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Expressão Tecidual e Reconhecimento Imune da Paramiosina do Carrapato *Rhipicephalus* (*Boophilus*) *microplus*

PORTO ALEGRE

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Dissertação apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular da Pontifícia Universidade Católica do Rio Grande do Sul como requisito para a obtenção do título de Mestre em Biologia Celular e Molecular.

Orientador: Prof. Dr. Carlos Alexandre Sanchez Ferreira

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Aprovado em_____de_____de_____

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RESUMO

O *Rhipicephalus microplus* é um carrapato que parasita bovinos, prejudicando a produção de leite e carne, além de ser um vetor para agentes de diversas doenças. O *R. microplus* causa sérios prejuízos em países que dependem da pecuária, e os métodos de controle utilizados são baseados no uso de acaricidas, os quais apresentam alto custo e contaminam o leite e a carne, além de causarem impactos ao meio ambiente. Desta forma, novos métodos de controle, como o desenvolvimento de vacinas, têm sido sugeridos. Antígenos protetores ao gado têm sido divididos em dois grupos: expostos, que interagem com o sistema imune do hospedeiro, e ocultos, que não interagem com o sistema imune do hospedeiro. A paramiosina (PRM) é uma proteína presente em invertebrados primariamente caracterizada pela sua função muscular, porém em diversos parasitos tem sido localizada em regiões não musculares, sugerindo que a mesma também possa apresentar funções envolvidas com a evasão do sistema imune do hospedeiro. A PRM de *Taenia solium* inibe "in vitro" a via clássica do sistema complemento, por meio do bloqueio da função de C1, e a de *R*. *microplus* liga a porção Fc de imunoglobulinas. Este estudo teve por objetivo avaliar o reconhecimento da paramiosina de *R. microplus* (RmPRM) por bovinos infectados com o carrapato e determinar os níveis de expressão do gene da paramiosina em tecidos e diferentes estágios de desenvolvimento do carrapato, tendo sido também avaliada a atividade anti-complemento da proteína recombinante. Os resultados mostraram que o soro de bovinos *Bos indicus* e *B*. *taurus* naturalmente infectados foram capazes de reconhecer paramiosina recombinante. Os níveis de anticorpos encontrados entre os bovinos apresentaram variações consideráveis, porém com os maiores títulos predominando entre indivíduos *B. indicus*. A transcrição do gene da RmPRM foi constatada na maioria dos tecidos, órgãos, ovos e larvas testados, com maior nível de expressão encontrado no corpo gorduroso, um órgão sem predominância de tecido muscular. Além disso, este trabalho mostra que a RmPRM é capaz de inibir o sistema complemento, indicando que esta proteína deve ser um importante componente para a sobrevivência do parasito, possivelmente envolvida na modulação do sistema imune do hospedeiro. Corroborando com outros trabalhos em parasitos, estes resultados sugerem que a RmPRM desempenha outras funções além das classicamente descritas na musculatura.

Palavras-chave: *Rhipicephalus* (*Boophilus*) *microplus*, paramiosina, evasão do sistema imune.

ABSTRAT

Rhipicephalus microplus is a tick that parasite bovines, damaging the milk and meat production, and constituting a vector for agents of various diseases. *R*. *microplus* cause serious losses in countries that depend on cattle production, and the control methods used are based on the use of acaricides, which present high cost and contaminate milk and meat, generating impacts on the environment. Therefore, new methods of control, as vaccines, have been suggested. Anti-parasite protective antigens can be divided into two groups: exposed, which interact with host immune system, and concealed, that do not interact with host immune system. Paramyosin (PRM) is a protein present in invertebrates primarily characterized by muscle function, but in various parasites it has been localized in non-muscle regions, suggesting that it may also perform functions involved in the evasion of the host immune system. The *Taenia solium* paramyosin inhibits “in vitro” the classical pathway of complement system by binding to C1, and *R*. *microplus* paramyosin (RmPRM) has been shown to bind immunoglobulins. This study aimed to evaluate the recognition of RmPRM by the sera of animals infected with *R. microplus* and determine the levels of paramyosin gene expression in tissues and different developmental stages of the tick, as well as to analyze the anti-complement activity of the recombinant protein. The results showed that sera from naturally infected *B*os *indicus* and *B*. *taurus* were able to recognize the recombinant PRM. Antibodies levels found among the bovines have considerable variation, but with higher titers predominating among *B*. *indicus* individuals. The RmPRM gene transcription was detected in most tissues, organs, eggs and larvae tested, with higher levels of expression found in the fat body, an organ without muscle tissue prominence. Furthermore, this study shows that RmPRM is able to inhibit the complement system, indicating that that this protein should be an important component for the parasite survival, possibly involved in modulation of the host immune system. Corroborating other studies in parasites, these results suggest that RmPRM performs other functions than those classically described in the musculature.

Key words: *Rhipicephalus* (*Boophilus*) *microplus*, paramyosin, evasion of the host immune system.

LISTA DE ABREVIAÇÕES

CDs – Células dendríticas

LPS - Lipopolissacarídeos

TLR – Receptor semelhantes a Toll

IL-12 – Interleucina-12

TNF-α - Fator de necrose tumoral-α

IL-10 – Interleucina-10

IGBPs – Proteínas que se ligam a imunoglobulinas

IgG – Imunoglobulina G

IgE – Imunoglobulina E

C1 – Componente 1 do sistema complemento

C1q – porção do componente 1 do sistema complemento

IFN-γ – Interferon-γ

IL-2 – Interleucina-2

Th1 – Linfócitos T auxiliares 1

Th2 – Linfócitos T auxiliares 2

qRT-PCR – Reação em Cadeia pela polimerase quantitativo em tempo real

PCR - Reação em Cadeia pela Polimerase

ELISA – "Enzyme-linked immunosorbent assay"

cDNA – DNA complementar

IPTG – isopropil-beta-D-tiogalactopiranosídeo

DTT - Ditiotreitol

PBS – Salina tamponada com fosfato

$CoCl_2$ – Cloreto de cobalto

H_2O_2 – Perssulfato de hidrogênio

H_2SO_4 - Ácido sulfúrico

BSA – Albumina do soro bovino

EDTA - Ácido etilenodiamino tetra-acético

SUMÁRIO

1 INTRODUÇÃO

1.1 *Rhipicephalus microplus*

Rhipicephalus é um gênero que inclui espécies de carrapatos que infectam o gado nas regiões tropicais e subtropicais do planeta. O *Rhipicephalus microplus* é um ectoparasito que causa grandes prejuízos econômicos, apresentando impactos na criação de gado e na produção de leite, afetando assim países em desenvolvimento na África, Ásia e América Latina que dependem da pecuária (Delgado et al., 1999). Este carrapato é um vetor para diversos patógenos que causam doenças como babesiose e anaplasmose (Peter et al., 2005; Willadsen, 2006).

O *R. microplus*, originário do sudoeste da Ásia, foi introduzido nos trópicos chegando na Austrália, Sul e Leste da África e América do Sul e Central, tornando-se uma das espécies de carrapato mais estudadas (Jongejan & Uilenberg, 2004).

1.1.1 Métodos de controle do *R. microplus*

Os métodos convencionais de controle são baseados no uso de acaricidas, porém os seus resíduos podem causar sérios impactos no meio ambiente e contaminar o leite e a carne (Willadsen, 2004; de la Fuente et al., 2007). Além disso, a utilização dos acaricidas apresenta um alto custo e o seu uso intensivo tem causado a seleção de populações resistentes (Davey & George, 1998), o que indica a necessidade do desenvolvimento de novos métodos de controle. A produção de uma vacina é considerada um dos métodos mais promissores, e para isso, a identificação e caracterização de uma ampla gama de antígenos protetores ao *R. microplus* é necessária (de la Fuente & Kocan, 2006).

