purkinje cells and number of spins correlation test

There was a strong correlation between the decrease in the number of Purkinje cells and the increase in the number of spins in 5 min in infected mice with *C. parapsilosis* (r = 0.9876) (Figure 5).

4.4. Immunofluorescence of Purkinje cells

The immunofluorescence (figure 6) complements the data obtained by counting the number of Purkinje cells, visually showing the reduction of these cells in the group with 56 days of infection compared with the control group.

4.5. Purkinje cells apoptosis analysis by Immunohistochemistry

No reactivity was observed with antibody anti-FasLigand, one of the apoptosis markers, in Purkinje cells by immunohistochemistry, suggesting that it is not the pathway responsible for the death of this cell (data not shown).

4.6. DTH assay

The delayed cutaneous hypersensitivity test used to evaluate the cellular immune response has shown increased response in infected mice during the course of infection, compared right paw (PBS) with left (CFA) ($P < 0.05$) (Figure 7).

4.7. Cerebellum CFU results

The CFU results from the cerebellum is shown in figure 8. CFUs recovered from the cerebellum showed a significant reduction ($p < 0.05$) in the number of colonies shown in 14 days (0.80 ± 0.30) and showing negative with 28 and 56 days infection (0.00 ± 0.00) compared with mice 7 days of infection (1.76 ± 0.51). The results are in logarithmic scale.

4.8. IgG anti-*C. parapsilosis* serum levels by ELISA

Increased serum IgG anti-CFA *C. parapsilosis* levels expressed in O.D. at 492nm was observed in 28 (0.26 ± 0.03) and 56 (0.41 ± 0.09) days post infection, compared with control (0.08 ± 0.00) and 7 (0.10 ± 0.01) or 14 (0.13 ± 0.02) days of infection (Figure 9).

4.9. Ag of *C. parapsilosis* levels in homogenized brain

Results of Ag levels in homogenized brain (figure 10) were observed a increased in 7 and 14 days post infection compered to other groups (7days post infection: 0.149 ± 0.04; 14 days post infection: 0.148 ± 0.04) (*$p<0.05$).

4.10. Cytokines levels

The cytokines IFN-γ, IL4. IL-17 and IL-10 levels, expressed as pg/ml, were higher in the homogenized cerebellum in group of 7 days post infection compared to other groups (IFN-γ

- control: 5.24 ± 0.58; 7 days of infection: 8.25 ± 2.14; 14 days of infection: 5.30 ± 1.50; 28 days of infection: 4.91 ± 0.47; 56 days of infection: 5.29 ± 0.52) (IL-4 – control: 4.78 ± 0.67; 7 days of infection: 7.58 ± 1.32; 14 days of infection: 4.97 ± 1.31; 28 days of infection: 4.39 ± 0.41; 56 days of infection: 5.06 ± 0.39) (IL-17 - control: 8.31 ± 1.41; 7 days of infection: 12.92 ± 1.35; 14 days of infection: 8.54 ± 1.24; 28 days of infection: 8.54 ± 0.44; 56 days of infection: 8.71 ± 0.53) (IL-10 - control: 12.97 ± 0.83; 7 days of infection: 22.22 ± 1.96; 14 days of infection: 12.88 ± 1.76; 28 days of infection: 12.96 ± 0.58; 56 days of infection: 13.76 ± 0.70) (figure 11). The serum IFN-γ levels was increased in 7 (3.61 ± 0.96) and 14 (4.45 ± 0.42) days of infection and no difference was detected in IL-4 level.
IL-17 (4.83 ± 0.60) and IL-10 (9.53 ± 1.65) levels were increased in 56 days post infection compared to 7 days of infection (7.17 ± 0.68). The cytokine profile in serum of animals demonstrated a significant increase in Th1 cytokines after 7 and 14 days of infection ($p < 0.05$). 28 and 56 days of infection had decreased levels of Th1 cytokines and a significant increase in Th2 and Th17 cytokines compared with the other groups ($p < 0,05$) (Figure 12).

4.11. IDO expression in cerebellum

IDO gene expression was measured by quantitative real time RT-PCR. Cerebellum IDO RNA expression was significantly ($p<0.05$) increased (7D - ΔΔCT 65.17 ± 3.22) comparing with other groups (14D - ΔΔCT 19.40 ± 0.31; 28D - ΔΔCT 8.71 ± 0.48; 56D - ΔΔCT 4.07 ± 1.54). The IDO RNA expression was decreased in mice treated with 1MT (28D + 1MT - ΔΔCT 2.48 ± 0.56) in relation to control untreated (28D - ΔΔCT 8.71 ± 0.48)(figure 13).

4.12. Kynurenine activity assay in cerebellum

Results of cerebellum IDO activity is shown figure 14. The results showed a significant reduction ($p<0.05$) of IDO activity during infection (7 days of infection: 49.84 ± 3.60; 14 days of infection: 47.20 ± 14.13; 28 days of infection: 42.18 ± 11.87; 56 days of infection: 25.40 ±6.47). The IDO activity was decreased in mice treated with 1MT (28 days of infection trated with 1MT: 22.40 ± 10.85) in relation to control untreated (28 days of infection: 42.18 ± 11.87).

4.13. IDO detection by Immunobloting

The presence of IDO was positive in all homogenized of cerebellum (control, 7, 14, 28 and 56 days post infection) detected by anti-IDO monoclonal, indicating that IDO is expressed

in all periods. However by this methodology it was not possible to observe the differences between the groups (figure 15).

5. Discussion

The property of *C. parapsilosis* in strongly adhere on different supports applied in hospitalized patients it enables for a systemic infection. Similar to human invasive infection occurring with catheter involvement, this study used experimental mouse model by intravenous route of *C. parapsilosis* infection. As expected there was dissemination of the fungus in several organs (data not shown). However the persistence of viable fungus was evident only in the initial phase of 7 -14 days, no detectable over 28 days of infection by CFU count. This result is in agreement with low virulence of *C. parapsilosis* described in the literature (TROFA, 2008). The mouse systemic model showed that *C. parapsilosis* remained unable to initiate progressive infections, even with addition of immunosuppressive treatments, although administration of a very high inoculums potentially allows some isolates to initiate disease (MACCALLUM, 2012) but recently, TREVINO-RANGEL *et al*., (2014) demonstrated that *C. parapsilosis* can induce disseminated infection in a murine model. The low virulence of *C. parapsilosis* as innate defense mechanism action by dendritic cells activated by exposure to *C. parapsilosis* inducing phagocytosis, killing and pro-inflammatory protein secretion has been demonstrated by NAGY *et al*., (2011).

However, 14 days post infection it was detected viable fungi in several organs, suggesting that innate immunity is not as effective to fully remove the *C. parapsilosis* clinical isolate used in this study. Increased DTH and serum levels of IgG antibodies to *C. parapsilosis* was detected at 28 and 56 days pi, coincident with negative CFU period, suggesting that the immune responses induced may be involved in host defense to this fungus. The protection by anti-mannan antibody was observed by ZHANG *et al*., (2006) in an experimental model of systemic *C. albicans* infection and as they detected the presence of mannose in *C. parapsilosis*, they suggested the possible protective action of this antibody in *C. parapsilosis* infection.

The cytokines derived from Th1 lymphocytes are essential for defense against intracellular microorganisms and for the vast majority of fungi, Th2 responses are deleterious (WÜTHRICH *et al*., 2012). In this work, all cytokines evaluated were increased in early phase of infection in the brain, the same period of highest fungal load, suggesting

the mix of Th1 and Th2 response in this phase locally. However in the serum it was detected higher INF-γ level in 7 and 14 days of infection suggesting the favorable response to the host that could be contributed for clearance of the fungi. Also high serum levels of IL-10 and IL-17 were detected in 56 days of infection. IL-17 cytokines are important in the defense against fungi (WÜTHRICH *et al*., 2012) but according to NETEA *et al*., (2004) resistance to *C. albicans* is related to decrease in IL-10 production.

