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CARACTERIZAÇÃO DE CISTATINAS E POSSÍVEIS FUNÇÕES NA FISIOLOGIA DO CARRAPATO *RHIPICEPHALUS (BOOPHILUS) MICROPLUS*

TESE DE DOUTORADO

LUÍS FERNANDO PARIZI

Porto Alegre, fevereiro de 2014

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Tese submetida ao Programa de Pós-graduação em Biologia Celular e Molecular (PPGBCM) da UFRGS como requisito parcial para a obtenção do grau de Doutor em Ciências.

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Dedico esse trabalho ao meu pai, Paulo Roberto Parizi, *in memorian*.

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BCA	Ácido bicinconínico
BCIP	5-bromo-4-cloro-3-indolil-fosfato
BmGI	Índice gênico do *Boophilus microplus*
cDNA	Ácido desoxirribonucleico complementar
$CoCl_2$	Cloreto de cobalto
DFCI	Instituto do câncer Dana-Farber
DNA	Ácido desoxirribonucleico
EDTA	Ácido etilenodiamino-tetracético
ELISA	Ensaio imunoenzimático
EST	Marcador de sequência expressa
GLPs	Glicoproteínas
GuHCL	Hidrocloreto de guanidina
H_2O_2	Peróxido de hidrogênio
HEPES	Ácido N-(2-hidroxietil)piperazina-N'-2-etanossulfónico
IgG	Imunoglobulina G
KI	Constante de inibição
MHC	Complexo principal de histocompatibilidade
mRNA	Ácido ribonucleico mensageiro
MW	Marcador de massa molecular
NBT	Cloreto de tetrazólio-nitroazul
ORF	Fase de leitura aberta
PARmS	Linhagem de *R. microplus* de Porto Alegre
PBMC	Células mononucleadas periféricas do sangue
PBS	Tampão fosfato-salina
PBS-T	Tampão fosfato-salina contendo tween
PCR	Reação em cadeia da polimerase
PVDF	Difluoreto de polivinilideno
qPCR	Reação em cadeia da polimerase quantitativa
RNA	Ácido ribonucleico
RNAi	Interferência pelo ácido ribonucleico
SDS-PAGE	Eletroforese em gel de poliacrilamida

SOB	Meio de cultura bacteriano "*Super Optimal Broth*"
TBDs	Agentes causadores de doenças transmitidos pelos carrapatos
UrRmP	Linhagem de *R. microplus* do Uruguai

RESUMO

Cistatinas constituem uma família de inibidores reversíveis de cisteíno-peptidases, estando envolvidas em diversos processos fisiológicos nos carrapatos. Contudo, até o momento, existem poucos trabalhos com cistatinas de *Rhipicephalus (Boophilus) microplus*, carrapato responsável por prejuízos na pecuária em diversos países. Nesse trabalho, foi analisado as sequências, o perfil transcricional, a localização tecidual, a afinidade por catepsinas, a antigenicidade e a imunogenicidade de prováveis cistatinas de *R. microplus*. As cinco sequências nucleotídicas analisadas contêm regiões codificadoras para os motivos característicos das cistatinas, além dos resíduos de cisteínas e o peptídeo-sinal pertencentes a esse grupo de inibidores. O perfil transcricional dos genes de cistatinas e a análise sorológica das cistatinas nativas detectaram diferentes níveis da presença desses inibidores em tecidos de ovo, larva, intestino, glândulas salivares, ovário e corpo gorduroso do carrapato. Três cistatinas foram expressas em *Escherichia coli*, sendo posteriormente purificadas por cromatografia de afinidade. Duas dessas cistatinas recombinantes apresentaram afinidades distintas para as catepsinas B, C e L, sugerindo o seu papel fisiológico diferencial durante o metabolismo do sangue ingerido e formação do ovo do carrapato, e também na modulação do sistema imune do hospedeiro. Através de análises *in silico*, regiões antigênicas das sequências de aminoácidos desses inibidores mostraram similaridade (54-92 %) com cistatinas homologas de *Rhipicephalus* spp., o que torna estas cistatinas de *R. microplus* possíveis alvos para o desenvolvimento de vacinas multi-espécies. A imunogenicidade das cistatinas recombinantes foi evidenciada por análises *in silico* e sorológicas, apresentando uma reatividade cruzada entre as cistatinas através de epitopos comuns. Esses resultados ajudam a esclarecer o papel das cistatinas na fisiologia

do *R. microplus*, e, assim, gerarem conhecimento para auxiliar no desenvolvimento de novas estratégias de controle desse parasito.

ABSTRACT

Cystatins belong to a family of tight-binding and reversible inhibitors of cysteine-peptidases. In ticks, these inhibitors are involved in a diversity of physiologic processes. At present, however, cystatins from *Rhipicephalus (Boophilus) microplus*, tick responsible for significant economic losses in livestock, are poorly characterized, limiting the elucidation of their physiological role and vaccine potential. Therefore, we investigated the sequences, tissue localization, enzyme targets and immunogenic properties of putative cystatins from *R. microplus*. The five nucleotide sequences analyzed encode the three cystatin motifs, cysteine residues and secretory signal peptides characteristic of these inhibitors, highly conserved. Transcription profiles and native protein expression analysis revealed differential gene transcription and protein expression patterns among cystatins in egg, larva, gut, salivary glands, ovary, and fat body tissues. Three cystatins were produced in the recombinant form in *Escherichia coli* and purified by affinity chromatography. Two of these cystatins showed distinct affinities to cathepsins B, C, and L, which are involved in tick and host physiological processes, suggesting cystatin role during tick blood digestion, egg development and host-immune system modulation. Furthermore, by *in silico* analysis, antigenic amino acid regions from these inhibitors showed a degree of homology of 54 to 92 % among *Rhipicephalus* spp. cystatins, suggesting the use of *R. microplus* cystatin in a multi-specie vaccine. Antigenicity and immunogenicity of the recombinant cystatins were determined by *in silico* and serological analysis, indicating cross-reactivity between cystatins in shared epitopes. Taken together, these results shed light of cystatins role in *R. miroplus* physiology, improving knowledge to the development of new vector control strategies.

1 INTRODUÇÃO

1.1 O carrapato *Rhipicephalus (Boophilus) microplus*

O carrapato *R. microplus* é um parasito hematófago da classe Arachnida, ordem Acarina, subordem Ixodida e família Ixodidae. Recentemente, através de análises moleculares e morfológicas, foi reclassificado como pertencente ao gênero *Rhipicephalus*, subgênero *Boophilus* (MURRELL & BARKER, 2003). O *R. microplus* pertence a família dos carrapatos duros, possuindo, portanto, uma placa dorsal denominada escudo, estrutura ausente na família dos carrapatos moles. Além disso, o *R. microplus* é monoxênico, ou seja, alimenta-se em um único hospedeiro ao longo do seu ciclo de vida. O principal hospedeiro do *R. microplus* é o bovino, embora outros hospedeiros ocasionais possam ocorrer, como os bubalinos, os ovinos, os equinos, cães, cervídeos e os humanos (GONZALES, 1995; FRANQUE *et al.*, 2007; SOARES *et al.*, 2007).