A saliva dos artrópodes hematófagos, entre estes os carrapatos, tem sido relatada como capaz de inibir respostas associadas com a homeostase do sistema fisiológico do

hospedeiro, podendo apresentar moléculas que impedem a agregação de plaquetas e a coagulação. Moléculas salivares também são conhecidas como moduladoras do sistema imunológico do hospedeiro, sendo essenciais, portanto, para a sobrevivência do parasito (Ribeiro, 1989). A inibição da agregação plaquetária foi demonstrada em bovinos infectados com *R. microplus*, apresentando máxima inibição 21 dias após a infecção, coincidindo com a fase de alimentação rápida dos carrapatos. O estudo suporta que as moléculas salivares do carrapato em contato com o sangue do hospedeiro foram responsáveis por inibir a agregação plaquetária (Reck Jr. et al., 2009). A coagulação sanguínea mostrou-se diminuída em bovinos infectados com o carrapato, porém a sua inibição foi neutralizada após infecções repetidas (Reck et al., 2009).

Oliveira et al. (2010) demonstrou que a saliva de *Rhipicephalus sanguineus* pode prejudicar a maturação de células dendríticas (CDs) quando estimuladas com LPS, através do aumento da expressão de “toll-like receptor” (TLR), em camundongos. O aumento da expressão do TLR-2 em CDs suprime a MAP-kinase p38, levando ao aumento da produção de Interleucina-10 e a redução da produção de IL-12 e TNF-α. Este pode ser um mecanismo utilizado pelo carrapato como uma estratégia de sobrevivência para evadir a resposta inflamatória do hospedeiro.

De acordo com Nuttall et al. (2006), os antígenos potencias estudados podem ser classificados como: antígenos “expostos” (moléculas secretadas na saliva do carrapato) e antígenos “ocultos” (moléculas não secretadas na saliva). Antígenos “expostos” também podem ser alternativamente considerados como aqueles que interagem com o sistema imunológico do hospedeiro durante a alimentação do parasito (Mulenga et al., 2000), e geralmente são proteínas sintetizadas nas glândulas salivares e presente na saliva (Nuttall et al., 2006). Os antígenos “ocultos” não estimulam uma resposta imunológica durante a infestação parasitária natural. Isto acontece provavelmente devido a sua localização física, já que, os antígenos ocultos estão associados a tecidos ou secreções do parasito que não são normalmente expostos ao sistema imune do hospedeiro (Opdebeeck et al., 1987,

Willadsen et al., 1993). Porém, caso o hospedeiro seja primeiramente vacinado com um antígeno “oculto” e, portanto, for induzida uma resposta imunológica contra este componente do parasito, a posterior exposição ao carrapato poderá resultar em menores níveis de infecção (Willadsen et al., 1993).

Quando o carrapato se alimenta, secreta antígenos expostos, os quais serão reconhecidos pelo sistema imune do bovino. Estas moléculas secretadas na saliva supostamente neutralizam os mecanismos homeostáticos, inflamatórios e imunológicos, os quais poderiam evitar que o carrapato se fixasse no bovino e se alimentasse de sangue (Ribeiro et al., 1987). A imunização de bovinos com antígenos expostos parece produzir uma resposta imunológica adquirida capaz de reduzir o número de carrapatos, mas não de evitar totalmente uma infecção mesmo depois de repetidas exposições. Portanto, uma resposta imunológica induzida por um único antígeno “exposto” não deve ser suficiente para a proteção efetiva (Nuttall et al., 2006).

Entre os sistemas de evasão já descritos, estudos mostraram que o carrapato possui um mecanismo para eliminar imunoglobulinas do hospedeiro via saliva (Wang & Nuttal, 1994). Proteínas que se ligam a imunoglobulinas (IGBPs) foram descobertas em carrapatos da família Ixodidae na hemolinfa e nas glândulas salivares (Wang & Nuttal, 1995), o que forneceu uma possível explicação do modo pelo qual o carrapato excreta anticorpos do hospedeiro via saliva durante a alimentação (Wang & Nuttal, 1994). Essa família de proteínas é produzida em abundância durante os estágios de alimentação e são armazenadas em grânulos secretores localizados na glândula salivar do carrapato (Wang et al., 1998). As imunoglobulinas do hospedeiro passam pela parede do intestino do carrapato até a hemolinfa (da Silva Vaz Jr et al., 1996), onde as IGBPs devem reconhecê-las e ligarem-se a elas. As IGBPs da hemolinfa transportam as imunoglobulinas G (IgG) do hospedeiro para a glândula salivar, as IGBPs da glândula se ligam ao IgG, e os anticorpo do hospedeiro são excretados na saliva. Este mecanismo parece proteger o carrapato dos efeitos prejudiciais das imunoglobulinas dos hospedeiros imunes (Wang & Nuttall, 1994).

Experimentos de vacinação com antígenos “ocultos” têm sido relatados, como, por exemplo, utilizando os antígenos Bm86 (Willadsen et al., 1989), Bm91 (Riding et al., 1994), BMA7 (McKenna et al., 1998) e Bm95 (García-García et al., 2000), BYC (Leal et al., 2006), VTDCE (Seixas et al., 2008) and GST (Parizi et al., 2011). O Bm86 foi identificado como uma glicoproteína intestinal protetora de bovinos infectados com o *R. microplus,* e uma vacina utilizando o antígeno foi introduzida no mercado em 1994 (TickGARD^{TM}) (Willadsen et al., 1995). As respostas imunológicas conferidas pelos antígenos Bm91 e BMA7 foram menos protetoras quando comparadas com a resposta ao Bm86. Uma nova vacina combinando o Bm86 ao Bm91 foi desenvolvida (TickGARD plus^{TM}), mostrando uma resposta imunológica mais eficiente quando comparada com a anterior, que utiliza somente o Bm86 (Willadsen et al., 1996). A próxima vacina a ser desenvolvida e comercializada foi a Gavac^{TM}, a qual utilizou o Bm86 expresso em *Pichia pastoris* e não em *Escherichia coli* como nas outras. A vacina reduziu em 60% os tratamentos com acaricidas (de la Fuente et al., 1999). Já uma linhagem de carrapatos da espécie *R. microplus* na Argentina apresentaram dois alelos (BmA1 e BmA2) para um gene homólogo ao Bm86, denominado Bm95. Os alelos possuem, respectivamente, 21 e 18 aminoácidos diferentes da proteína Bm86 originalmente descrita. O Bm95 extraído de larvas desta linhagem de *R. microplus* mostrou proteger o hospedeiro dos carrapatos, que apresentavam baixa suscetibilidade à vacina contendo o Bm86. A eficiência do Bm95 foi similar a apresentada pela Gavac^{TM} (García-García et al., 2000). Em outros organismos, também tem sido descrita a utilização de antígenos “ocultos” para o desenvolvimento de vacinas, incluindo o PM44, um antígeno intestinal da larva de *Lucilia cuprina,* que mostrou proteger as ovelhas contra o parasito (Willadsen et al., 1993). Além disso, antígenos “ocultos” de outros parasitos como *Chrysomya bezziana* (Willadsen, 1999)*, Ctenocephalides felis* (Willadsen & Billingsley, 1996) *e Pediculus humanus* (Willadsen & Billingsley, 1996) foram relatados.

Recentemente, um terceiro grupo de antígenos que combina as propriedades dos antígenos “expostos” e “ocultos” foi caracterizado. Como exemplo, pode ser citada a

proteína 64P, isolada da glândula salivar de *R. appendiculatus*, a qual foi utilizada em uma vacina de dupla ação, destinada aos antígenos “expostos” presentes na saliva do carrapato mas que reagiram adicionalmente de forma cruzada contra epitopos antigênicos “ocultos” no intestino do mesmo (Trimnell et al., 2002). A vacina consiste na indução da resposta humoral e de hipersensibilidade tardia, afetando tanto o local de alimentação do carrapato, quanto a integridade do seu intestino médio, causando a morte do parasito (Nuttall et al., 2006). A vacinação com a proteína 64P recombinante induziu reatividade cruzada com diferentes espécies de carrapatos, mostrando ser uma vacina de amplo-espectro (Trimnell et al., 2005).

A maioria, se não todos, os antígenos usados como vacina contra ectoparasitas conferem somente uma proteção parcial ao hospedeiro, e mostram a necessidade de integrar outras estratégias de controle (de la Fuente et al., 2007). O desenvolvimento de vacinas combinando dois ou mais antígenos tem se mostrado mais eficaz para a proteção do mesmo (Willadsen, 2006).