Surprisingly, in the negative CFU phase we observed behavioral change in infected mice. Changes started from 20 days and at 28 days all infected mice presented a complex neurological syndrome, including head tilt, circling, twirling when raised by the tail and ataxia. As this change occurred after the induction of the immune response and in absence of viable fungi, the immune response could be involved in this complex neurological syndrome. In this context the IL-10 production could be important for regulating the immune response.

Considering that even with different accessibility to the CNS as the presence of a blood brain barrier, lack of lymphatic vessels, and the absence of parenchymal cells (RANSOHOFF *et al*., 2003), various pathogens can induce inflammatory response in the CNS (FURTADO *et al*., 2009) and as a result of inflammation, myelin and microglia can be phagocytosed by activated macrophages (KWIDZINSKI & BECHMANN, 2007), which could induce myelin autoimmune antibodies and tissue damage. However, in the present study it was not detected inflammatory response in the brain and the analysis of plasma levels of IgG anti-myelin showed similar level of anti-myelin IgG (data not shown), suggesting that the behavioral change is not derived by brain tissue inflammation or due to the induction of anti-myelin auto-antibodies.

Interestingly, in this study it was detected decreased number of Purkinje cells in brain of *C. parapsilosis* 56 days infected mice. As these cells are responsible for the equilibrium (PORRAS-GARCÍA *et al*., 2013) and also the detection of correlation (r=0.9876) between decreased number of these cells and increased number, suggest that behavioral change could be due to decreasing in Purkinje cells. As no inflammatory response observed, the removal of these cells could be due to apoptosis. But the brain tissue analysis with anti-FasL antibody, showed no reactivity, suggesting that this pathway not taking part in changing the number of Purkinje cells or this event may have already occurred in the previous phase or may have occurred by another independent Fas pathway, which requires further study.

The IFN-γ cytokine can induce the expression of IDO in various cell types (CARLIN *et al*., 1989), and this expression represents an important mechanism of antimicrobial resistance to parasites (KWIDZINSKI & BECHMANN, 2007; PFEFFERKORN, 1984; SANNI *et al*., 1998; SILVA *et al*., 2002) and bacteria (NETTELNBREKER, 1998; BEATTY *et al*., 1994; MACKENZIE *et al*., 1998). The expression of IDO can occur in the brain under IFN-γ treatment (ALBERATI - GIANI *et al*., 1996; HEYES *et al*., 1996) and the kynurenine pathway metabolites that presents two degradation products of tryptophan (Trp), quinolinic acid (Quin) and 3- Hydroxyanthranilic acid (3-HAA), present neurotoxic properties (STONE & PERKINS, 1981; SCHWARCZ *et al*., 1983; CHIARUGI *et al*., 2001). According to BOZZA *et al.,* (2005) in *C. albicans* infections, IFN-γ may have a central role in the activation of IDO and KWIDZINSKI & BECHMANN (2007) speculated that the trauma induced by IDO activation induces secondary neuronal damage through accumulation of neurotoxic metabolites. In the present study higher IDO expression and its activity were observed in 7 days of infection but suppression occurred in 56 days, suggesting that IDO could be involved in the early phase of infection.

This study shown for the first time that systemic infection with *C. parapsilosis* induce humoral and cellular immune responses, brain and systemic cytokines modulation and decrease in brain Purkinje cells number associated to behavioral change.

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7. Figures.

Figure 1. Behavior changes in mice infected with *C. parapsilosis* strain.

Mice were infected (iv) with 1x10^{8} yeast *C. parapsilosis* (strain), control was inoculated PBS and monitored daily for behavioral alterations. The signs began to appear 20 days after infection. (a) non-infected, (b) 28 days post infection and (c) 56 days post infection.

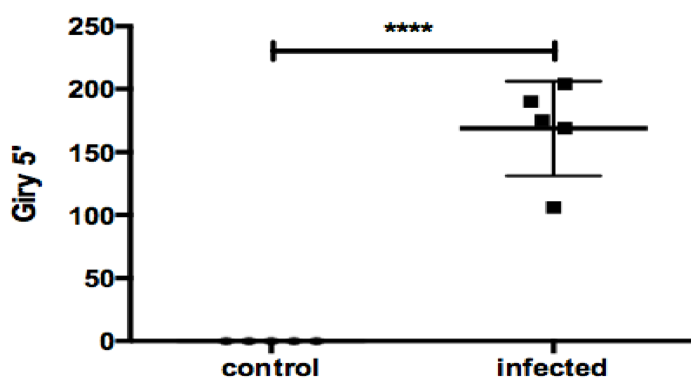

Figure 2. Counting of the number of turns/ 5 minutes in infected mice.

Swiss mice control and infected. Results were expressed as means ± standard error of turns in 5' (n=5 each group). (* $p<0.05$).

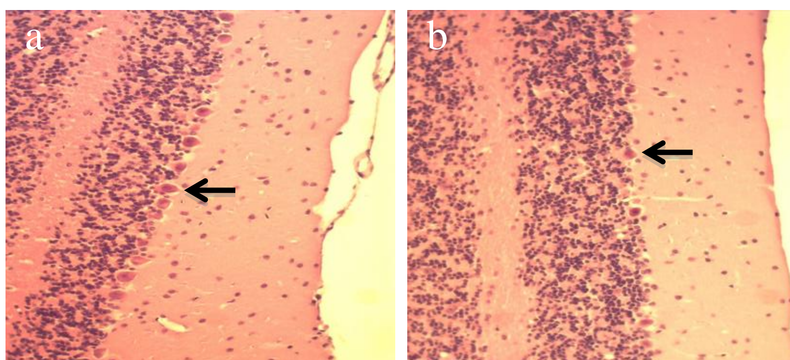

Figure 3. Histopathological analysis of cerebellum in *C. parapsilosis* strain infected mice.

Sections of cerebellum from non-infected animal (a) and infected animal (b) after 56 days. Purkinje cells indicated with arrows. Images captured with objective 100x optical microscope.

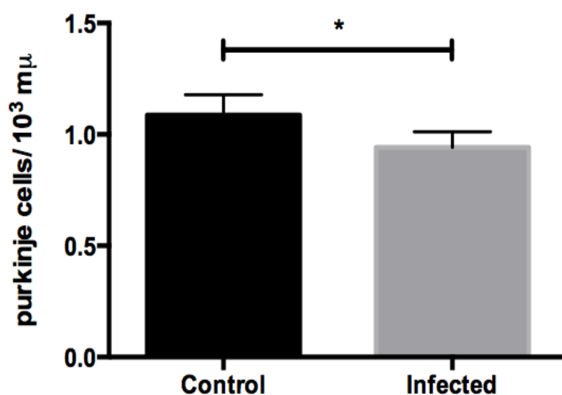

Figure 4. Number of Purkinje cells in *C. parapsilosis* strain infected mice.

Cerebellum Swiss control (PBS) and infected with *C. parapsilosis* (n=5 each group). (decrease * $p < 0.05$).

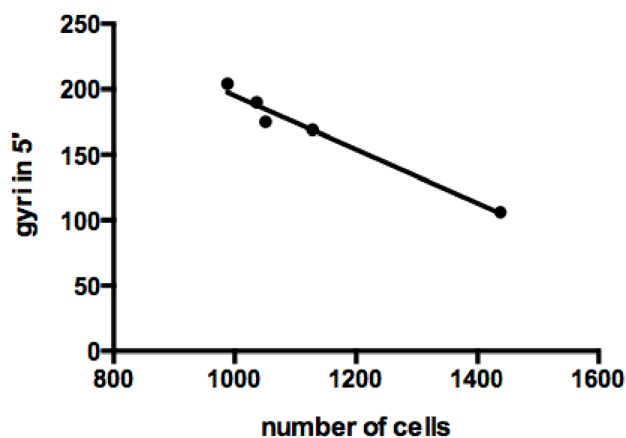

Figure 5. Person correlation between number of Purkinje cells and number of turns.