Originário da Ásia, a introdução desse carrapato nos outros continentes ocorreu nos últimos séculos através de diferentes linhagens originárias da Índia e Indonésia, levantando-se a hipótese de haverem pelo menos duas espécies classificadas como *R. microplus* ao redor do mundo (LABRUNA *et al.,* 2009). A difusão mundial do *R. microplus* foi facilitada pela frequente introdução de raças bovinas Europeias (*Bos taurus*) em diversos países, as quais são mais suscetíveis ao parasitismo por esse carrapato quando comparadas às raças zebuínas (*Bos indicus*) (FRISCH, 1999). Atualmente, esse carrapato é encontrado em zonas tropicais e subtropicais, afetando economicamente o sistema agropecuário dessas regiões (FRISCH, 1999; JOHNSTON *et al.*, 1986; JONSSON, 2006). Entre os prejuízos relacionados estão a anemia e a diminuição na produção de leite e carne causados aos animais parasitados (SUTHERST *et al.*, 1983), a desvalorização do couro pelo processo inflamatório desencadeado no local da fixação (SEIFERT *et al.*, 1968),

assim como a transmissão de agentes infecciosos, como os protozoários *Babesia bovis* e *Babesia bigemna* e da riquétsia *Anaplasma marginale* (MCCOSKER, 1981; YOUNG & MORZARIA, 1986), causadores da tristeza parasitária bovina. Somado a esses prejuízos estão os gastos relacionados ao controle do carrapato, como à mão-de-obra, instalações, compra e aplicação de acaricidas, etc (JAMROZ *et al.*, 2000). No Brasil, país possuidor do maior rebanho comercial de bovinos do mundo, as perdas econômicas relacionadas ao parasitismo pelo *R. microplus* foram estimadas em 2 bilhões de dólares americanos (GRISI *et al.*, 2002).

1.1.1 Ciclo de vida

Podemos dividir o ciclo de vida do *R. microplus* em fase de vida livre e parasitária. Após terminarem o processo de ingurgitamento, as fêmeas adultas totalmente alimentadas, também denominadas teleóginas, se desprendem do hospedeiro e caem ao solo para início da postura. Inicia-se então a fase de vida livre para essas fêmeas, de duração variável dependendo de condições climáticas, como temperatura e umidade. Por possuírem geotropismo positivo, as fêmeas se deslocam para ambientes protegidos no solo para início da postura, com um período de 2 a 3 dias de pré-postura em condições ideais (ROCHA, 1998). Cada teleógina realiza a postura média de 3.000 ovos durante aproximadamente 12 a 15 dias, em condições ideais de 27 ºC e umidade superior a 70% (GONZALES, 1995). Após a eclosão dos ovos, as larvas necessitam de aproximadamente 7 dias para se tornarem capacitadas a infestação. Apesar de poderem sobreviver em condições ambientais de baixas temperaturas por meses, a capacidade infestante dessas larvas é reduzida para aproximadamente 90 dias (GONZALES, 1995). Quando a larva sobe no hospedeiro, inicia-se a fase de vida parasitária, que dura em torno de 21 dias para as fêmeas do carrapato. As larvas alimentam-se de plasma e, para se fixarem, procuram nos hospedeiros a região

perianal, perivulvar e posterior das coxas, devido à espessura, vascularização e temperatura da pele, bem como à diminuída capacidade de autolimpeza pelo hospedeiro dessas áreas (WAGLAND, 1978; CORDOVÉS, 1996). Sobre o hospedeiro, ocorre o desenvolvimento da larva para as fases de metalarva, ninfa, metaninfa e adulto. Os carrapatos adultos copulam e as fêmeas, denominadas neóginas, se alimentam de 2 a 3 mL de sangue, completando o ciclo de vida. Os machos adultos sexualmente maduros, denominados gonandros, permanecem no hospedeiro a procura de novas fêmeas (ROBERTS, 1968).
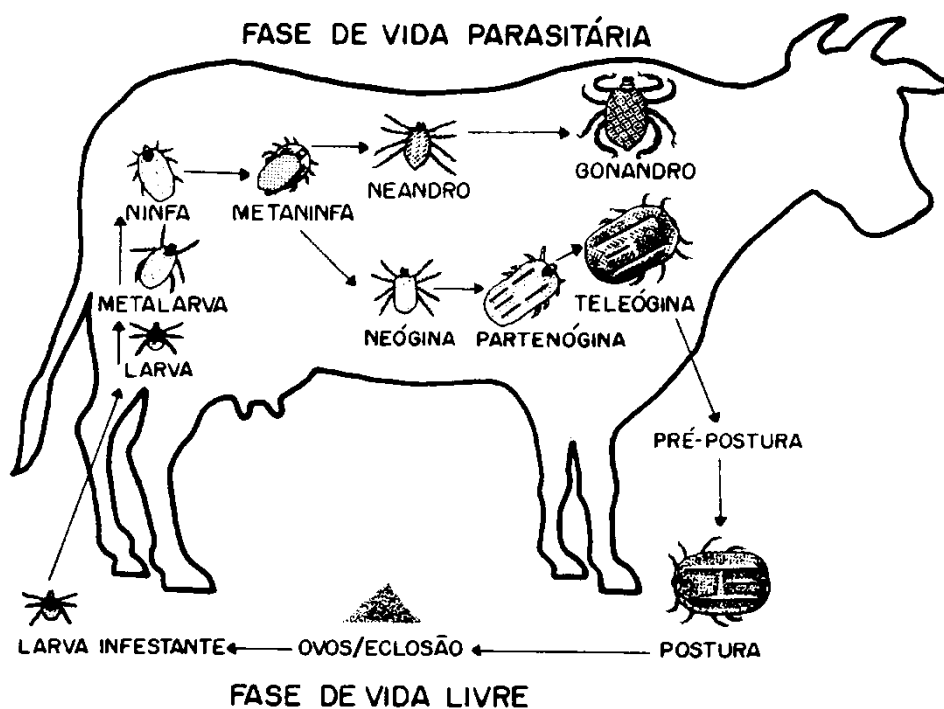

Figura 1 - Ciclo de vida do carrapato *R. microplus* (Fonte: GONZALES, 1975).

1.1.2 Métodos de controle

Na prática, o controle do carrapato *R. microplus* é realizado basicamente através da aplicação de acaricidas. Diversas classes de compostos já foram aplicadas para esses fins, como, por exemplo, os arsenicais, os organoclorados, os organofosforados, os carbamatos, as nitroguanidinas, os fenilpirazoles, as formamidinas, os piretróides, as avermectinas, as lactonas macrocíclicas e as fenil-uréias (GEORGE *et al.*, 2004). No entanto, populações de carrapatos resistentes a esses compostos têm sido descritas com uma frequência cada vez maior (MARTINS & FURLONG, 2001; DUCORNEZ *et al.*, 2005; PRUETT & POUND, 2006; WILLADSEN, 2006; GUERRERO *et al.*, 2012). Além disso, esses compostos causam a contaminação do leite e da carne bovina, assim como também do meio ambiente (DAVEY & GEORGE, 1998). Em uma sociedade preocupada cada vez mais com questões como a sanidade animal e a obtenção de alimentos livres de acaricidas, a persistência desses compostos nos produtos animais representa um grave entrave para bovinocultura. Por essas razões, torna-se cada vez mais necessária a pesquisa e o desenvolvimento de métodos alternativos de controle contra a infestação do *R. microplus*.