1.1.2 Resistência naturalmente induzida por infecções de carrapatos.

Infecções com *R. microplus* apresentam efeitos fisiológicos adversos no hospedeiro, resultando na diminuição do peso e anemia. Os bovinos exibem fenótipos divergentes de resistência e suscetibilidade a infecção por *R. microplus*. Estas divergências apresentam base hereditária e foram constatadas entre as diferentes raças de bovinos (Piper et al., 2009). Por outro lado, ao longo da sucessão das infecções os bovinos tendem a apresentar níveis gradativamente menores de parasitos que completam o ciclo parasitário. Esta resistência ao carrapato pode ser afetada por muitos fatores incluindo idade, sexo, lactação, estresse e estação do ano (de Castro & Newson, 1993) e pode ser evidenciada por uma diminuição da produção dos ovos, assim como a sua viabilidade, a redução de fêmeas

ingurgitadas, a inibição da muda e o aumento da mortalidade dos carrapatos (Rechav et al., 1991).

Estudo realizado por Piper et al. (2010) mostra que a resistência às infestações de carrapatos deve estar ligada a regulação diferencial da expressão de vários genes no tecido cutâneo de bovinos infectados. Seus resultados sugerem a diminuição da atividade celular na pele de animais resistentes no local da fixação da larva, mostrando diferenças na expressão de alguns genes e nas secções histológicas da pele de indivíduos das raças Holstein–Friesian (*Bos taurus*) e Brahman (*Bos indicus*) durante a fixação. A expressão aumentada de alguns genes de Holstein–Friesian infectados com carrapatos parecem ser referentes à anafilatoxina derivada da quebra do componente 3 da cascata do complemento (C3a), que recruta e ativa fagócitos, e a determinadas imunoglobulinas, indicando uma maior atividade celular no local.

Em relação à variabilidade relatada no nível de resistência dos bovinos a infestações de carrapatos, Kashino et al. (2005) mostrou que após infestações pesadas com carrapatos da espécie *R. microplus* em bovinos, houve diminuição dos níveis de anticorpos anti-saliva de isotipos IgG1 elicitados em bovinos suscetíveis, mas não em bovinos resistentes. Já os níveis de anticorpos IgG2 foram similares entre os grupos experimentais, mas apresentaram níveis significativamente maiores do que em hospedeiros não infectados. Piper et al. (2009), por outro lado, observou maiores níveis de anticorpos IgG1 específicos para diferentes extratos do carrapato em bovinos Holstein-Friesian do que em Brahman, e todos os animais apresentam níveis moderadamente altos ou não houve significativo aumento de IgG2 específicos. Já Cruz et al. (2008), comparando infestações leves e pesadas, mostrou que durante as infestações pesadas os animais apresentaram níveis maiores de IgG contra extrato de glândula salivar, de intestino e de extrato de larva de *R. microplus*, além de variação nos antígenos majoritariamente reconhecidos, indicando que o reconhecimento de antígenos e a variação dos níveis de IgG pode ser grandemente influenciado pelos níveis de infecção por carrapatos.

1.2 Paramiosina

A paramiosina (PRM) é uma proteína encontrada no tecido muscular de invertebrados, a qual é constituída em sua maior parte por α-hélice, apresenta um massa molecular de aproximadamente 97 kDa e apresenta a forma geral de bastão. Esta proteína é formada adicionalmente por duas regiões com estruturas secundárias distintas: N-terminal e C-terminal.

A PRM foi primeiramente isolada de filamentos grandes em músculo não estriado de moluscos (Cohen et al., 1970), está localizada no centro dos filamentos e determina o comprimento e a estabilidade dos mesmos (Mackenzie & Epstein, 1980). A paramiosina aparece também como uma proteína importante na condição fisiológica conhecida com "catch mechanism" em moluscos (Watabe & Hartshorne, 1990), na qual os músculos "catch" podem permanecer contraídos por longos períodos com baixo gasto de energia (Johnson & Kahn, 1959).

A PRM tem sido isolada em diversos invertebrados, incluindo vários parasitos como *Schistosoma japonicum (*Zhou et al., 2000*), S. mansoni* (Berriman et al., 2009), *Taenia solium* (Laclette et al., 1991), *T. saginata* (Ferrer et al., 2003), *Trichinella spiralis* (Yang et al., 2008), *Echinococcus granulosus* (Muhlschlegel et al., 1993), *Sarcoptes scabiei* (Mattsson et al., 2001), *Brugia malayi* (Langy et al., 1998), *Dirofilaria immitis* (Limberger & McReynolds, 1990)*, Onchocerca volvulus* (Limberger & McReynolds, 1990), entre outros. Esta proteína pode ser considerada multifuncional, pois além de seu papel estrutural, ela tem sido incluída em outras funções distintas envolvidas na modulação do sistema imunológico de hospedeiros durante a infecção parasitária (Landa et al., 1993).

A paramiosina de *T. solium* parece ser capaz de inibir, "in vitro", a via clássica do sistema complemento, através do bloqueio da função de C1, por meio da sua ligação ao colágeno (Laclette et al., 1991; Laclette et al., 1992), podendo agir como moduladora da resposta imune do hospedeiro. Foi demonstrado também que as paramiosinas de *S.*

mansoni (Loukas et al., 2001), *T. crassiceps* (Kalianna & McManus, 1993), além de *R. microplus* (Ferreira et al., 2002b) são capazes de ligar-se a porção Fc de imunoglobulinas, mostrando mais uma vez o seu envolvimento na supressão do sistema imune dos hospedeiros.

Tem sido demonstrado por alguns autores a existência de isoformas não musculares da paramiosina, encontradas no tegumento e ou na superfície de vermes como o *S. mansoni* (Matsumoto et al., 1988), *S. japonicum* (Gobert et al., 1998), *T. solium* (Laclette et al., 1995), na superfície da lamela do intestino e no tegumento da *Fasciola hepatica* (Cancela et al., 2004). Em *Paragonimus westermani,* a proteína foi encontrada em todos os estágios de desenvolvimento e foi localizada no intestino e no ovário (Zhao et al., 2006), e em *R. microplus,* a mesma foi encontrada em regiões não-musculares como intestino e glândula salivar (Ferreira et al., 2002b). A paramiosina expressa na superfície dos organismos tem sido relacionada à estratégia de evasão do sistema imune (Jiz et al., 2008), por isso, esta proteína aparece como forte candidata a antígeno na vacina contra diferentes parasitoses como, por exemplo, a esquistossomose. Seu envolvimento com a produção de imunidade protetora, descrita pela primeira vez por Pearce et al. (1988), vem sendo confirmada por diversos autores. Por exemplo, camundongos imunizados, tanto com a forma nativa quanto com a forma recombinante da proteína, foram significativamente protegidos contra a infecção por metacercária de *S. japonicum* (Ramirez et al., 1996). Além disso, a paramiosina mostrou estimular linfócitos T de camundongos vacinados com o antígeno, para produção de citocinas como IFN-γ, que podem ativar os macrófagos para atacar a schistossomula (Lanar et al., 1986). Zhou et al. (1999) mostrou que camundongos imunizados com a paramiosina recombinante de *S. japonicum* tiveram alta produção de citocinas como IFN-γ e IL-2, indicando a presença de resposta imune do tipo Th1. Já camundongos imunizados com a paramiosina recombinante de *P. westermani,* produziram altos níveis de respostas IgG contra a proteína. O IgG1 foi o anticorpo proeminente, sugerindo a indução da resposta imunológica Th2 (Zhao et al., 2006). A paramiosina tem

sido considerada uma potencial candidata a antígeno vacinal não só contra a esquistossomose, mas também contra infecções como filarioses (Nanduri & Kazura, 1989; Li et al., 1993) e cisticercoses (Vázquez-Talavera et al., 2001).