Person correlation test showed a strong correlation between number of Purkinje cells and number of turne in the period 5' in Swiss mice infected with *C. parapsilosis* (n= 5, r= 0.9876).

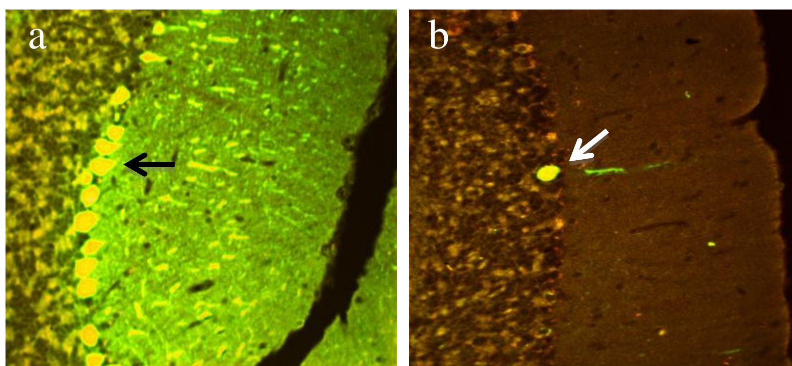

Figure 6. Immunofluorescence of Purkinge cells in mice infected with *C. parapsilosis*. Marking of Purkinje cells in the cerebellum of Swiss mice by anti-calbindin (green). Non-infected animal (a), infected animal (b) after 56 days. Purkinje cells indicated with arrows. Images captured with LEIKA immunofluorescence microscope and 200x objective.

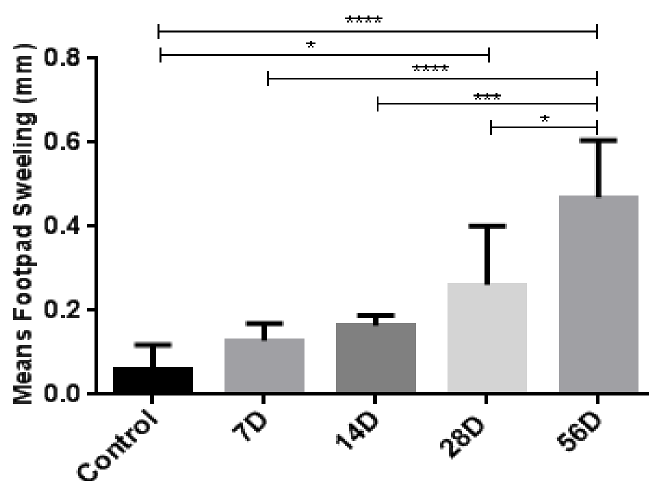

Figure 7. DTH test with total soluble *C. parapsilosis* antigens.

The reactions were evaluated employing a footpad test. The mice were inoculated with CFA (or PBS as control), and footpad thickness was measured almost 24h later. The graph shows, in mm, the difference between CFA and PBS (*p<0.05).

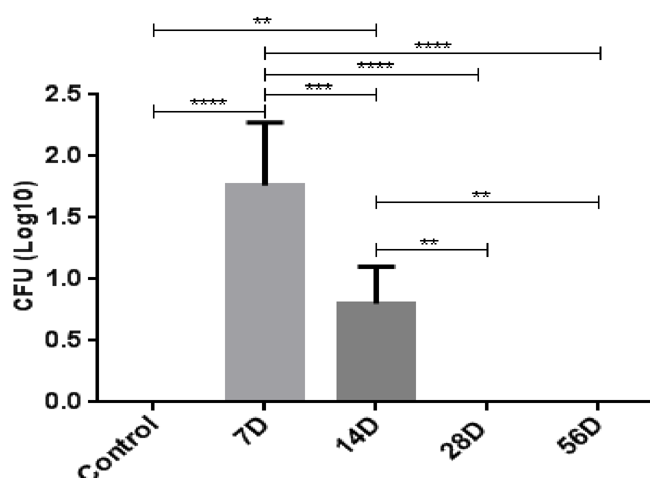

Figure 8. ***C. parapsilosis*** **in cerebellum of infected mice.**

During experimental infection with *C. parapsilosis* at days 7, 14, 28 and 56 post infection cerebellum homogenates (0.2g tissue/mL) were plated on Sabouraud and colonies counted after 48 h of incubation 37ºC (*p<0.05).

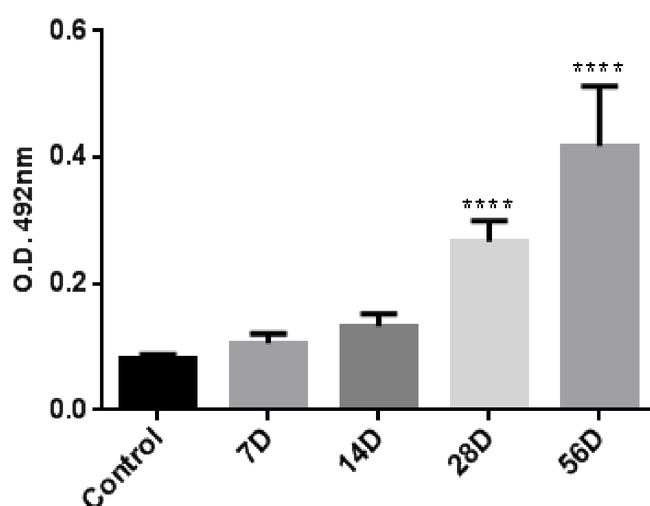

Figure 9. Serum anti-CFA of ***C. parapsilosis*** **IgG levels determination.**

Serum samples of infected mice with *C. parapsilosis* and control non-infected were analyzed by ELISA for specific IgG (* p< 0.05). O.D. optical density.

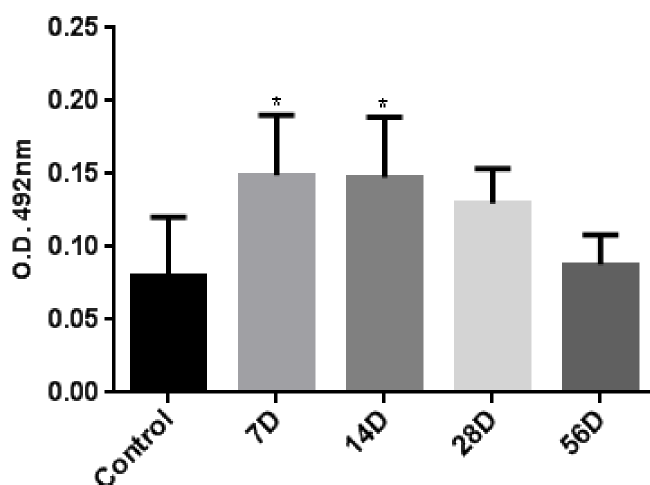

Figure 10. Ag levels of *C. parapsilosis* in homogenized brain.

Homogenized brain samples of infected micewith *C. parapsilosis* and control non-infected were analysed by CUPTURE ELISA (*p<0.05). O.D. optical density.