O controle biológico representa uma alternativa ao uso de acaricidas contra infestações pelo carrapato, valendo-se de interações desse parasito com o meio ambiente onde ele se encontra. Um exemplo dessa forma de controle seria a predação do carrapato por outras espécies de animais. São exemplos de predadores naturais para o *R. microplus* a garça vaqueira *Egretta ibis* (ALVES-BRANCO *et al.*, 1983), os pássaros vira-bosta (*Molothrus bonariensis*) e o quero-quero (*Vanellus chilensis*), assim como formigas carnívoras (GONZALES, 1975). Pode-se ainda recorrer à utilização de agentes infecciosos que têm se mostrado eficientes no controle biológico de insetos (SAMISH & GLAZER, 2001), como as bactérias *Escherichia coli*, *Cedecea lapagei* e *Enterobacter agglomerans*

(BRUM, 1988) e fungos entomopatogênicos, como o *Metarhizium anisopliae* (SANTI *et al*, 2009; FERNANDES *et al*., 2012).

Outras formas de controle incluem a criação de raças bovinas mais resistentes a infestação por *R. microplus*, assim como a utilização de compostos naturais (DAVEY *et al*., 2001), como, por exemplo, extratos de plantas (BORGES *et al*., 2011; KISS *et al*., 2012) ou feromônios (SONENSHINE, 2004). A manipulação de fatores como as condições climáticas de temperatura e umidade do campo (GONZALES, 1995; SUTHERST & BOURNE 2006), o tipo de vegetação (SUTHERST *et al*., 1982; FARIAS *et al*., 1986) e a rotação de pastagens (ELDER *et al*., 1980; NORTON *et al*., 1983) são capazes de influenciar negativamente no ciclo de vida do carrapato, podendo reduzir consideravelmente o número desses parasitos no ambiente.

1.1.2.1 Controle imunológico

Outra possibilidade para a substituição do uso de acaricidas no controle do carrapato seria o uso do controle imunológico. Essa estratégia baseia-se na vacinação do hospedeiro com antígenos capazes de desencadear uma resposta imune protetora, podendo assim prejudicar o parasitismo do carrapato. Os efeitos no parasitismo acarretados pela vacinação são normalmente estimados através da variação no número, peso e postura das teleóginas, assim como na eclodibilidade dos ovos (WIKEL & BERGMAN, 1997; FRANCISCHETTI *et al*., 2009). Entre as vantagens do uso do controle imunológico estão o custo-benefício (CANALES *et al*., 2010), a ausência do período de carência e a maior segurança na aplicação da vacina e consumo dos produtos animais devido à ausência de toxicidade das formulações administradas.

Diversos fatores determinam a proteção conferida pelo sistema imune dos hospedeiros frente à infestação por uma espécie de carrapato, dependendo de características tanto do parasito como do animal parasitado. Por exemplo, para realizarem a alimentação sanguínea, as diferentes espécies de carrapatos permanecem fixadas nos seus hospedeiros por períodos variados de tempo, abrangendo poucos minutos até vários dias (SONENSHINE, 1991). Quanto menor o período de alimentação, menor será o contato do carrapato com o sistema imune do hospedeiro.

Algumas espécies de carrapato estão amplamente adaptadas a uma determinada espécie de hospedeiro, como é o caso do parasitismo por *R. microplus* em bovinos. A resposta imunológica inata e adquirida está presente em bovinos infestados pelo *R. microplus* através da ação de anticorpos, do sistema complemento ou de reações de hipersensibilidade (WIKEL, 1988; VALENZUELA, 2004), envolvendo, entre outras, granulócitos, células apresentadoras de antígenos, citocinas, linfócitos B e T (WIKEL, 1996). Foi demonstrado o envolvimento de anticorpos (ROBERTS & KERR, 1976) e linfócitos (WIKEL & ALLEN, 1976) na resistência adquirida através da transferência do soro e de células de linfonodos de hospedeiros resistentes aos carrapatos para hospedeiros sensíveis, acarretando em ambos os casos a transferência de parte dos níveis de proteção. No caso do parasitismo de bovinos pelo *R. microplus*, há a produção de linfócitos B e T de memória (WIKEL, 1996), apesar de a participação na proteção conferida ao hospedeiro de cada tipo de resposta (humoral ou celular) necessitar de maiores investigações (WIKEL & BERGMAN, 1997; WILLADSEN, 2004; RUIZ *et al.*, 2006). Estas observações suportam o desenvolvimento de vacinas visando o controle do carrapato.

O sucesso do parasitismo do carrapato se deve em parte à adaptação desse na modulação do sistema imune do hospedeiro, capacidade essa adquirida gradualmente

durante a co-evolução dos parasitos e seus hospedeiros. Para tanto, os carrapatos possuem diversas moléculas imunossupressoras secretadas pelas glândulas salivares, que inibem o sistema complemento, a resposta inflamatória e os fatores hemostáticos e vasoconstritores dos hospedeiros, além de algumas dessas moléculas desviarem a resposta imune através de competição antigênica (RIBEIRO, 1989; BARRIGA, 1999; NUTTALL & LABUDA, 2004). Moléculas com funções semelhantes estão presentes na saliva dos carrapatos, como as serpinas que atuam em diversos pontos inibindo a cascata de coagulação sanguínea, evidenciando a importância da modulação dos sistemas de defesa dos hospedeiros.

O desenvolvimento de uma vacina comercial contra o *R. microplus* possui como passo fundamental a identificação de antígenos capazes de induzir uma resposta imune protetora reduzindo a carga parasitária por esse carrapato em níveis adequados a prática da bovinocultura (WILLADSEN & KEMP, 1988). Atualmente, apenas um antígeno derivado de uma proteína do intestino de fêmeas do carrapato está disponível comercialmente em uma vacina, sendo denominado Bm86. A proteção conferida é variável, dependendo de fatores como o isolado do carrapato ou a condição nutricional dos bovinos analisados (RODRIGUEZ *et al.*, 1995; PATARROYO *et al.*, 2002). Dependendo do isolado de carrapato, pode haver diferenças na sequência de aminoácidos da Bm86 e sua localização no intestino desse parasito, refletindo nos níveis de proteção conferida (GARCIA-GARCIA *et al.*, 2000). Assim sendo, embora essa vacina proteja adequadamente contra a infestação por *R. microplus* em algumas regiões do mundo, ela não assegura o grau de proteção necessário para suspender o uso de acaricidas em países como o Brasil (WILLADSEN *et al.*, 1996; JONSSON *et al.*, 2000). Portanto, faz-se necessária a pesquisa de novos antígenos imunoprotetores isolados de linhagens de carrapatos pertencentes à regiões próximas a serem aplicada a vacina visando-se obter melhores índices de proteção.

A procura de candidatos a antígenos protetores inclui não somente moléculas presentes unicamente no intestino do carrapato, pois, uma vez ingeridos os anticorpos do hospedeiro, esses podem ser transportados para outros tecidos a partir do intestino do carrapato (DA SILVA VAZ JR. *et al.*, 1996).

O potencial de diversas classes de proteínas já foi analisado no uso em formulações vacinais contra a infestação pelo *R. microplus*, resultando em proteções parciais (DA SILVA VAZ JR. *et al.*, 1998; LEAL *et al.*, 2006; SEIXAS *et al.*, 2008; ALMAZÁN *et al.*, 2010; HAJDUSEK *et al.*, 2010; PARIZI *et al.*, 2011, 2012a). Alguns desses antígenos protegeram não somente a própria espécie de carrapato, mas mostram-se capazes de conferir proteção cruzada para diferentes gêneros de carrapatos (KUMAR *et al.*, 2012; PARIZI *et al.*, 2012b). Entre as diversas funções desempenhadas por essas proteínas encontram-se as relacionadas com a modulação do sistema imune e hemostático do hospedeiro, a digestão sanguínea, a detoxificação celular, a embriogênese, etc. Dessa forma, o estudo de moléculas desempenhando papéis cruciais nessas vias é uma estratégia que mostrou identificar potenciais candidatos na composição de uma vacina para carrapatos, embora ainda abaixo dos níveis de proteção exigidos para a sua aplicação comercial.