Além disso, tem sido descrita a presença de anticorpos que reconhecem a paramiosina dos parasitos no soro dos hospedeiros infectados, como por exemplo, a paramiosina recombinante de *S. scabiei,* que foi reconhecida pelo soro de cães e suínos que apresentavam o parasito (Mattsson et al., 2001). O soro de pacientes infectados com *S. japonicum* (Nara et al., 1997)*, T. saginata* (Ferrer et al., 2003) *e T. solium* (Vazquez-Talavara et al., 2001) revelou o reconhecimento da região carboxi-terminal da paramiosina. Já no caso de pacientes infectados com *Dirofilaria immitis*, o soro reconheceu principalmente a porção N-terminal da proteína (Steel, 1990). Identificando-se a porção da proteína mais importante para ser alvo de interferência por anticorpos, pode-se usar como imunógeno apenas este fragmento.

A paramiosina está presente em vários órgãos e tecidos do *R. microplus,* como glândula salivar, intestino e corpo gorduroso, porém, não foi localizada na saliva (Ferreira et al., 2002b). A princípio, a ausência da paramiosina na saliva sugere que a mesma não é secretada para o hospedeiro durante a alimentação do carrapato. Alternativamente, porém não de forma excludente, este fato pode refletir a importância da RmPRM nas funções relacionadas a evasão do sistema imune do hospedeiro, sendo possivelmente considerada um novo antígeno “oculto”.

2 OBJETIVOS

2.1 Objetivo Geral:

Caracterizar os sítios de síntese da paramiosina, determinar seu possível reconhecimento imune por animais infectados e avaliar possíveis atividades imunomoduladoras.

2.2 Objetivos específicos:

2.2.1 Clonar e expressar a RmPRM em vetores procarióticos.

2.2.2 Purificar a paramiosina recombinante do *R. microplus*.

2.2.3 Detectar e quantificar o nível de expressão do gene codificante para a paramiosina de *R. microplus* em órgãos e tecidos utilizando a técnica de qRT-PCR.

2.2.4 Avaliar a modulação da RmPRM sobre a via clássica do sistema complemento.

2.2.5 Analisar o grau de reconhecimento da paramiosina por soros de bovinos infectados por western-blot e ELISA.

3 ARTIGO

Tissue expression and host immunological recognition of a *R. microplus* paramyosin

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SUMMARY

Rhipicephalus microplus is a parasite that causes economic losses in cattle herds, and control methods using acaricides present a high cost and contaminate the environment. The paramyosin, a muscular protein, has been found in non-muscle tissues and characterized as presenting activities that enable the evasion of the host immune system in various parasites. This report investigated the recognition level of paramyosin by sera of infected bovines, its expression in tissues, organs and different life stages of the tick, and verified its capacity to inhibit the complement cascade. Paramyosin and salivary gland extract were recognized by both infected *B. taurus* and *B. indicus* sera by ELISA. Paramyosin gene expression was evaluated in eggs and larvae at different tick developmental stages, adult male, and several tissues of partially and fully engorged females by qRT-PCR, showing the higher expression level in fat body. In addition, recombinant paramyosin inhibited the complement system in a dose dependent manner in an erythrocyte lyses assay. These results show that *R. microplus* paramyosin is able to perform immunomodulatory roles and is immunologically recognized during the tick infection. In addition, its presence in organs that do not present a highly developed musculature, specially the high transcription rate in adult fat body, suggests that paramyosin may possess additional, non-muscle functions in the tick-bovine relationship.

Key words: *Rhipicephalus* (*Boophilus*) *microplus*, paramyosin, bovine resistance.

INTRODUCTION

Rhipicephalus microplus is an ectoparasite that causes important economic losses in cattle herds (Jonsson, 2006; Willadsen, 2006). Conventional methods of control are based on the use of acaricides, however, their residues can cause serious impacts in the environment and contaminate meat and milk (Willadsen, 2004; de la Fuente *et al*. 2007). Moreover, acaricides present a high cost and their intensive use has caused the selection of resistant populations (Davey and George, 1998). The production of a vaccine is considered one of the most promising alternative methods for tick control, which demands the identification and characterization of protective antigens.

Vaccination experiments with “concealed” antigens (which are not recognized by the host immune system), such as Bm86 (Willadsen *et al*. 1989), Bm91 (Riding *et al*. 1994), BMA7 (McKenna *et al*. 1998), VTDCE (Seixas *et al*. 2008), BYC (Leal *et al.* 2006), and GST (Parizi *et al*. 2011), have shown to partially protect the host. When antigens were combined, this protection was increased (Willadsen *et al*. 1996; McKenna *et al*. 1998), which indicates that antigen combinations are potentially more effective to elicit a protective immune responses against tick infections. Exposed antigens (recognized by the host immune system) have also been investigated for host protection against ticks (Wang *et al*. 1998; Trimnel *et al*. 2002; Bishop *et al*. 2002).

Paramyosin (PRM) is a muscle protein found in invertebrates that presents in the majority of its extension a coil-coiled form, and, beyond its structural function, it has been implicated in the modulation of the host’s immune system during different parasitic infections (Landa *et al*. 1993; McManus *et al*. 1998; Zhao *et al*. 2006). The PRM of parasites have been shown to inhibit “in vitro” the classical pathway of complement system (Laclette *et al*. 1992), and bind IgG (Ferreira *et al*. 2002*b*; Strube *et al*. 2009; Loukas *et al*. 2001). Corroborating the importance of the described activities in parasite-host relationships, PRM has been suggested as a candidate antigen to compose vaccines against diseases such as

schistosomiasis (Pearce *et al*. 1988, Lanar *et al*. 1986, Zhou *et al*. 1999), filariasis (Nanduri and Kazura, 1989; Li *et al*. 1993) and cysticercosis (Vázquez-Talavera *et al*. 2001).

In this work, it was evaluated the recognition of paramyosin by the sera of infected bovines and it was measured the levels of the PRM gene expression in different *R. microplus* tissues and developmental stages. Additionally, it was tested the anti-complement activity of a prokaryotic recombinant PRM.

MATERIALS AND METHODS

RmPRM cDNA cloning

Two recombinant forms of *R. microplus* paramyosin were used. The cDNA coding sequence of *R. microplus* PRM was previously cloned into pGEX-4T3 vector (GE-Life Sciences) as described by Ferreira *et al.* (2002*b*), generating a recombinant form named rBmPRM. In order to increase the recombinant protein production, to diminish the number of carboxyl-terminal truncated forms and avoid thrombin contamination, that could not be easily cleared from the rBmPRM preparations, a second construct was designed. The cDNA coding sequence of *R. microplus* PRM was amplified by PCR using *Pfu* DNA polymerase (Fermentas) and the primers 5'-CACCATGTCTAGCAGGAGCAGCAAG-3' and 5'-AAAAAGAATTCTTAGTGGTGGTGGTGGTGGTGGAAGTTCTGGCTGGACTCCTCG-3', with the insertion of a sequence coding for six histidines (underlined) in the carboxyl-end to enable nickel-affinity chromatography purification of the recombinant protein. Cloning was performed with Champion^{TM} Pet Directional TOPO Expression Kit (Invitrogen) and the integrity of the construction was confirmed by sequencing. The second recombinant PRM was named rRmPRM-His.

Protein expression and purification

rRmPRM-His was expressed in the BL21 *Escherichia coli* strain. Protein expression was obtained from growth induced with IPTG 0.5 mM in Luria-Bertani at 37ºC for 3h. Culture

cells were disrupted in a French press and centrifuged for 20 min at 18000 X *g*. Supernatant and pellet were separately collected and purified. Protein in the insoluble fraction was resuspended in 10 mM dithiothreitol (DTT), 8 M Urea, 10 mM Tris-Cl, pH 8.0 and incubated for 1h with stirring, following protocol by Jiz *et al*. (2008). Samples were filtered and purified using a column containing nickel (HisTrap HP - GE Healthcare) according to manufacturer's instructions. rBmPRM expression and purification was followed according to Ferreira *et al*. (2002). Efficiency of purification was monitored by SDS-PAGE 10% stained with coomassie brilliant blue G-250.