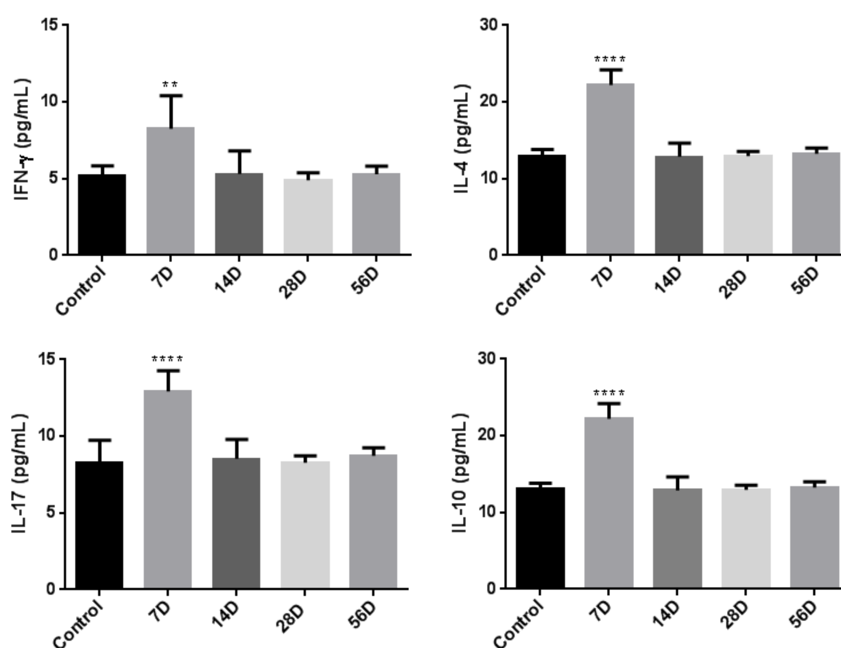

Figure 11. Cytokines levels in the cerebellum of infected mice.

IFN-γ, IL-4; IL-17 and IL-10 were measured in cerebellum homogenates from control mice and mice infected (7, 14, 28 e 56 days) with *C. parapsilosis.* The levels are shown in pg/mL, and they were calculated based on a standard curve. (*p < 0,05).

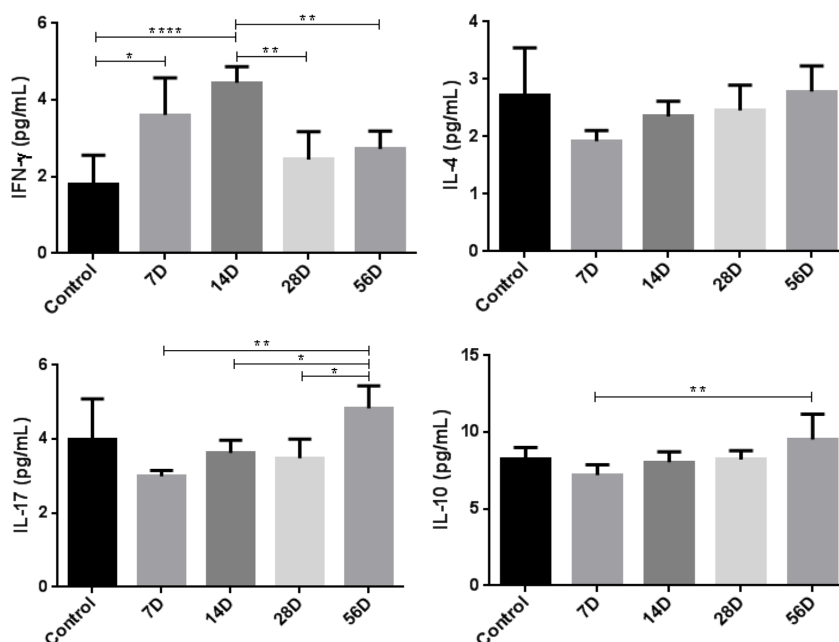

Figure 12. Cytokines levels in serum of infected mice.

INF-γ, IL-4, IL-17 and IL-10 1 were measured in serum from mice infected with *C. parapsilosis* and from the control group at 7, 14, 28, and 56 days after infection. The levels are shown in pg/ml, and they were calculated based on a standard curve. (*p<0.05).

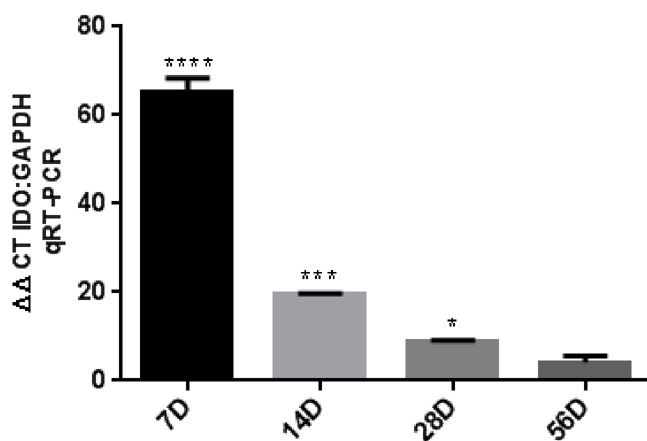

Figure 13. qRT-PCR of IDO cerebellum

Cerebellum IDO RNA expression in during the infection with *C. parapsilosis* (7, 14, 28 and 56 days of infection) (*p<0.05).

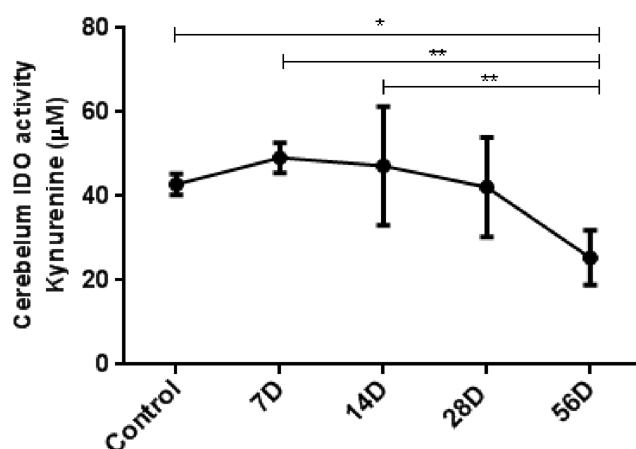

Figure 14. IDO activity in cerebellum.

Time course of cerebellum IDO activity during infection with *C. parapsilosis* (7, 14, 28 and 56 days of infection) (*p<0.05).

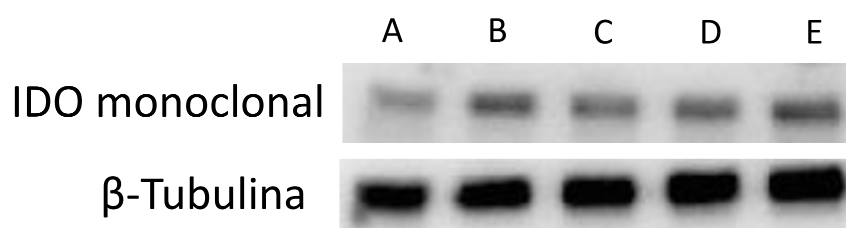

Figure 15. Immunobloting analisys in *C. parapsilosis* strain infected mice.

Results of immunobloting to homogenates of cerebellum (A. control, B. 7, C. 14, D. 28 e E. 56 days post infection) (SDS-PAGE gel 12,5%) with monoclonal and policlonal antibodies anti-IDO (β-Tubulin with control).

2º ARTIGO: Hemolytic factor isolated from *Candida parapsilosis* and specific IgG antibodies during experimental systemic murine infection.

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1. Abstract

The hemolytic activity of *Candida ssp.* has been considered an important virulence factor for survival in the host, but there is no data about its immune response during infection, mainly with *C. parapsilosis*. The objective of this study was to isolate *C. parapsilosis* hemolytic factor and evaluate its immune response induced in experimental systemic infection in mice. The hemolytic factors was obtained from *C. parapsilosis* (IFM 663581) cell free antigen (CFA) by differences in its MM (Sephadex G-100 and Amicon 50k and 100k) and its hemolytic activity determined by mouse erythrocyte lysis (550 nm). Additionally, specific antibodies were obtained for use in western blotting and hemolysis inhibition test. The experimental systemic infection ($1x10^7$ IFM 663581 yeast, i.v.) was performed in BALB/c mice. Fungal organs (spleen and liver CFUs) and IgG anti-hemolytic fraction was analyzed after 7, 14, 28 and 56 days p.i. The hemolytic fractions reacted with both antibodies and ConA, presented two components with high MM (~ 280, ~ 300kDa) and antibodies presented inhibitory activity ($p<0.05$). Higher specific antibodies levels ($p<0.05$) and decreased fungi CFUs ($p<0.05$) were detected at 28 and 56 days p.i. ($p<0.05$). In conclusion, hemolytic factors present in *C. parapsilosys* CFA preparations present high MM, possible with mannose and consisting of two immunogenic components. This work demonstrated for the first time the immune response to hemolytic fraction and possible fungi clearance by specific immune response during experimental murine infection, that require further study.