Uma ferramenta de seleção de antígenos vacinais cada vez mais usada é a vacinologia reversa, em que a partir de critérios como altas taxas de expressão, importância fisiológica, baixa similaridade para com os genes ortólogos do hospedeiro, etc, chega-se a candidatos a antígenos com maiores probabilidades de conferir proteção (MARITZ-OLIVIER *et al.*, 2012). Outra metodologia que vem crescendo atualmente é a seleção de epitopos protetores e remoção de epitopos que não são imunogênicos e/ou interferem na resposta imune protetora, aumentando assim a proteção conferida contra a infestação pelo

carrapato. Este conjunto de análises vem sendo recentemente aplicado (PATARROYO *et al*, 2002; PATARROYO *et al*, 2009; NUCCITELLI *et al*, 2011; SINGH *et al*, 2012; SKWARCZYNSKI *et al*., 2012; SHRIVASTAVA *et al*., 2013), resultando em uma estratégia racional para identificação de polipeptídios para a composição de uma vacina.

1.2 Cisteíno-peptidases

Cisteíno-peptidases são enzimas presentes em uma ampla variedade de organismos, estando envolvidas em uma variedade de processos fisiológicos, como, por exemplo, o processamento intracelular proteico, a resposta imunológica, o processamento antigênico, a ativação de pró-proteínas e hormônios, o remodelamento da matriz extracelular e a apoptose (TURK *et al*., 2000,2002a; TURK *et al*., 2001; FRIEDRICHS *et al*., 2003). Essas enzimas são hidrolases que possuem uma díade catalítica no centro ativo contendo resíduos de cisteína e histidina, sendo agrupadas em duas superfamílias: a da papaína (BERTI & STORER, 1995) e a das enzimas relacionadas à enzima conversora de interleucina 1β (ICE) (CHAPMAN *et al*., 1997). Entre as cisteíno-peptidases podemos encontrar uma variedade de catepsinas lisossômicas (catepsinas B, C, H, K, L, M, N, S, T, V e W) (OTTO & SCHIRMEISTER, 1997; RZYCHON *et al*., 2004). A maioria das císteino-peptidases são endopeptidases (catepsinas L, V, F, S, K, B, H), embora algumas, como as catepsinas X e C, sejam exopeptidases. As catepsinas B e H possuem atividade de exo e endopeptidases (TURK *et al*., 2000; TURK *et al*., 2001). Além de similaridades na sequência e estrutura tridimensional, cisteíno-peptidases compartilham outras características, como as de serem proteínas monoméricas pequenas, serem instáveis em pH neutro e a de requererem condições redutoras para a sua atividade, além da especificidade relativamente baixa para seus substratos. Após a sua síntese, as catepsinas são transportadas para os lisossomos pela via do receptor da manose-6-fosfato, onde podem

chegar a concentrações tão altas quanto 1 mM (XING *et al.*, 1998). Para regular a sua ação hidrolítica, essas enzimas estão sujeitas a um controle estrito. Diferenças significativas nos níveis e proporções entre essas proteínas em vários tecidos foram descritos (TWINING, 1994), indicando funções distintas para catepsinas individuais. Todas as catepsinas são sintetizadas como pró-enzimas inativas, requerendo ativação por hidrólise proteolítica. Isso pode ocorrer por autocatálise em condições especificas, como em baixo pH, ou ser facilitado por outras peptidases ou glicosaminoglicanos. Uma vez ativada, a atividade enzimática pode ser perdida pela degradação ou oxidação da cisteína do centro reativo (TURK *et al.*, 2000). Um dos principais reguladores endógenos da atividade das cisteíno-peptidases são os inibidores da família das cistatinas, que se ligam ao redor do sítio ativo da enzima-alvo, bloqueando a interação e, consequentemente, a hidrólise do substrato (TURK *et al.*, 2002b).

1.2.1 Cisteíno-peptidases na fisiologia dos carrapatos

A digestão do conteúdo sanguíneo ingerido pelos carrapatos ocorre intracelularmente, dentro dos endossomos de células digestivas do intestino, sendo parte da função do lúmen do intestino o estoque de nutrientes durante a ovogênese (SONENSHINE, 1991). As células digestivas do carrapato medeiam, através de receptores e endocitose, a internalização do sangue armazenado no lúmen do intestino (TARNOWSKI & COONS, 1989). A degradação proteolítica ocorre no pH 3,0 do ambiente endossômico, bem abaixo do pH de 6,3 – 6,5 do lúmen do intestino (SAUER & HAIR, 1986). O pH dos endossomos, onde ocorre a digestão, é consistente com a atividade de cisteíno-peptidases específicas identificadas em *R. microplus* (REICH & ZORZOPULOS, 1978).

Nas últimas décadas, o papel das cisteíno-peptidases durante a digestão sanguínea tem sido reportado em várias espécies de carrapato. A expressão de uma catepsina L de *R. microplus* (BmCL1) foi identificada no intestino desse carrapato unicamente durante estágios de alimentação (RENARD *et al*., 2000). Foi demonstrada a presença dessa catepsina em vesículas secretoras de células epiteliais do intestino (RENARD *et al*., 2002). Similarmente, identificou-se cisteíno-peptidases com alta homologia com catepsinas L no intestino de *Haemaphysalis longicornis* (MULENGA *et al*., 1999a, 1999b; YAMAJI *et al*., 2009a). Uma dessas peptidases, denominada HlCPL-A, tem sua expressão regulada durante a alimentação nos hospedeiros vertebrados, sendo sua forma recombinante produzida em *E. coli* capaz de hidrolisar substratos comerciais e hemoglobina bovina em pH de 3,6 (YAMAJI *et al*., 2009a). Finalmente, uma catepsina B de *H. longicornis* denominada longipaina, também está regulada positivamente durante a alimentação do carrapato, estando localizada nos vacúolos lisossômicos e na superfície de células digestivas do intestino, apresentando, contudo, atividade frente a substratos sintéticos em pH mais básicos (TSUJI *et al*., 2008).

Similar a outros parasitos (WILLIAMSON *et al*., 2003; CAFFREY *et al*., 2004; DELCROIX *et al*., 2006), a degradação do conteúdo ingerido pelos carrapatos é caracterizado por um sistema complexo de digestão envolvendo não somente cisteíno-peptidases, mas também aspártico e leucina aminopeptidases (BOLDBAATAR *et al*., 2006; SOJKA *et al*., 2008). O perfil transcricional do intestino dos carrapatos *Dermacentor variabilis* e *Ixodes ricinus* demonstraram que entre as cisteíno-peptidases envolvidas na digestão sanguínea estão as catepsinas B, C e L (ANDERSON *et al*., 2008; HORN *et al*., 2009). No caso do *I. ricinus*, a transcrição dessas catepsinas foi observada simultaneamente durante e após a alimentação desse carrapato. Através da análise de

degradação de substratos comerciais e inibição enzimática, o papel durante a digestão de cada uma dessas catpsinas foi detalhadamente descrito em *I. ricinus* (HORN *et al.*, 2009). De acordo com esse estudo, a degradação inicial das globinas se daria pela catepsina L, conjuntamente a outras peptidases. Em sequência, a catepsina B degrada os peptídeos em fragmentos menores e, conjuntamente com a catpsina C, haveria a hidrólise até dipeptídeos.