Antigen preparation

Antigens were obtained according to da Silva Vaz *et al*. (1994). Briefly, partially engorged females from the Porto Alegre strain were dissected in PBS and salivary glands were separated from other organs and frozen at -70 ºC. Salivary glands were macerated with a solution containing Tris/HCl 10 mM pH 8.2, 1 % deoxicolato, leupeptina (8 mg/ml), pepstatina A (1 mg/ml) and TPCK (0.1 mM) and centrifuged at 32,000 x g for 40 min at 4 ºC. The supernatant was then collect and stored at - 70 ºC.

Western-blot

rBmPRM was submitted to SDS-PAGE 10 % (58 µg/cm) and transferred to nitrocellulose membrane at 70 V for 1h at 4 ºC (Dunn, 1986). Nitrocellulose strips of 4 mm were blocked for 1 h at room temperature with blotto 5 % (cow non-fat dry milk 5 %-PBS). Prior to the overnight incubation at 4 ºC with the antigens, all sera were diluted in an *E. coli* BL21 strain lysate expressing the pGEX-4T3 vector and incubated for 2 h at room temperature for absorption of anti-*E. coli* and anti-vector derived protein antibodies. Preparation of the *E. coli* BL21 strain lysate was performed according to Rott *et al*. (2000). After 3 washes with blotto 5 %, the strips were incubated for 1 h with anti-bovine IgG peroxidase conjugate (Sigma), diluted 1:6000 in blotto 5 %. The strips were then washed

three times with PBS, and the development buffer (5 mg 3,3-diaminobenzidine in 30 ml PBS plus 150 µl H_2O_2 30 % and 100 µl $CoCl_2$ 1 %) was added.

Sera of Infected Bovines

Sera from six *Bos taurus* (Hereford) and eight *Bos indicus* (Nelore) bovines of a farm from Pelotas (Brazil), within a region naturally infected with *R. microplus*, as well as the sera from uninfected *B. indicus* animals (negative controls) were kindly provided by the Departamento de Veterinária Preventiva, from the Universidade Federal de Pelotas, (Brazil). *B. taurus* negative controls (non-infected bovines) were the same described previously (Cruz *et al*. 2008).

ELISA

The plate was incubated overnight at 4 ºC with 3 µg of antigen diluted in 50 mM carbonate/bicarbonate buffer pH 9.6 The plate was washed for three times with blotto 5 %, blocked for 1 h with blotto 5 % at 37 º C, and then incubated with bovine sera diluted in blotto 5 % for 1h at 37 º C. The plate was washed with blotto 5 % and incubated with anti-bovine IgG peroxidase conjugated, and after, wash with PBS for three times. Then, 3.4 mg o-phenylenediamine, 5 µl H_2O_2 in 0.1 M citrate-phosphate buffer, ph 5.5 were added in the wells and incubated for 20 min in a dark room. Reaction was stopped with 12.5 % H_2SO_4. The Optical densities were determined at 492 nm.

Paramyosin gene expression in different tick tissues and developmental stages by qRT-PCR

Ticks were dissected in PBS and/or macerated and tissue RNA extraction was performed using TRIzol (Invitrogen) according to the manufacturer's instructions. 500 ng RNA were submitted to reverse transcription, which was performed using superscript III (Invitrogen) according to manufacturer’s instructions. qRT-PCR was performed with RNAs from gut, fat body, ovary and salivary gland of partially- and fully-engorged females, 1, 3, 6,

12, 18-day-old eggs, 5, 10, 15-day-old larvae and adult males. The primers 5'-CCAGCGCACGATTGTTGA-3' and 5'-CACTTGAAGGTTGCGGACTTC-3' for RmPRM gene were used in qRT-PCR. The samples were also amplified with primers 5'-GGACGACCGATGGCTACCT-3' and 5'-TGAGTTGATTGGCGCACTTCT-3' for the 40S ribosomal protein, used as control as already described (Pohl *et al*. 2008). The reactions were performed using the Platinum® SYBR® Green qPCR SuperMix kit (Invitrogen), 10 pmol primers and 100 ng cDNA. The qRT-PCR reactions were performed in an Applied Biosystens Step One Plus thermocycler, and the Relative Expression Software Tool (REST) was used for data analyses (Pfaffl *et al*. 2002).

Hemolytic assay

For classical pathway complement activation, sheep erythrocytes were washed in PBS and sensitized with rabbit anti-sheep erythrocyte antibody (1:1000) (Laborclin) at 37 ºC for 20 min in complement buffer (CB; $MgCl_2$ 5mM, $CaCl_2$ 2mM, NaCl 150mM, Tris-HCL 10mM, pH 7,2). Prior to the hemolytic assay, 2 µl, 5 µl and 10 µl rBmPRM-His (2.09 µg/µl) dialyzed in PBS, and 2, 5 and 10 µl bovine serum albumin (BSA) (1 µg/µl in PBS) were incubated for 20 minutes at 37 ºC, minimal amount of guinea pig serum (Laborclin) to generate 100% of hemolysis (usually 1:30). Sensitized erythrocytes and CB were then added in a total volume of 100µl per reaction and incubated for 20 minutes at 37 ºC. Reaction was stopped with the addition of 200µl of ice-cold saline-EDTA (150 mM NaCl, 10mM EDTA). Samples were centrifuged for 1 minute at 7000 x g, supernatants were transferred to a microplate (in duplicates) and the optical densities were measured at 405 nm.

RESULTS

Paramyosin is recognized by sera of infected bovines

ELISA was performed to evaluate the rBmPRM recognition by the sera of infected *B. indicus* and *B. taurus* bovines (Fig. 1–A). The specificity of the antibody recognition was confirmed by western-blot (a representative western-blot from a positive *B. indicus* bovine serum is shown in fig. 1B). Sera from uninfected bovines were used as negative control. Sera from individuals of both *B. indicus* and *B. taurus* showed to recognize rBmPRM. In order to evaluate the overall recognition of tick antigens by the same sera, an ELISA test using salivary glands protein extract as antigen was performed (Fig. 1–C). Accordingly, *B. indicus* and *B. tau*rus sera showed to recognize the salivary extract.

Paramyosin gene expression

Figure 2 shows a qRT-PCR analysis of the expression of the paramyosin gene (*Rmprm*) within eggs, larvae, adult males and adult female organs and tissues. Comparing the results it can be noted a decreasing expression of *Rmprm* in 3, 6 and 12-day-old eggs, reaching a no detectable expression in 15-day-old eggs, contrasting to a high expression on 18-day-old eggs. Comparing to the 18-day-old eggs expression, 5 and 10-day-old larvae showed lower expression levels, with total absence of expression in 15-day-old larva. Gut and salivary gland showed higher *Rmprm* expression in partially-engorged than fully-engorged females; while in ovary a higher expression was detected in fully-engorged females. The highest *Rmprm* expression levels detected in all developmental stages and tick tissues tested were observed in fat body, with minimal difference comparing fully-engorged and partially-engorged females.

Complement Inhibition by *R. microplus* paramyosin

Complement mediated lysis of erythrocytes via the classical pathway of the complement system was inhibited by adding different amounts of rRmPRM-His in a dose

dependent manner (Fig.3). No erythrocytes lyses were observed in samples without complement. BSA addition showed to do not influence complement activity (Fig. 3).

DISCUSSION

A prototypical parasite concealed antigen is considered not being able to generate an adaptative immune response under a natural infection (Wiladsen *et al*. 1993; Nuttall *et al*. 2006). In this regard, parasite muscle proteins are candidates to comprise such definition and, if they are able to modulate the host immune system, they may also turn into candidates to take part in a vaccine cocktail, what have been showed initially against the stable fly *Stomoxys calcitrans* (Schlein and Lewis, 1976). So, *R. microplus* PRM seemed to deserve further attention, as it was shown to be present in many *R. microplus* tissues that take contact with the host immune system (Ferreira *et al*. 2002) and PRMs have been shown to induce protective responses when used as immunogen against different parasite infections (Li *et al*. 1993; McKenna *et al*. 1998; Vazquez Talavera *et al*. 2001). Contrarily to our initial expectations, considering that RmPRM was not identified in saliva (Ferreira *et al*. 2002), but consistent with the described host immune response against PRM from other acari (Mattsson *et al*. 2001; Tsai *et al*. 2000; Lee *et al*. 2004), rRmPRM was recognized by sera of infected bovines, turning it from a probable concealed into an exposed antigen.