Key Words: antibodies, fungi, systemic infection, virulence factor.

2. Introduction

The fungi *Candida parapsilosis* was described as a relatively non-pathogenic yeast in the normal flora of healthy individuals that was of minor clinical significance in the past (WEEMS 1992). However in the last decade, *C. parapsilosis* has emerged as an important pathogen of bloodstream infections and is currently the second or third most commonly isolated *Candida* species from blood cultures worldwide (SAN MIGUEL *et al*., 2005; VAN ASBECK *et al*., 2009; MARRA *et al.*, 2011). *C. parapsilosis* infections are a significant problem in the premature neonate and contribute significantly to neonatal mortality and morbidity and, according to PAMMI *et al.,* (2013) it is responsible for a third of neonatal *Candida* infections and have a mortality rate of approximately 10%. The hemolytic ability of *Candida sp* has been considered an important virulence factor for survival in the host. The hyphal cells of *C. albicans* use hemoglobin as a source of iron (TANAKA *et al*., 1997). Iron is an essential micronutrient for most organisms and in addition, independent of iron acquisition, according to PENDRAK *et al*., (2004), hemoglobin may induces expression of several genes that act as virulence factor important for several steps in the pathogenesis of disseminated *C. albicans* infections. The hemolytic activity is often observed in clinical isolates of *C. albicans* and non *albicans* (TROFA *et al.,* 2008) but there are few studies on hemolytic activity in *C. parapsilosis* and usually with small sample size. Hemolytic activity of *C. parapsilosis* has been reported to be absent or with weak hemolytic activity (YİGİT, N.; AKTAS, E., 2009; ROSSONI *et al*., 2013, ISSA *et al*., 2011, SENEVIRATNE *et al*., 2011). FRANÇA *et al.,* (2011) determined *in vitro* hemolytic activities of *C. parapsilosis* isolates from anatomically distinct sites and noted that tracheal isolates had increased hemolytic activity compared with blood isolates, indicating that perhaps hemolytic ability plays a role in colonization of specific sites.

The hemolytic factor of *C. albicans* is detected in the culture supernatant of hyphal cell (TANAKA *et al.,* 1997) and is characterized as mannoprotein with high MM (WATANABE *et al.,* 1999). Also it has been demonstrated that secreted hemolytic factor of *C. parapsilosis* may be a mannoprotein (FAVERO *et al*., 2014). A simple method for obtaining antigen from the surface of *Paracoccidioides brasiliensis*, which was designated "cell free antigen" (CFA) was introduced by CAMARGO *et al*., (1991).

This study evaluated the hemolytic activity of CFA from *C. parapsilosis,* partially characterized the hemolytic factor and investigated humoral immune response to hemolytic factor in experimental murine systemic infection with *C. parapsilosis*.

3. Materials and Methods

3.1. Fungal Isolates

C. parapsilosis IFM 663581 was initially recovered from a patient from the State University Hospital of Londrina, Londrina, PR, Brazil, with histoplasmosis. It was identified through selective medium, Chrom agar Candida (CA221, Paris, France), followed by confirmation by polymerase chain reaction (PCR), using the first primer ITS-5 e ITS-4 and second primer ITS-5, ITS-4, ITS-2 and ITS-3, two consecutive PCRs and analysis by sequencing (Applied Biosystes) at Chiba University, Chiba, Japan.

3.2. Cell Free Antigen (CFA) and Hemolysis Test

CFA was obtained according to CAMARGO *et al*., (1991) and concentrated to 1500 μg/mL and incubated in "V" bottom plates for 3 h at 35 °C with mouse erythrocyte suspension (1% in PBS). Hemolysis was determined by absorbance at. 550 nm (Multiskan EX, Uniscience – Labsystems, Helsinki, Finland) in supernatants collected after centrifugation. By same way CFA and heated CFA (56 °C, 30 min) samples were diluted in series (ratio 1:2, 1:4 and 1:8) and then incubated 1:1 with a 1% erythrocyte suspension. PBS was used as a negative control, and distilled water as a positive control. Furthermore, CFA fractions obtained by gel filtration chromatography (3.4) and CFA fractions retained in Amicon 100k filter (Millipore) and Amicon 100 k eluate plus Amicon 50k were analyzed by this hemolytic assay.

3.3. Antibodies against hemolytic factor from *C. parapsilosis*

Antibodies against hemolytic factor were obtained according to VIVAN *et al.,* (2010) with some modifications. Autologous erythrocytes (1%) were incubated with CFA from *C. parapsilosis* (v/v) for 1 h at 37ºC and 1 h at 4ºC, washed with ice-cold HBSS and resuspended in HBSS. Sensitized erythrocytes emulsified in complete Freund's adjuvant (v/v) were inoculated subcutaneously into sheep. Incomplete Freund's adjuvant was used for second and third inoculations at 2-week intervals. Antiserum (anti-E-CFA) was secured 2 weeks later. Also antiserum against total *C. parapsilosis* CFA was produced in rabbit as described above by using total CFA without erythrocytes treatment.

3.4. CFA sephadex G-100/120 gel filtration chromatography and reactivity with antibodies anti-E-CFA

CFA samples (3-4 mL) were applied to a Sephadex G-100/120 column (0.65 x 30 cm) (Sigma, St. Louis, MO, USA) and eluted with PBS. Two milliliter fractions were collected with an automatic fraction collector (FC203B Gilson Inc., Middleton, WI, USA), and the absorbance was read at 280 nm (SP2000UV Ultrospec-200, Shanghai, P.R, China). CFA fractions reactivity was performed by ELISA with plates coated with fractions and incubated with sheep anti-E-CFA (1:50 for 2 h at 35 °C) and then with anti-sheep IgG (A3415, Sigma, St, Louis, MO, USA), diluted 1:4000 for 1.5 h at 35 °C. The revelation was made with OPD and H_2O_2 (100 mL/well) and read at 492 nm (Multiskan EX,Uniscience-Labsystems, Helsinki, Finland).

3.5. Reactivity of chromatography fractions with Concanavalin A (Con A) and carbohydrate level determination

ELISA plates were coated with Con A (50 μg/ml) in a carbonate buffer, pH 9.6, for 1 h at 35 °C and overnight at 4 °C. The plates were washed, blocked with blocking buffer and incubated with Sephadex chromatography fractions for 3 h at 35 °C. Then it was incubated with sheep anti-E-CFA (1:50 for 2 h at 35 °C) followed by anti-sheep IgG (A3415, Sigma, St, Louis, MO, USA), diluted 1:4000 for 1.5 h at 35 °C and processed as above. The carbohydrate level in fractions was determined by the phenol-sulfuric acid method in a microplate. The analyses were performed with 7.5 μg of protein in each fraction. The total carbohydrate level was determined by absorbance at. 492 nm and D-mannose was used as standard.