Um segundo papel na fisiologia dos carrapatos em que as cisteíno-peptidases foram descritas é na embriogênese, ou, mais especificamente, na degradação das proteínas de vitelo. Durante o desenvolvimento embrionário, há a metabolização das vitelinas em espaços similares a organelas lisossômicas onde há a maquinaria de degradação e o substrato (vitelina). O conhecimento das enzimas envolvidas nesse processo é escasso, exceto pela caracterização de aspártico-peptidases e catepsinas L. Em *Ornithodorus moubata*, uma catepsina L foi identificada estando armazenada como zimogênio, mostrando-se inativa a pH neutros e sendo ativada em pH 3-4 (FAGOTTO, 1990a,1990b). Uma catepsina L de *R. microplus*, denominada VTDCE, foi identificada em ovos desse carrapato (SEIXAS *et al.*, 2003), apresentando a propriedade de ligar-se fortemente a vitelina (SEIXAS *et al.*, 2008). Outra enzima degradadora de vitelina denominada RmLCE foi identificada em extratos de larva de *R. microplus* (ESTRELA *et al.*, 2007). Foi proposto que a VTDCE é uma peptidase do ovo do carrapato que possui atividade específica restrita, enquanto a RmLCE é sintetizada na larva para completar a hidrólise das vitelinas restantes, provendo assim a larva de energia até a sua primeira alimentação sanguínea.

1.3 Inibidores de cisteíno-peptidases da família das cistatinas

Cistatinas são inibidores reversíveis de cisteíno-peptidases (TURK & BODE, 1991), embora também encontremos exemplos na literatura da capacidade de inibição de

metalo e serino-peptidases pelas cistatinas (VALENTE *et al.*, 2001; CORNWALL *et al.*, 2003). O controle proteolítico desempenhado pelas cistatinas é essencial para a viabilidade celular e, consequentemente, dos organismos. Além de regularem catepsinas lisossômicas, cistatinas humanas possuem a propriedade de inibir peptidases de microrganismos e parasitos, participando dos mecanismos de defesa contra esses agentes. Além disso, a transcrição de genes que codificam para cistatinas foi induzida por lipopolissacarídeos em monócitos e células dendríticas humanas (HASHIMOTO *et al.*, 2000; SUZUKI *et al.*, 2000).

No final da década de 1960, uma cistatina isolada do ovo da galinha capaz de inibir as enzimas papaína e ficina foi descoberta, se tornando o protótipo do tipo 2 das cistatinas (FOSSUM & WHITAKER, 1968). A partir de então, muitos membros novos da família das cistatinas foram descobertos, e suas funções regulatórias propostas e estabelecidas. Essa família de inibidores deriva de um ancestral comum e inclui um grupo diverso de proteínas contendo distintos perfis de inibição para as enzimas-alvo. Estudos de difração de raios X e mutagênese revelaram três regiões conservadas entre as cistatinas, responsáveis pela formação de uma estrutura em forma de cunha, a qual bloqueia o sítio ativo das cisteínos-peptidase (TURK & BODE, 1991; BODE *et al.*, 1988; STUBBS *et al.*, 1990). Essas regiões incluem o motivo da glicina N-terminal (G), o motivo glutamina, valina e glicina (QXVXG) e o motivo da prolina e do triptofano C-terminal (PW).

A classificação pelo banco de dados MEROPS coloca as cistatinas na Família I25 do Clan IH, baseado na similaridade das sequências de aminoácidos (RAWLINGS *et al.*, 2012). Baseado na similaridade de sequências entre as cistatinas homólogas, o número de domínios de cistatinas presentes e o número de pontes dissulfeto, podemos dividir esses inibidores em diferentes tipos. O tipo 1, ou estefinas, são na sua maioria intracelulares,

embora a sua presença tenha sido relatada também em fluidos corpóreos (ABRAHAMSON *et al.*, 1986). Elas possuem uma cadeia polipeptídica única, com uma massa molecular ao redor de 11 kDa, não possuindo pontes dissulfeto, glicosilação ou peptídeo-sinal. As do tipo 2 são denominadas de cistatinas, possuindo uma cadeia polipeptídica única, com uma massa molecular ao redor de 13-15 kDa. Elas são sintetizadas com peptídeo-sinal, o que possibilita o seu transporte através de vesículas para espaços extracelulares. Cistatinas do tipo 2 possuem duas pontes dissulfeto conservadas na porção C-terminal e são, na sua maioria, não glicosiladas (DICKINSON, 2002), embora existam exceções. Finalmente, o tipo 3, das estatinas, ou cininogênios, inclui proteínas multi-domínio com alta massa molecular (60- 120 kDa), sendo compostas por uma cadeia leve e outra pesada. Os cininogênios possuem três domínios típicos de cistatinas na cadeia pesada (OHKUBO *et al.*, 1984; MÜLLER-ESTERL *et al.*, 1985), embora somente dois desses domínios possuam a capacidade de inibir cisteíno-peptidases (MÜLLER-ESTERL, 1987; SALVESEN *et al.*, 1986). Esses inibidores são glicosilados, possuem 8 pontes dissulfeto (SALVESEN *et al.*, 1986), são expressos no fígado e estão presentes no sangue em concentrações na ordem de μM (ADAM *et al.*, 1985). Se as cistatinas contendo um único domínio são os inibidores de cisteíno-peptidases predominantes nos tecidos, os cininogênios constituem a maior fonte de inibição dessas enzimas na corrente sanguínea de mamíferos, provendo uma proteção sistêmica contra a liberação patológica de peptidases lisossômicas, assim como peptidases advindas de microrganismos invasores. Em carrapatos, cistatinas do tipo 1 e 2 já foram descritas, não sendo identificada até o momento qualquer uma do tipo 3 (SCHWARZ *et al.*, 2012).

1.3.1 O papel das cistatinas na fisiologia dos carrapatos

Trabalhos com diversos tipos de parasitos vêm demonstrando não somente a função das cistatinas no controle enzimático em processos endógenos, mas também a interação desses inibidores com as peptidases dos hospedeiros. Em parasitos nematódeos, já foi estabelecida a importância cardinal desempenhada pelas cistatinas na evasão do sistema de defesa pela modulação da resposta imune do hospedeiro (HARTMANN & LUCIUS, 2003). As cistatinas secretadas por esses parasitos interferem na produção de citocinas, no processamento antigênico e na apresentação de antígenos pelas APCs. No parasitismo por *Fasciola gigantica*, constatou-se que uma cistatina específica desse trematódeo constitui o maior componente do produto de secreção/excreção produzido durante o processo de invasão no seu hospedeiro (TARASUK *et al*, 2009). Interessantemente, constatou-se modulação diferencial do sistema imune do hospedeiro entre cistatinas de nematódeos parasitos e de vida livre (SCHIERACK *et al*, 2003). Foi sugerido que as cistatinas dos nematódeos parasitos adquiriram a capacidade de inibir a resposta proliferativa e a produção de citocinas por PBMC em resposta adaptativa ao estilo de vida parasitário.