PRM was initially described as an internal muscular protein of invertebrates, but many parasites, mainly mites and helminths, show to generate an humoral immune response in their hosts (Zhao *et al*. 2006; Nara *et al*. 2007; Ramos *et al*. 2003*a*) indicating that they take direct contact with it. In the mites *Dermatophagoides pteronyssinus* (Tsai *et al*. 2005), *Dermatophagoides farinae* (Tsai *et al*. 1999) and *Blomia tropicalis* (Ramos *et al*. 2003*b*), PRM showed to represent an important allergen. In *Schistosoma japonicum*, IgE responses to PRM predict resistance to reinfection (Jiz *et al*. 2009). Anti–PRM IgG is present against acari and helminths, such as *T*. *saginata* (Ferrer *et al*. 2003), *Trichostrongylus colubriformis* (Kiel *et al*. 2007), *T*. *spiralis* (Yang *et al*. 2008), *Blomia tropicalis* (Ramos *et al*. 2003*a*), and

Sarcoptes scabiei (Mattsson *et al*. 2001). The data presented herein showed that both *B. taurus* and *B. indicus* infected bovines presented IgG against rRmPRM as well as recognize salivary antigens at different titers. Recently, Rodriguez-Valle *et al*. (2012) reported the recognition of the RMS-3, a salivary *R*. *microplus* protease inhibitor, by sera of infected bovines, showing the development of higher IgG titers in resistant than susceptible individuals. Antigens with relative mobility in SDS-PAGE consistent with RmPRM that are recognized by sera of infected and vaccinated bovines have already been described in different adult tissues and developmental stages (da Silva Vaz *et al*. 1994; Kimaro and Opdebeeck, 1994; Cruz *et al*. 2008), but were not characterized. Interestingly, Pruett *et al*. (2006) described the recognition of a 102.3 kDa antigen by the sera of *B. taurus* bovines successively infected with *R. microplus* larvae, suggesting it may be a specific marker of *R. microplus* larvae exposure. If the 102.3 kDa antigen described represents RmPRM, which presents 102 kDa, the host immune recognition of BmPRM may initiate in the larvae stage, not necessarily depending on its presence in female adult saliva.

The cDNA coding for the mature RmPRM has been previously cloned and expressed using an N-terminal GST-fusion protein and purified by affinity chromatography (Ferreira *et al*. 2002). Unfortunately, the expression levels obtained were very low, as already described for other recombinant PRMs, not only in *Escherichia coli* (Ramos *et al*. 2001), but also in *Pichia pastoris* expression systems (Ramos *et al*. 2003*a*). Also, the size of the resulting fusion protein containing GST (102 kDa of PRM + 26 kDa of GST = 128 kDa) was suggested as a possible factor interfering with the correct folding of *B. tropicalis* PRM (Ramos *et al*. 2003*a*). The presence of truncated products found in rRmPRM-His preparations was greatly diminished comparing to the results found from expressing RmPRM from the previous construct containing GST at the N-terminal, although it was not completely abolished (data not shown). In this regard, Jiz *et al*. (2008) reported the probable dimerization of complete and truncated recombinant PRM forms, what may allow the affinity co-purification of the truncated rRmPRM-His. rRmPRM is found entirely in the soluble fraction while rRmPRM-His

is found in a great proportion in the insoluble fraction, what indicates that the recombinant RmPRMs may possess differences on their folding. Corroborating the results obtained by Jiz *et al*. (2008), after the removal of urea rRmPRM-His remained soluble.

Complement inhibition by paramyosin has been reported by binding to C8 and C9 (Deng *et al*. 2007; Zang *et al*. 2011) and by binding to C1 (Laclette *et al*. 1992). Studies with *T*. *solium* PRM suggest that it binds to the collagen-like region present in C1q, subcomponent of C1, which should prevent activation of the classical complement cascade (Landa *et al*. 1993; Laclette *et al*. 1992). rRmPRM-His was shown to share this activity presented by other PRMs from parasites, corroborating the already described capacity of RmPRM to bind type I collagen (Ferreira *et al*. 2002). The ability of RmPRM to inhibit the complement system, and bind collagen and IgG, makes it a possible modulator of host immune system. Additionally, it was described that *R*. *microplus* saliva was able to inhibit collagen-induced platelet aggregation (Reck et al, 2009). Therefore, molecules present in the salivary glands able to bind collagen, such as RmPRM, can be involved in this activity.

The multifunctional nature of parasite PRMs, specially accounted by their possible immunossupressive properties, has been assigned to explain, at least partially, their distribution within the organisms. PRM was recognized in the surface of *S*. *mansoni* (Matsumoto *et al*. 1988) and *S. japonicum* (Gobert *et al*. 1998). In *Paragonimus westermani*, the protein was found in all development stages and was localized in gut and ovary (Zhao *et al*. 2006). In *R*. *microplus*, RmPRM was found in all tissues and developmental stages tested, excepting saliva (Ferreira *et al*. 2002), what was corroborated by the data of the qRT-PCR performed in this study which showed that the BmPRM gene is expressed in very different levels at 3, 6, 12, and 18-day-old eggs, 5, and 10-day-old larvae, gut, salivary gland, fat body and ovary of partially- and fully-engorged adult females, and at adult male, but not at 1-day-old egg and 15-day-old larvae. The large difference of expression found between 12 and 18-day-old eggs indicates that changes preceding hatching need higher quantities of RmPRM. The presence of RmPRM in tick tissues without a prominent musculature, such as

adult female salivary gland and fat body, corroborate the indications that RmPRM plays additional roles beyond the muscle. Considering that, (i) when *R. microplus* feeds, antibodies are ingested retaining functional activity (da Silva Vaz Jr. *et al*. 1996), (ii) the wide distribution of RmPRM in adult tick tissues, and (iii) rRmPRM has been shown to bind immunoglobulins (Ferreira *et al*. 2002), the possible involvement of RmPRM in IgG clearance is reinforced, as already suggested (Ferreira *et al*. 2002). It has been shown that immunoglobulin binding proteins (IGBPs) of *Rhipicephalus appendiculatus*, *Amblyomma variegatum* and *Ixodes hexagonus* mediate the binding of IgG in gut, the transportation by the hemolymph to the salivary glands, and its secretion in the saliva (Wang and Nuttall, 1994, 1995, 1999). Indeed, the proportionally high expression of the RmPRM gene in adult female fat body indicates the high demand of the protein in the rapid ingurgitation and post-detachment tick phases and raises the possibility on the presence of RmPRM in hemolymph, what remains to be analyzed.

RmPRM has been shown to possess similar characteristics presented by helminthic PRMs beyond the contractile apparatus, which are considered important for parasite survival. As ticks and helminths evolved independently their parasitic relationships, it is tempting to speculate that the non-muscle functions performed by these orthologous proteins are the result of convergent evolution. Also, helminths, mites and ticks (or at least *R. microplus*) show to generate humoral responses in their hosts, although none PRM present a signal peptide sequence to enable their secretion. Taken together, all these facts indicates that RmPRM presents the same attributes that turned PRMs into promising targets for the development of vaccines against helminth parasites (Pearce *et al*. 1988; Chen *et al*. 1999; Nanduri and Kazura, 1989; Vázquez-Talavera *et al*. 2001) and, therefore, represents a candidate to compose a cocktail vaccine against *R. microplus*.