3.6. Immunoblotting

Total *C. parapsilosis* CFA and fractions retained in Amicon 100k filter were treated with mercaptoethanol sample-buffer (BIO-RAD, Bio-Rad Laboratories, Inc. USA) and submitted to SDS-polyacrilamide gel electrophoresis (SDS-PAGE) according to Rigobello *et al*., (2013), with some modifications. After transfer to a nitrocellulose membrane it was incubated individually with each immune serum (anti-E-CFA and anti-total CFA), diluted 1:50, for 2 h at 37ºC. Then incubated with anti-Sheep IgG (A3415, Sigma, St, Louis, MO, USA) or monoclonal anti-rabbit IgG-peroxidase (A1949, Sigma, St, Louis, MO, USA),

respectively and the reaction detected by 3,3,5,5-tetramethylbenzidine (TMB solution – BLOT, Life Technologies, Novex®). As molecular weight protein standards it was used pre-stained protein (Precision Plus Protein Kaleidoscope Standards, Catalog No.: 161-0375. BioRad),

3.7. Hemolysis inhibition test with anti-CFA anti-E-CFA antibodies

CFA serum, anti-E-CFA serum (1:40) previously inactivated (56 °C for 30 min) were incubated with CFA and erythrocytes (1%) at 35 °C for 3 h in a "V" bottom plate. The plate was centrifuged at 155 x g for 10 min at 4°C (5804R Eppendorf Centrifuge, Hamburg, Germany). The supernatants were collected and analyzed at 550 nm (Multiskan EX Uniscience Labsystems, Helsinki, Finland). CFA without serum and PBS were used as positive and negative controls, respectively.

3.8. Mouse infection with *C. parapsilosis* and analysis of infection degree (organs CFU) and serum IgG anti-hemolytic fraction levels determination

BALB/c mice (6- to 8-week-old female) were divided into five groups with ten animals each and inoculated with 100 uL of *C. parapsilosis* isolated ($1x10^8$ viable yeast cells/ml in PBS) by intravenous route and as control with PBS. The spleen and liver were removed after 7, 14, 28 and 56 days post infection, weighed, homogenized, and plated on sabouraud agar. The plates were incubated at 35ºC and read after 2 days. The results were expressed as the number of viable CFUs per mg of tissue per mouse. Also bloods samples were collected in the same period of infection. All the procedures applied to animals in this study were approved by the Animal Care and Use Committee of the State University of Londrina (CEEA nº. 100/09). For IgG anti-hemolytic fraction, ELISA plates were coated with *C. parapsilosis* hemolytic fraction (25 mg/mL, 100 mL/well) obtained by Amicon 100k and incubated with serum samples of mice (100 mL/well diluted 1:10) for 2 hours at 37°C followed by anti-mouse IgG peroxidase conjugate 1:4000 (A8924, Sigma, St. Louis, MO USA) and processed as described in 3.4.

3.9. Statistical Analysis

The data were analyzed by GraphPad Prism 6.0 software using ANOVA and Tukey's test or t-test; we considered $p<0.05$ to be statistically significant.

4. Results

4.1. Hemolytic activity assay

The hemolytic activity was detected only with CFA derived by the proportion higher than v/v used by CAMARGO *et al.,* (1991) or after CFA concentration (Figure 1). Hemolytic activity was detected even after heating the sample for 30 min at 56 °C. When native CFA was compared to heated by dilution (both pure and diluted 1:2, 1:4 and 1:8 at final concentrations of 1500, 750, 375 and 187 µg/mL, respectively) greater hemolytic activity was detected in heated CFA (Figure 2). The CFA fractions obtained by gel filtration chromatography didn't show hemolytic activity but fractions retained in Amicon 100k filter shown 100% of hemolysis. On the other hand Amicon 100k eluate retained in Amicon 50k didn't present hemolytic activity, suggesting that hemolytic factor has high molecular mass (MM).

4.2. Sephadex G-150/120 chromatography and fractions analysis

The CFA Sephadex G-100/120 spectrophotometric profile at 280 nm is shown in Figure 3. The fractions analysis with anti-E-CFA resulted in greater intensity of reaction with high MM fractions by ELISA (Figure 3). The total carbohydrate amount showed that the higher carbohydrate content is located in fraction of low MM and little in high MM fractions (Figure 4). The fractions that most bound to Con A and recognized by anti-E-CFA antibodies are located in fraction with higher MM (Figure 5).

4.3. Immunoblotting

The immunobloting of total CFA with anti-total CFA antibodies resulted in several bands with distinct MM (~300, 280, 70 and 47 kDa) and with anti-E-CFA antibodies resulted in two major bands: one about 300 kDa and the other to 280 kDa. The similar bands were recognized in fractions retained in Amicon 100k (Figure 6).

4.4. Hemolysis inhibition test with anti-CFA and anti-E-CFA antibodies

Considering 100% of the hemolytic activity with CFA, when incubated with anti-CFA resulted in 59% of hemolysis and in presence of anti-E-CFA resulted in 50% hemolysis. (Table 1).

4.5. Infection degree (organs CFU) and serum IgG anti-hemolytic fraction levels determination in infected mouse with *C. parapsilosis*

Higher *C. parapsilosis* CFUs were detected in 7 and 14 days post infection in spleen and liver in relation to control or other groups of animals ($p<0.05$). On the other hand IgG anti-hemolytic fraction serum levels was higher in 28 and 56 days post infection, mainly 56 days ($p<0.05$) (Figure 7).

5. Discussion

This study used the same CFA preparations described by CAMARGO *et al.,* (1991) for *C. parapsilosis* hemolytic activity evaluation and it was possible to detect this activity only in higher proportion of fungi or after antigen concentration. Then it is possible that *C. parapsilosis* present the hemolytic factor but produce it in lower concentrations than others *Candida sp*. If our analysis was performed in normal conditions without concentration, it would be considered negative for hemolytic activity. This is in accordance for absent or very low hemolytic activity of *C. parapsilosis* described in the literature (ISSA *et al*., 2011; SENEVIRATNE *et al.*, 2011).

According to Watanabe *et al.,* (1999), the hemolytic activity was not lost even after heat treatment and in this study, surprisingly, higher hemolytic activity was detected in heated CFA preparations. It may be that the protein denaturation exposes more hemolytic active sites, that require further study. Also its not be the protein nature as discussed by WATANABE *et al.,* (1999).

Chromatography was performed in order to isolate the hemolytic factor, but no activity was detected in any fraction possibly due to its low concentration. Hemolytic activity was detected in amicon 100 k retained and concentrated sample, therefore higher than 100 kDa, corresponding to first chromatography peak. This peak showed higher reactivity with Con A and according to FAVERO *et al.,* (2014) the hemolytic factor of *C. parapsilosis* react with Con A. Also this peak was recognized by antibodies produced against hemolityc factor and showed two components with MM ~280 and 300 kDa by western blotting. LUO *et al*., (2001) described alpha (incomplete) and beta (complete) hemolysis, that developed in 24h and 48h of incubation, respectively and suggested that *Candida* strains has two or more different hemolytic factors sequentially produced by the yeast. For hemolytic assay, the authors used sheep blood agar medium that require the diffusion of the hemolytic components. For this diffusion, if they are two components with

distinct MM, probably the higher molecular mass require more time for diffusion. In this context, our results are concordant to LUO *et al*., (2001) and reinforce the idea of existence of more than one component for the full hemolytic activity also in *C. parapsilosis*.
This study also investigated if antibodies have the hemolytic inhibitory ability and at least partial hemolytic blocking effect was observed. It may be that not all active epitopes have accessibility to antibodies, that require further study.

Sheep, rabbit and human blood have been used and best source found by YİGİT and AKTAS (2009) was sheep followed by rabbit. Here we used mouse blood and this may have contributed to the failure to detect hemolytic activity in non concentrated samples.

The mouse systemic *C. parapsilosis* infection was followed for immune-response investigation and higher antibodies levels were detected in the 28 and the 56 days post infection and not earlier. On the other hand viable fungi was detected in the begning and not after immune response development.

Moreover, whereas the hemolytic factor induces humoral immune response, this factor would have the potential to be used for immunodiagnosis, considering that there is still no antigen investigated for diagnostic purposes in *C. parapsilosis* infection.

In conclusion, hemolytic factors are present in *C. parapsilosis* CFA preparations and they present high MM, possibly two components containing mannose, that has potential to induce specific humoral response during experimental murine infection. This work demonstrates for the first time the immune response to hemolytic fraction and if this response contribute for clearance of *C. parapsilosis* during experimental murine infection, require further study.