Em carrapatos, apenas recentemente a caracterização bioquímica, molecular e fisiológica das cistatinas tem sido alvo de estudos. Esses trabalhos demonstraram que existem diversas cistatinas sendo expressas tanto em carrapatos duros (KARIM *et al*., 2005; LIMA *et al*., 2006; KOTSYFAKIS *et al*., 2007; YAMAJI *et al*., 2010; IMAMURA *et al*., 2013) como em moles (SALÁT *et al*., 2010), sendo expressas em diferentes momentos do ciclo de vida e em diferentes tecidos, principalmente nas glândulas salivares e intestino durante a alimentação (LIMA *et al*., 2006; GRUNCLOVÁ *et al*., 2006; ZHOU *et al*., 2006, 2010; KOTSYFAKIS *et al*., 2007; YAMAJI *et al*., 2009b, 2010; IBELLI *et al*., 2013). Essas cistatinas mostraram inibir diversas classes de catepsinas envolvidas na

digestão sanguínea, no desenvolvimento embrionário do carrapato e na resposta imunológica do hospedeiro (LIMA *et al*., 2006; GRUNCLOVÁ *et al*., 2006; ZHOU *et al*., 2006, 2009, 2010; KOTSYFAKIS *et al*., 2006, 2007, 2010; YAMAJI *et al*., 2009b, 2010; SALÁT *et al*., 2010). Foi sugerido que ao inibir catepsinas L secretas por macrófagos, a sialostatina poderia interferir na resposta imunológica pelo remodelamento tecidual dos hospedeiros no local de fixação do carrapato, facilitando a alimentação hematófaga do *Ixodes scapularis* (KOTSYFAKIS *et al*., 2007). Além disso, experimentos *in vitro* e *in vivo* demonstraram a modulação de células do sistema imune pela inibição de proliferação celular de células T e dendríticas, pela diminuição da produção de citocinas e pela diminuição de processos inflamatórios (KOTSYFAKIS *et al*., 2006; SÁ-NUNES *et al*., 2009; SALÁT *et al*., 2010). Através de técnicas de RNAi, verificou-se que as cistatinas são importantes para o sucesso da alimentação dos carrapatos *Amblyomma americanum* (KARIM *et al*., 2005) e *I. scapularis* (KOTSYFAKIS *et al*., 2007). Durante o período em que estiveram fixadas no hospedeiro, o bloqueio da expressão das cistatinas prejudicou a sobrevivência e o ganho de peso das fêmeas. Apesar das peptidases-alvo e as rotas metabólicas envolvidas permanecerem ainda amplamente desconhecidas, os resultados já obtidos demonstram que as cistatinas desempenham funções, ainda que parciais, na regulação dos processos digestivos dos carrapatos e modulação do sistema imune dos hospedeiros.

1.3.2 Potencial vacinal das cistatinas dos carrapatos

Trabalhos recentes vêm demonstrando a possibilidade do desenvolvimento do controle imunológico através do uso das cistatinas de carrapatos. O bloqueio das cistatinas através de anticorpos poderia impactar processos fisiológicos importantes durante todo o ciclo de vida do carrapato, devido a esses inibidores estarem presentes em diversos tecidos

e estádios, assim como por desempenharem funções fisiológicas durante a embriogênese e a alimentação hematófoga do carrapato. Ensaios de vacinação em animais de laboratório utilizando-se como antígenos vacinais cistatinas de *I. scapularis* (KOTSYFAKIS *et al*., 2008) e *O. moubata* (SALÁT *et al*., 2010) resultaram em proteções parciais, reduzindo a sobrevivência e peso médios dos carrapatos. Cabe ressaltar que tanto carrapatos duros como moles foram impactados por anticorpos anti-cistatinas, sugerindo um potencial de proteção amplo entre as espécies de carrapato.

Outro dado interessante apoiando o uso das cistatinas em uma vacina foi o aumento da resistência ao parasitismo adquirido por coelhos que foram infestados com *A. americanum* ou *I. scapularis* cujas cistatinas presentes na saliva desses carrapatos foram silenciadas por RNAi (KARIM *et al*., 2005; KOTSYFAKIS *et al*., 2007). Os coelhos se tornaram resistentes a infestações posteriores com carrapatos normais, indicando a participação das cistatinas nos mecanismos de evasão desses parasitos do sistema imunológico dos hospedeiros. Apesar de evidências mostrando a secreção desses inibidores no hospedeiro pela saliva do carrapato (KOTSYFAKIS *et al*., 2007), os coelhos não desenvolvem anticorpos anti-cistatinas quando naturalmente infestados, embora as formas recombinantes sejam imunogênicas (KOTSYFAKIS *et al*., 2008; SALÁT *et al*., 2010). Isso sugere que as cistatinas secretadas não são reconhecidas pelo sistema imune do hospedeiro, embora infestações por carrapatos em animais vacinados com cistatinas recombinantes poderiam estimular a produção de anticorpos anti-cistatina sem a necessidade de repetidas imunizações.

Existem evidências que associam a expressão de cisteíno-peptidases de *H. longicornis* com a transmissão de patógenos como *Babesia* spp. (TSUJI *et al*., 2008). Em

outro trabalho, cistatinas de *H. longicornis* mostraram inibir a multiplicação *in vitro* de *B. bovis*, sugerindo o envolvimento desse inibidor no sistema imune inato do carrapato (ZHOU *et al*., 2006). Esses trabalhos indicam a importância tanto das cisteíno-peptidases como das cistatinas na sobrevivência de agentes infecciosos no carrapato e seus hospedeiros, tornando essas proteínas alvos potenciais para métodos de controle de patógenos como *Babesia* spp.

2 OBJETIVOS

Diversos trabalhos comprovam que as cistatinas estão envolvidas em diferentes processos fisiológicos nos carrapatos. Apesar disso, até o momento, poucos trabalhos caracterizaram as cistatinas de *R. microplus*. Nesse trabalho, através de análises moleculares e funcionais de cistatinas de *R. microplus*, buscou-se uma maior compreensão do papel desempenhado por esses inibidores na fisiologia desse carrapato e o seu potencial uso em uma vacina anti-carrapato. Para tanto, a tese dividiu-se em dois capítulos em formato de artigos, apresentando no primeiro uma caracterização das sequências, do perfil transcricional e da antigenicidade das cistatinas e, no segundo, a localização tecidual e a afinidade por catepsinas. Além disso, dois artigos publicados durante o doutorado relativos a questão do desenvolvimento de uma vacina anti-carrapato estão em anexo.

2.1 Objetivo Geral

Avaliar o papel das cistatinas na fisiologia de *R. microplus*.

2.2 Objetivos Específicos

a) Anotar as sequências de cistatinas de *R. microplus*;

b) Analisar o perfil transcricional em diferentes tecidos e fases de vida do carrapato;

c) Prever *in silico* as regiões antigênicas das cistatinas;

d) Expressar e purificar as cistatinas recombinantes de *R. microplus*;

e) Definir as constantes de inibição das cistatinas para catepsinas comerciais;

f) Determinar a imunogenicidade e antigenicidade cruzada das cistatinas;

g) Analisar a presença das cistatinas nativas nos tecidos de *R. microplus*.

3 CAPÍTULO 1: "Sequence characterization and immunogenicity of cystatins from the cattle tick *Rhipicephalus (Boophilus) microplus*"

Este capítulo apresenta os dados do artigo publicado: "Sequence characterization and immunogenicity of cystatins from the cattle tick *Rhipicephalus (Boophilus) microplus*".