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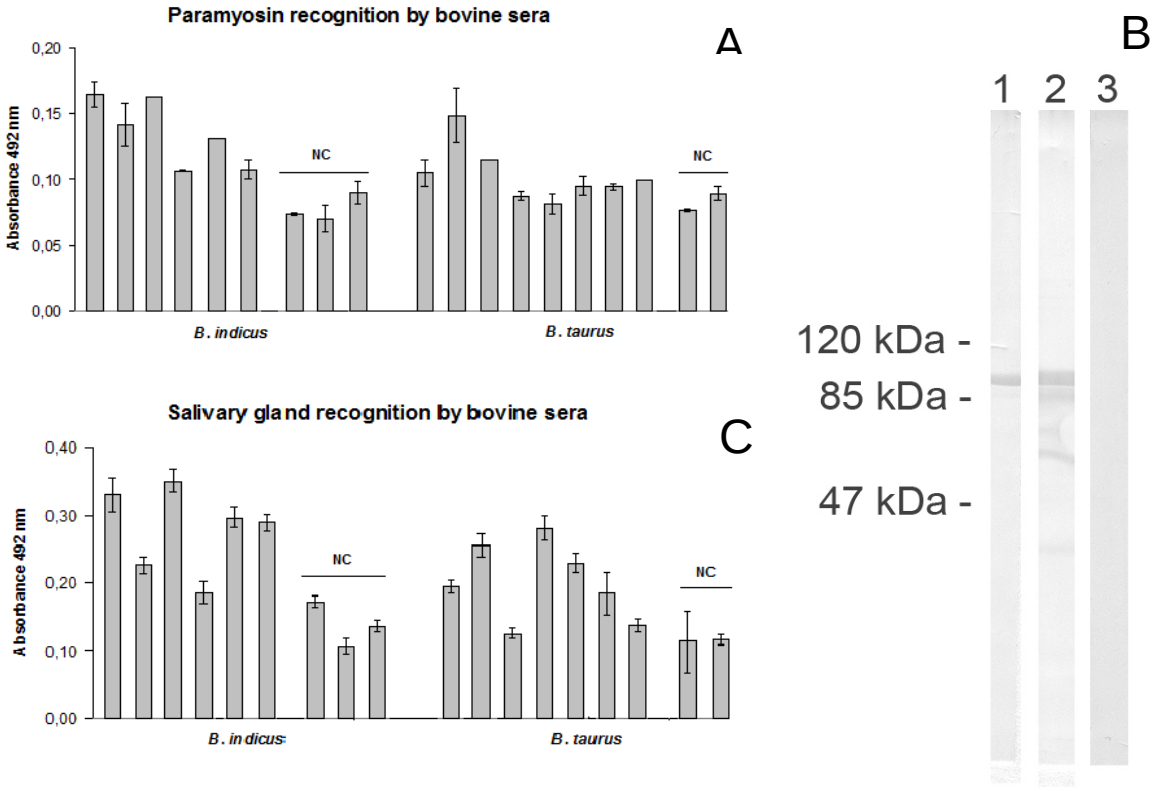

Fig.1 – Recognition of paramyosin and salivary gland extract by infected *Bos indicus* and *Bos taurus* sera. A: ELISA showing rBmPRM recognition by bovine sera. B: Western-blot of rRmPRM recognition by *B. indicus*; 1- bovine infected; 2- anti-paramyosin serum (positive control); 3- bovine not infected. C: ELISA of salivary gland recognition. NC: negative control.

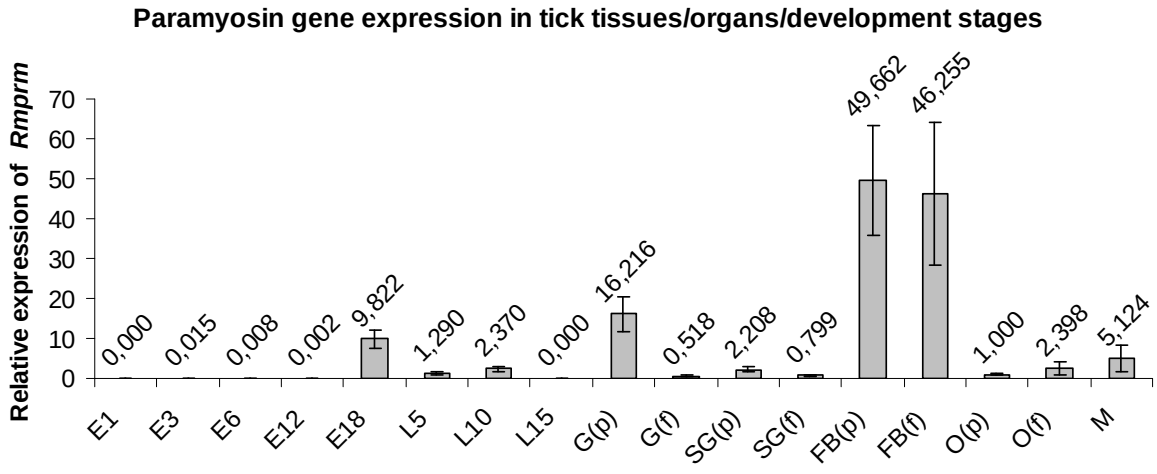

Fig. 2 – Paramyosin gene expression in tick tissues and organs detected by RT-PCR. E1: 1-day-old egg; E2: 3-day-old egg; E6: 6-day-old egg; E12: 12-day-old egg; E18: 18-day-old egg; L5: 5-day-old larva, L10: 10-day-old larva; L15: 15-day-old larva; G(p): partially-engorged female gut; G(f): fully-engorged female gut; SG(p): partially-engorged female salivary glands; SG(f): fully-engorged female salivary gland; FB(p): partially-engorged female fat body; FB(f): fully-engorged female fat body; O(p): partially-engorged female ovary; O(f): fully-engorged female ovary; M: male. Paramyosin gene expression was found in all tissues and organs tested, but was not detected in 1-day-old egg and 15-day-old larva.

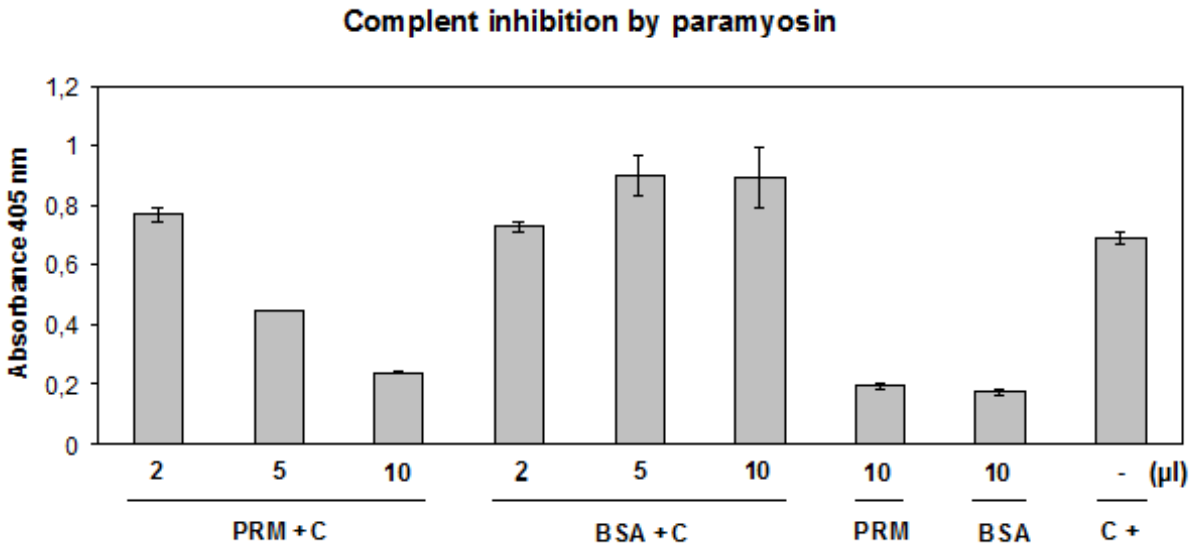

Fig. 3: Complement inhibition by 5 µl and 10 µl of rRmPRM-His (2,09 µg/µl). The higher inhibition was verified using 10 µl of PRM. PRM+C: paramyosin with complement; BSA+C: BSA with complement; PRM: PRM without complement; BSA: BSA without complement; C+: positive control.