6. References

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7. Figure

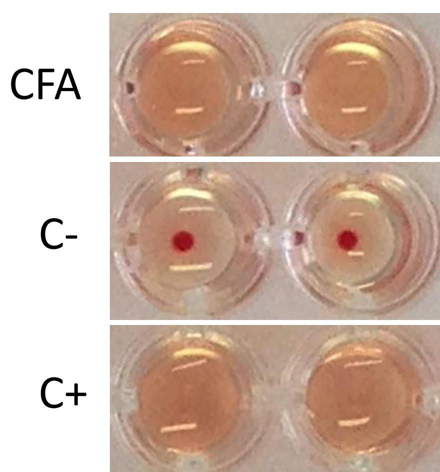

Figure 1. Hemolysis test with native CFA

Hemolysis test with native CFA. The native CFA pure were incubated in duplicate with a 1% mouse erythrocytes. C-: negative control (PBS); C+: positive control (distilled water).

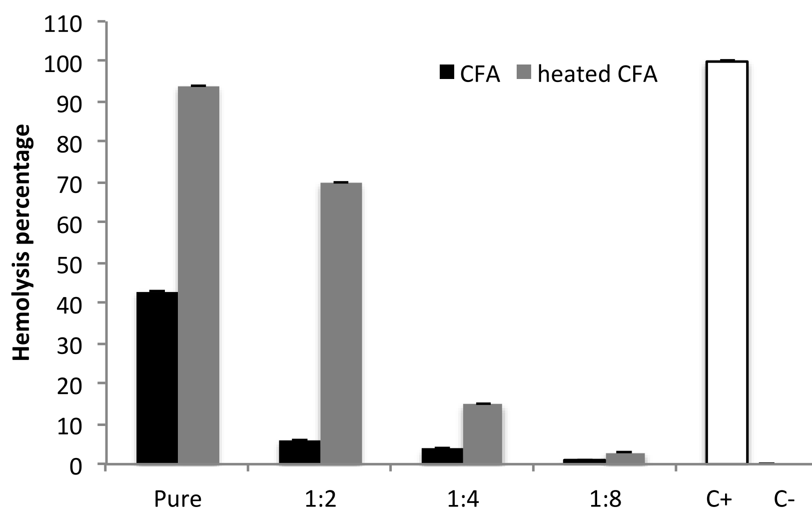

Figure 2. Analysis of the effect of heating sample CFA of *C. parapsilosis* in hemolytic activity

The results are expressed as percentage of hemolysis, the native and heated CFA (56 °C, 30 minutes), pure and diluted 1:2, 1:4 and 1:8, were incubated in duplicate with a 1% mouse erythrocytes. C-: negative control (PBS); C+: positive control (distilled water).

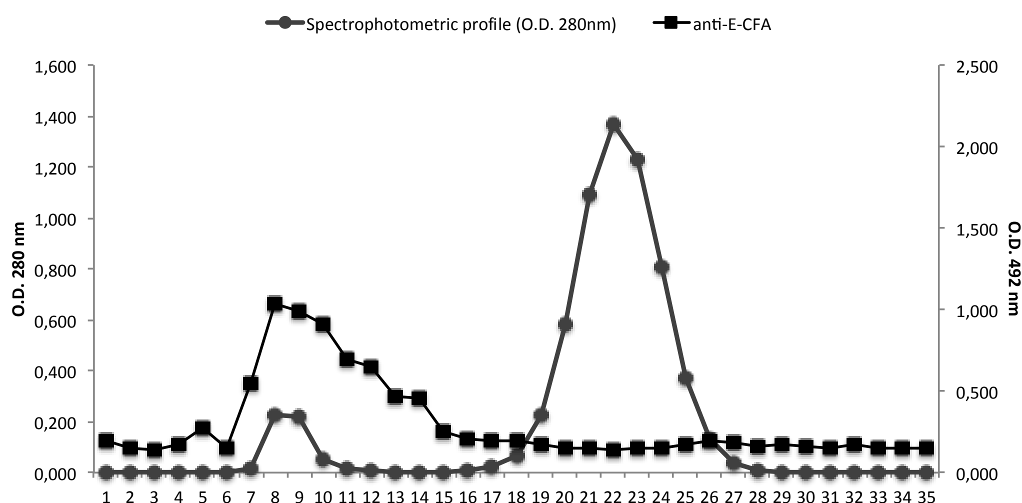

Figure 3. Spectrophotometric profile of *C. parapsilosis* CFA chromatography and analysis of CFA fraction reactivity with anti-E-CFA antibodies by ELISA

CFA chromatography was performed with Sephadex G-100/120 and fractions were read at 280 nm. Anti-E-CFA antibodie was tested by ELISA using Sephadex G-100/120 CFA fractions showing that antibody have reactivity with the fractions.

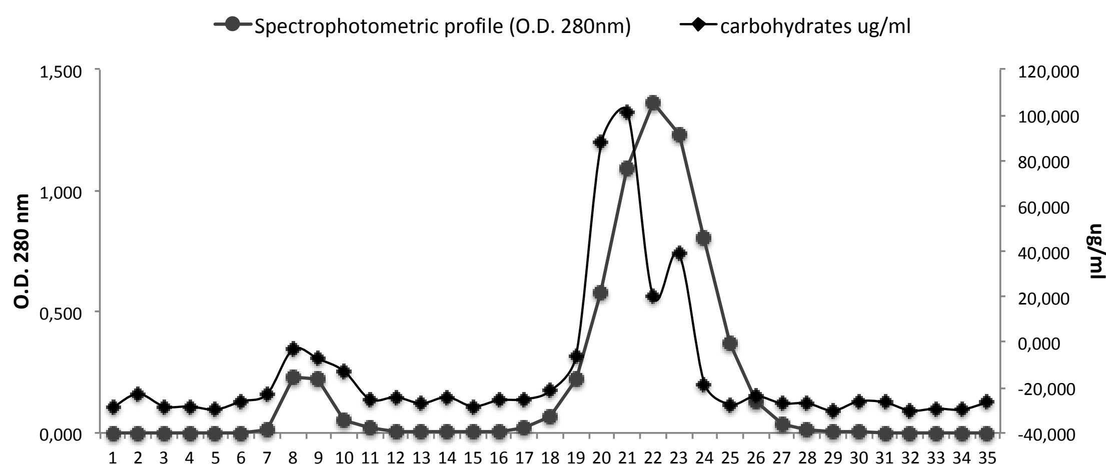

Figure 4. Carbohydrate levels in the *C. parapsilosis* CFA fractions

The line shows the carbohydrate levels in the fractions from Sephadex chromatography.

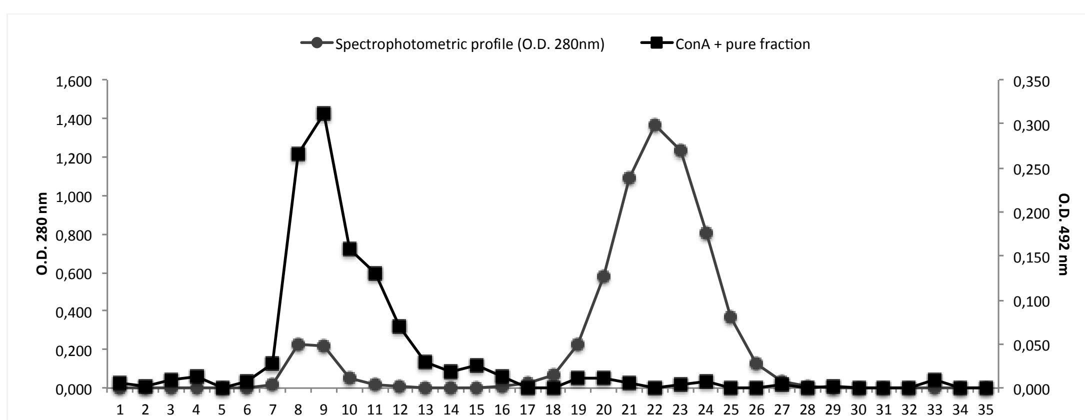

Figure 5. Analysis of CFA fraction reactivity with Con A

Anti-E-CFA antibodies was tested by ELISA using Sephadex G-100/120 CFA fractions recognized Con A showing that antibodies have the same profile of reactivity.