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Participação dos autores no desenvolvimento do trabalho:

Luís F. Parizi: delineamento, realização e análise dos experimentos das cistatinas do isolado brasileiro; redação do manuscrito.

Naftaly W. Githaka: delineamento, realização e análise dos experimentos sorológicos; redação do manuscrito.

Carolina Acevedo: delineamento, realização e análise dos experimentos das cistatinas do isolado uruguaio; redação do manuscrito.

Uruguaysito Benavides, Adriana Seixas, Carlos Logullo, Satoru Konnai, Kazuhiko Ohashi, Aoi Masuda, Itabajara da Silva Vaz Jr.: delineamento e análise dos experimentos; redação do manuscrito.

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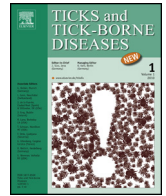
Original article

Sequence characterization and immunogenicity of cystatins from the cattle tick *Rhipicephalus (Boophilus) microplus*

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ABSTRACT

Various classes of endopeptidases and their inhibitors facilitate blood feeding and digestion in ticks. Cystatins, a family of tight-binding and reversible inhibitors of cysteine endopeptidases, have recently been found in several tick tissues. Moreover, vaccine trials using tick cystatins have been found to induce protective immune responses against tick infestation. However, the mode of action of tick cystatins is still poorly understood, limiting the elucidation of their physiological role. Against this background, we have investigated sequence characteristics and immunogenic properties of 5 putative cystatins from *Rhipicephalus (Boophilus) microplus* from Brazil and Uruguay. The similarity of the deduced amino acid sequences among cystatins from the Brazilian tick strain was 27–42%, all of which had a secretory signal peptide. The cystatin motif (QxVxG), a glycine in the N-terminal region, and the PW motif in the second hairpin loop in the C-terminal region are highly conserved in all 5 cystatins identified in this study. Four cysteine residues in the C terminus characteristic of type 2 cystatins are also present. qRT-PCR revealed differential expression patterns among the 5 cystatins identified, as well as variation in mRNA transcripts present in egg, larva, gut, salivary glands, ovary, and fat body tissues. One *R. microplus* cystatin showed 97–100% amino acid similarity between Brazilian and Uruguayan isolates. Furthermore, by in silico analysis, antigenic amino acid regions from *R. microplus* cystatins showed high degrees of homology (54–92%) among *Rhipicephalus* spp. cystatins. Three Brazilian *R. microplus* cystatins were expressed in *Escherichia coli*, and immunogenicity of the recombinant proteins were determined by vaccinating mice. Western blotting using mice sera indicated cross-reactivity between the cystatins, suggesting shared epitopes. The present characterization of *Rhipicephalus* spp. cystatins represents an empirical approach in an effort to evaluate the physiological role of cystatins in a larger context of targeting them for use in future tick control strategies.

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Introduction

Rhipicephalus (Boophilus) microplus is a highly invasive tick species, spreading to all tropical and subtropical regions of the world (Labruna et al., 2009) and causing enormous economic losses to the livestock industry (de la Fuente et al., 1998; Jonsson, 2006). The emergence of chemical-resistant ticks has hampered the use of acaricides to reduce *R. microplus* infestations, creating the need for new control strategies (George et al., 2004). In an effort to minimize the amounts of acaricides currently used, development of anti-tick vaccines has been prioritized worldwide (Willadsen, 2004). A sizeable number of candidate vaccine targets have been identified (reviewed by Parizi et al., 2012; Seixas et al., 2012); however, poor antigenicity and limited protection against

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unrelated tick species have shown the importance of additional antigens.

The cystatin family is composed of inhibitors of papain-like cysteine proteases and legumains (Abrahamson et al., 2003) and is involved in biological processes such as protein turnover, antigen processing and presentation, phagocytosis, bone remodeling, and cytokine expression (Zavasnik-Bergant, 2008). In parasites such as helminths, secreted cystatins are involved in active modulation of the host immune response (Hewitson et al., 2009). Alongside functional characterization of several tick cysteine endopeptidases (Seixas et al., 2003, 2010; Estrela et al., 2010; Franta et al., 2010), increasing research efforts have been made on the physiological role of protease inhibitors in ticks in recent years (Karim et al., 2005; Grunclová et al., 2006; Kotsyfakis et al., 2006, 2007, 2008, 2010; Zhou et al., 2006, 2009; Yamaji et al., 2009, 2010; Salát et al., 2010), and some of these studies suggest that cystatins and other enzyme inhibitors are candidate antigens for inclusion in future anti-vector vaccines (Mulenga et al., 2001; Imamura et al., 2005; Franta et al., 2010).

In ticks, several type 1 (intracellular proteins) and type 2 (secretory proteins) cystatins have been identified and characterized; however, vaccinating with tick cystatins has only been attempted with two type 2 cystatins: sialostatin 2 from *Ixodes scapularis* (Kotsyfakis et al., 2007) and OmC2 from *Ornithodoros moubata* (Salát et al., 2010). Immunizing rodents with sialostatin 2 or OmC2 decreased blood feeding and survival of *I. scapularis* and *O. moubata*, respectively. Furthermore, blockade of cystatins with antibodies or through RNAi renders the hosts resistant to tick infestation (Karim et al., 2005; Kotsyfakis et al., 2007, 2008; Salát et al., 2010). These novel observations confirm the physiological importance of cystatins to control ticks and point to the potential use of these inhibitors in future anti-tick vaccine formulations (Ibelli et al., 2013). Although *R. microplus* is a major disease vector, no type 2 cystatin has been characterized. Additionally, since field populations of *R. microplus* are highly heterogenous (Labruna et al., 2009), we have hypothesized that sequence heterogeneity may likewise exist in cystatins as a consequence of geographic isolation of *R. microplus* populations. Using ticks originating from Brazil and Uruguay, we characterized several cystatins and describe sequence variants of these proteins. Also, in the context of potential vaccination, we describe the expression and purification of cystatin recombinant proteins and the evaluation of their immunogenicity.

Materials and methods

Animals and ticks

Porto Alegre *R. microplus* strain (PARmS) was maintained on Hereford (*Bos taurus taurus*) cattle housed at Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Brazil. Cattle care was in accordance with the institutional guidelines (FASS, 1999). Partially and fully engorged PARmS female ticks were collected from cattle for tissue dissection. Partially engorged *R. microplus* females weighing between 25 and 60 mg were recovered manually from calves (Gonsioroski et al., 2012). To obtain PARmS eggs and larvae of different stages, fully engorged adult ticks were maintained at 28 °C and 85% relative humidity until egg laying and egg hatching. Uruguayan *R. microplus* populations (UrRmP) were collected in different tick endemic areas in Uruguay (Montevideo, Rivera, and Cerro Largo). The Mozo population collected in Montevideo was supplied by Departamento de Parasitología, División de Laboratorios Veterinarios Miguel C. Rubino, Uruguay.

Immunized mice were maintained in a P3 animal facility at Graduate School of Veterinary Medicine, Hokkaido University, in accordance with the guidelines of the Institutional Animal Care and Use Committee.