4 CONSIDERAÇÃOES FINAIS

O *R*. *microplus* é um dos principais parasitos de importância econômica que acomete a criação de gado em muitos países (Delgado et al., 1999). A necessidade de combater o carrapato e os danos causados pelo uso de acaricidas incentivaram a busca por novos métodos de controle. Desta forma, antígenos têm sido identificados e suas atividades potencialmente protetoras caracterizadas. A paramiosina, uma proteína muscular, foi caracterizada e avaliada protetora em diversos animais contra diferentes parasitos, como *S. mansoni* (Berriman et al., 2009), S. *japonicum* (Zhou et al., 2000) e *T*. *solium* (Vázquez-Talavera et al., 2001), e apresentou atividades como a inibição do sistema complemento (Laclette et al., 1992; Zang et al., 2011), ou ligação a imunoglobulinas, como em *S*. *mansoni*, *S*. *japonicum* (Loukas et al., 2001), *P*. *westerman*i (Zhao et al., 2006) e *R*. *microplus* (Ferreira et al., 2002b), indicando que ela pode agir na modulação do sistema imune. Além disso, a presença de resposta imune humoral contra a PRM está bem estabelecida em helmintos (Ferrer et al., 2003; Zhao et al., 2006). Respostas imunes contra a PRM também foram relatadas em ácaros, onde ela mostrou-se um importante alergeno (Ramos et al., 2003b; Tsai et al., 2000). Ramos et al. (2003a) e Mattsson et al. (2001) adicionalmente mostraram que a PRM de ácaros também pode ligar-se a IgG.

Apesar de inicialmente considerar-se improvável que a PRM de *R*. *microplus* induzisse uma resposta humoral em bovinos infectados, pela ausência na saliva de carrapatos fêmeas adultos (Ferreira et al., 2002b), a presença de anticorpos contra paramiosina de ácaros em hospedeiros infectados (Ramos et al., 2003a; Lee et al., 2004) motivou a avaliação presente na figura 1 do artigo científico. O ELISA mostrou que rBmPRM foi naturalmente reconhecida pelos soros de *B*. *indicus* e *B*. *taurus,* identificando-se que os maiores níveis de anticorpos identificados estavam presentes no soro de animais *B*. *indicus*, o que também foi observado na glândula salivar. Os dois testes também revelaram um reconhecimento diferenciado entre os indivíduos. Esta variação nos níveis de anticorpos pode ser explicada por diferentes níveis de infestações de carrapatos, como proposto por

Cruz et al. (2008), pelo tempo de exposição dos hospedeiros, ou mesmo por diferenças genéticas entre as raças bovinas.

Diferentes fenótipos de resistência e suscetibilidade a infecção por *R*. *microplus* têm sido relatados entre espécies e raças de bovinos (Kashino et al., 2005; Piper et al., 2009). Após muitos anos de co-evolução com o carrapato, o *B*. *indicus* tornou-se mais resistente ao *R*. *microplus*. O *B*. *taurus*, exposto mais recentemente ao carrapato comparado com o *B*. *indicus*, tornou-se, portanto, mais suscetível ao parasito (Tatchell, 1987). A história de co-evolução do *B*. *indicus* com o *R*. *microplus* pode ser um dos possíveis fatores para explicar a diferença de reconhecimento da rBmPRM e do extrato de glândula salivar entre *B*. *indicus* e *B*. *taurus*.

A ausência da PRM na saliva (Ferreira et al., 2002b) caracterizou a proteína como um possível antígeno oculto, porém, a partir dos resultados obtidos neste estudo, sugere-se que a mesma deve ser um antígeno exposto. Assim como a 64P (um antígeno que combina características de antígenos expostos e ocultos) (Trimnell et al., 2002), a paramiosina pode constituir um antígeno exposto que apresenta epítopos antigênicos em vários tecidos do carrapato, podendo estarem acessíveis ou não ao sistema imune de hospedeiros. Outros antígenos, por outro lado, parecem apresentar a mesma propriedade, como a Bm91, encontrada na glândula salivar e no intestino, mostrou ser protetora após a vacinação de bovinos, porém animais expostos ao carrapato não desenvolveram anticorpos contra a proteína (Riding et al., 1994). A calreticulina, uma proteína secretada pela saliva, também não foi naturalmente reconhecida por soros de bovinos infectados (Ferreira et al., 2002*a*; Parizi et al., 2011). Este grupo diferenciado de antígenos tem sido considerado promissor para compor uma vacina de amplo-espectro e mais efetiva na proteção do hospedeiro (Nuttall et al., 2006).

A identificação da transcrição do gene que codifica a paramiosina (*Rmprm*) em tecidos, órgãos e diferentes estágios de desenvolvimento do *R*. *microplus*, corroboram com diferentes estudos que encontraram PRM no tegumento (Matsumoto et al., 1988; Cancela et

al., 2004), intestino, ovário (Zhao et al., 2006), e nos diversos estágios de desenvolvimento (Wang et al., 2012) de parasitos. A grande diferença de expressão entre ovos de 12 e 18 dias indica que haja possivelmente necessidade de grandes quantidades de PRM no momento da eclosão da larva. Quando o carrapato se alimenta, anticorpos são ingeridos e proteínas específicas ligam-se as IgG do hospedeiro (IGBPs), as quais atravessam a parede do intestino e retornam pra a glândula salivar e são secretadas (Wang and Nuttall, 1995), desta forma, a expressão de *Rmprm* no intestino poderia ser explicada por esta atividade. A expressão da PRM na glândula salivar já foi descrita, enquanto que no ovário, pode estar relacionada com a musculatura vinculada a ovoposição. A expressão de *Rmprm* na glândula salivar, intestino, ovário e o maior nível no corpo gorduroso, observados por qRT-PCR, sugerem que a paramiosina deve ter outras funções não envolvidas com a musculatura. Outro indicativo desta provável função adicional da proteína é a inibição do sistema complemento pela rRmPRM-His, constatada neste trabalho. A inibição do complemento pela paramiosina, descrita em *T*. *solium* (Laclette et al., 1992), parece resultar da ligação da proteína a C1q, que apresenta uma porção semelhante ao colágeno. A ligação da paramiosina de *R*. *microplus* ao colágeno descrita por Ferreira et al. (2002b) corrobora com a possível forma de inativação da cascata do complemento descrita por Laclette et al. (1992) e Landa et al. (1993).

Os resultados deste estudo indicam que a paramiosina constitui-se em uma proteína multifuncional, envolvida tanto na formação muscular quanto na evasão do sistema imune do hospedeiro. Os dados obtidos mostram que a paramiosina de *R*. *microplus* apresenta as atividades já descritas em helmintos. Além disso, em relação ao reconhecimento das diferentes regiões da PRM por anticopos, foi demonstrado que a região carboxi-terminal de S*. japonicum* (Nara et al., 1997) e *T. saginata* (Ferrer et al., 2003) e a região N-terminal de *D. immitis* (Steel et al., 1990) das respectivas PRM são preferencialmente reconhecidas pelos soros de pacientes infectados. Estas características mostram que talvez uma região específica da proteína possa ser mais importante no seu reconhecimento dependendo do

parasito, a qual não necessariamente corresponda a região que desempenha as atividades mais importantes para serem inibidas. Portanto, a utilização de uma pequena porção da PRM pode mais adequada para a utilização em uma vacina. Consequentemente, a caracterização imunológica e funcional das diferentes porções estruturais da RmPRM, assim ensaios de vacinação com bovinos utilizando estas diferentes regiões da proteína, necessitam ser realizados. Desta forma, assim como PRMs vêm sendo estudadas contra helmintos, sugere-se que a RmPRM pode ser também uma possível candidata a compor uma vacina coquetel contra o carrapato.

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ANEXO 1 - Guia para autores

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Journal references

Higgs, S., Snow, K. and Gould, E. A. (2003). The potential for West Nile virus to establish outside of its natural range: a consideration of potential mosquito vectors in the United Kingdom. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **98**, 82-87. doi: 10.1016/S0035-9203(03)00004-X.

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Jenkins, D. J. and MacPherson, C. N. L. (2003). Transmission ecology of *Echinococcus* in wild-life in Australia and Africa. *Parasitology* **127** (Suppl.) S63-S72. doi: 10.1017/S0031182003003871.

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