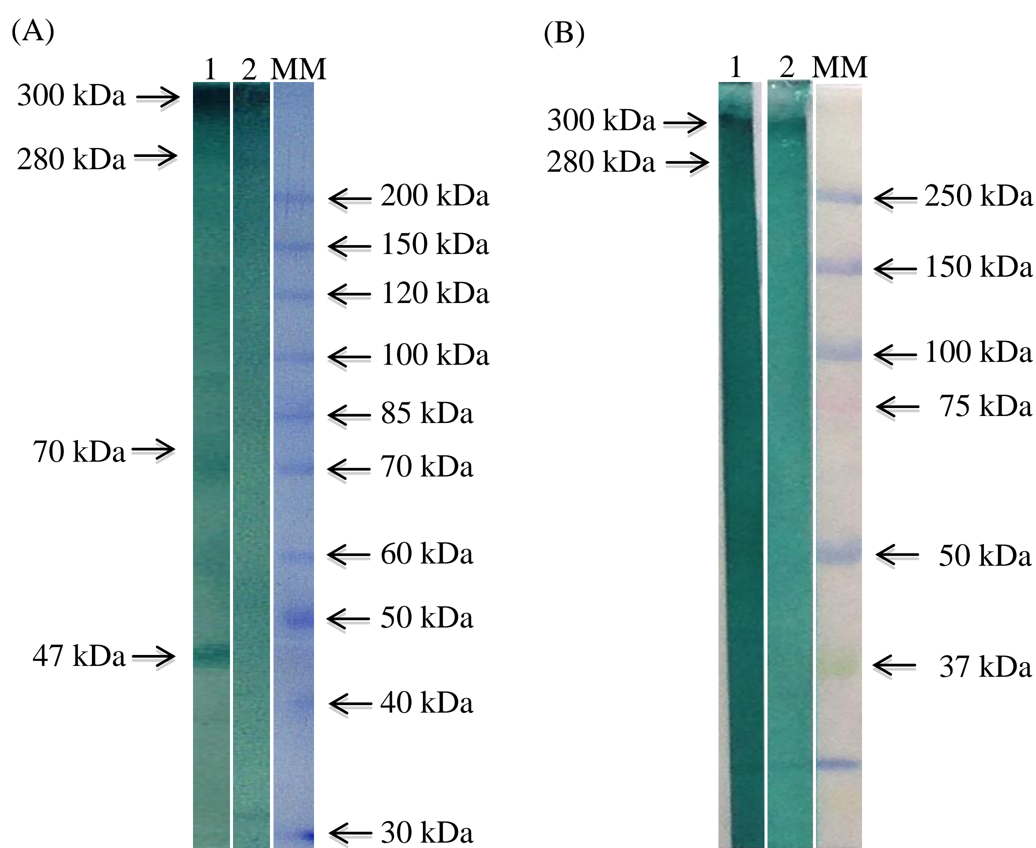

Figure 6. Immunoblotting of CFA of *C. parapsilosis* with policlonal antibodies

Results of immuniblotting to CFA of *C. parapsilosis* (SDS-PAGE gel 7,5%) with polyclonal antibodies anti-CFA (1) and anti-E-CFA (2). MM= molecular mass standard. (A) CFA total (B) CFA purified in Amicon 100k.

Table 1 – Hemolysis inhibition tests

Hemolysis inhibition tests were performed using anti-CFA and anti-E-CFA. The antigen (CFA) without antibodies was considered to be 100% hemolysis. (*p<0.05).

	Sample			
	CFA	CFA + anti-CFA	CFA + anti-E-CFA	PBS
O.D. average	0,166	0,098	0,083	0,037
standard deviation	0,005	0,001	0,019	0,002
Hemolysis percentage	100%	59%	50%	0%

Hemolysis inhibition with anti-CFA and anti-E-CFA serum (1:50), previously incubated with CFA os *C. parapsilosis* for 1h at 37°C and after addition of mouse erythrocyte (1%), followed by incubation.

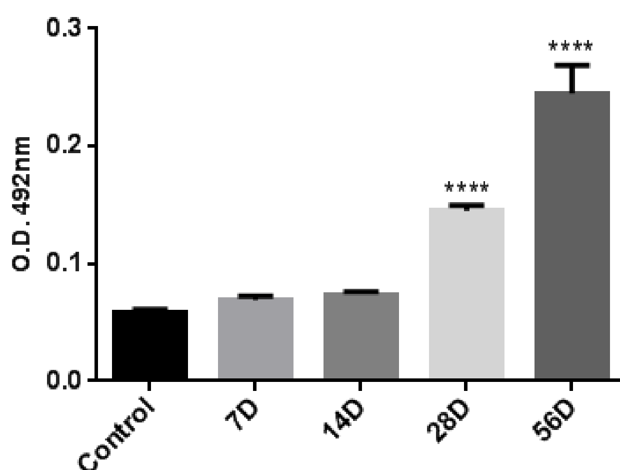

Figure 7. Analysis of CFA purified in amicon 100k with antibodies of mice infections by ELISA

Serum samples of infected mice with *C. parapsilosis* (7, 14, 28, 56 days post infection and control no infection) were tested by ELISA using CFA isolated by amicon 100k (*p<0.05). O.D. optical density.

CONCLUSÕES

A detecção do fungo apenas no período inicial de 7 e 14 dias pós infecção sugere que o isolado *C. parapsilosis* apresenta baixa virulência.

No decorrer de infecção de camundongos por isolado *C. parapsilosis* ocorre aumento na resposta imune celular e humoral, ocorrendo no mesmo período diminuição e negativação de fungemia, o que poderia sugerir a importância da imunidade adaptativa na proteção.

No decorrer de infecção de camundongos por isolado *C. parapsilosis* ocorre modulação nos níveis de citocinas local e sistemico, possivelmente com desvio para Th1 e modulação de atividade (produção) de IDO.

O isolado (663581) *C. parapsilosis* apresenta a capacidade de induzir alteração comportamental no período tardio de infecção em camundongos.

A alteração comportamental no período tardio de infecção possivelmente seja devido à diminuição das células Purkinje no cerebelo.

A alteração comportamental no período tardio de infecção, na ausência de fungos viáveis, sugere que a modulação de citocinas e resposta imune induzidas tenham algum papel na patogenese, o que requer estudos adicionais.

C. parapsilosis libera fator solúvel com capacidade de lisar hemácias de camundongos, todavia a sua produção in vitro é baixa, o que poderia explicar os dados da literatura como a espécie que apresenta atividade baixa ou nenhuma atividade hemolítica.

O(s) fator(es) hemolítico(s) solúvel (eis) de *C. parapsilosis* é (são) termoestável (eis.), possivelmente é (são) glicoproteína(s) contendo manose e constituída de dois componentes com MM ~ 280 kDa e 300kDa.

Os anticorpos policlonais anti-E-CFA reconhecem componentes de alta MM e os anticorpos anti-CFA total além de reconhecerem os mesmos de anti-E-CFA reconhecem também componentes menores, sugerindo maior especificidade dos anticorpos anti-E-CFA.

No decorrer de infecção de camundongos por isolado *C. parapsilosis* ocorre aumento no nível sérico de IgG à fração contendo o(s) fator(es) hemolítico(s). Como no mesmo período ocorre negativação de fungemia e como os anticorpos policlonais anti-E-CFA apresentam a capacidade de inibir parcialmente a atividade hemolítica *in vitro*, esses anticorpos poderiam estar envolvidos na proteção, o que requer estudos adicionais.