RNA extraction and cDNA synthesis

Gut, salivary glands, ovary, and fat body tissues from partially and fully engorged female ticks were dissected in PBS. Dissected tissues, larvae (12, 18, and 21 days post-hatching) and eggs (one-day and 5-day-old) were macerated and total RNA extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. The quantity and quality of the recovered RNA was determined spectrophotometrically at A280 nm and by the ratio A260/A280 nm, respectively. To produce cDNA, RNA was reverse transcribed using the superscript III kit (Invitrogen), following the manufacturer's instructions.

Cloning of cystatins coding cDNA

The PARmS and UrRmP cystatin-coding regions were amplified by PCR from *R. microplus* salivary glands (BrBmcys2a–d), ovary (BrBmcys2e), and gut (UrBmcys2c 1–6) cDNA. The amplicons were separated by agarose gel electrophoresis, purified using Glassmilk DNA purification kit (BIO 101 Systems), and cloned into pGEM-T vector (Promega). Primers (Table 1) were based on cystatin sequences retrieved from the DFCI *Boophilus microplus* Gene Index (http://compbio.dfci.harvard.edu/index.html). Plasmids were purified and the nucleotide sequences of the inserts determined on a 16-capillary 3130xl (Life Technologies) automated sequencer. The coding regions of BrBmcys2a–c were then subcloned into pET-5a expression vector (Novagen) using primers containing restriction sites of *Nde* I and *Hin*d III (Table 1).

Bioinformatic analyses

DNA sequences coding for *R. microplus* cystatins and the deduced amino acid sequences were analyzed with BioEdit version 7.1.3.0 software program (Hall, 1999). Signal peptides were determined with the SignalP 4.0 server of the Technical University of Denmark (server: http://www.cbs.dtu.dk/services/SignalP/). An unrooted neighbor-joining phylogenetic tree was created using the MEGA version 5.10 (Tamura et al., 2011) with 1000 replicates bootstrap support. The antigenic index analysis of *R. microplus* cystatins was performed with the Jameson–Wolf algorithm by software LASERGENE version 7.0.0 for predicting antigenic determinants by combining existing methods for protein structural predictions (Jameson and Wolf, 1988). The cystatin sequences included in the phylogenetic analyses are listed under GenBank accession numbers: *R. microplus*, KC816579 (BrBmcys2a), KC816580 (BrBmcys2b), KC816581 (BrBmcys2c), KC816582 (BrBmcys2d), KC816583 (BrBmcys2e); *Rhipicephalus pulchellus*, JAA54337.1, JAA54340, JAA54250, JAA54339.1, JAA54154.1, JAA53901.1; *Rhipicephalus sanguineus*, ACX53922, ACX53862.1; *Rhipicephalus appendiculatus*, AGB35873.1, KC816584 (QnRacys2a).

Expression analysis of cystatins mRNA by qRT-PCR

Relative quantification was carried out with 100 ng of cDNA prepared from the gut, salivary glands, ovary, and fat body of partially and fully engorged females, 12-, 18-, 21-day-old eggs and 1-, 5-day-old larvae cDNA. To normalize the obtained gene expression, tick 40S ribosomal locus was selected as a housekeeping gene, as described previously (Pohl et al., 2008). The specific primers used to quantify the cystatins and 40S ribosomal transcripts are listed in Table 1. The amount of mRNA transcripts for each target gene present in 6-day-old eggs were considered equivalent to 1 and were used as reference for expression in other tissues. Cycling

Table 1
Primers used for cloning into pGEM-T (pG), pET5-a (pE), and for the qRT-PCR assays (qR) of *Rhipicephalus (Boophilus) microplus* cystatins.

Gene	Primer	Sequence 5'–3'	T_m (°C)	Amplicon size (bp)
BrBmcys2a	Sense (pG)	ATGATAGCGATCAAGCAGACCTGCT	60.0	
	Antisense (pG)	TCAATTTGTTGCGCCGGAACAT	61.3	
	Sense (pE)	TTTTT<u>CATATG</u>ATAGCGATCAAGCAGACCTGCT	59.4	
	Antisense (pE)	AAAAA<u>GGATC</u>CC*TAGTGGTGGTGGTGGTGGT*GATTTGTTGCGCCGGAACATGT	59.0	
	Sense (qR)	GGTTTGTGGACAAGCGCCTTGC	62.4	100
	Antisense (qR)	TTCAGGTACTTGGGGCTTCCCTG	60.2	
BrBmcys2b	Sense (pG)	ATGGCTTCTTTGAGAATCACCCCG	61.0	
	Antisense (pG)	TTAGGTAGATGTGCTGCTTCCTTCGA	59.4	
	Sense (pE)	TTTTT<u>CATAT</u>GGCTTCTTTGAGAATCACCCCG	61.2	
	Antisense (pE)	AAAAA<u>GGATC</u>CC*TAGTGGTGGTGGTGGTGGT*GGGTAGATGTGCTGCTTCCTTCGCA	61.3	
	Sense (qR)	GCGTGGCTTCGCAGTCCTGA	60.8	106
	Antisense (qR)	CCTCAAACATGGCGTTGTCGC	60.4	
BrBmcys2c/UrBmcys2c 1–6	Sense (pG)	ATGGCTCGTATGGCAGTTAGCATTG	62.7	
	Antisense (pG)	CTAAGCGCATCGAAGAGAAGTCAGAGA	60.0	
	Sense (pE)	TTTTT<u>CATATG</u>GCTCGTATGGCAGTTAGCATTG	60.0	
	Antisense (pE)	AAAAA<u>GGATC</u>CC*TAGTGGTGGTGGTGGTGGT*GAGCGCATCGAAGAGAAGTCAGAGAC	59.0	
	Sense (qR)	TCGTATGGCAGTTAGCATTGCAGTTC	60.4	99
	Antisense (qR)	TAGGGCTCGACGAGGGTTCTTTTG	60.3	
BrBmcys2d	Sense (pG)	ATGAAAGCTGTTGAAATGGCCCATT	60.3	
	Antisense (pG)	TGTATATAGTGCCGTTGTGTACACTACCCAG	59.6	
	Sense (qR)	GCCAGTCATGCGCTGACTACCTTA	60.2	102
	Antisense (qR)	TCCTGCGTTGTGATGAGGGTCTTG	59.5	
BrBmcys2e	Sense (pG)	ATGAAGGGAGCGGTTGTACTTCTCG	60.4	
	Antisense (pG)	TTAACTGTGGCACTGCAGCGTTACA	61.2	
	Sense (qR)	GGGGAGCGGTTGTACTTCTCGTCG	60.8	100
	Antisense (qR)	CTTTGGATCACTCAACGGGTCCTG	60.5	
Ribosomal 40S	Sense (qR)	ACGACCGATGGCTACCTCCTCCGC	59.1	106
	Antisense (qR)	TGAGGCGAACCTGGTTGTGCTGAGCG	59.2	

T_m, primer melting temperature; restriction enzyme sites are underlined; histidine tag sites are in italics.

parameters for all amplification were 5 min at 95 °C followed by 30 cycles of 15 s at 95 °C, 30 s annealing at 60 °C, and extension at 72 °C for 30 s. qPCR was performed using Platinum SYBR Green qPCR SuperMix kit (Invitrogen) using Step One Plus thermocycler (Applied Biosystems), and data analyzed by Relative Expression Software Tool (REST) (Pfaffl et al., 2002). To ensure primer fidelity, dissociation curve analysis and gel electrophoresis of target gene amplicons were performed for each tissue sample following the qPCR step. Primer efficiency was determined with serial-diluted (fourfold) cDNA extracted from gut (BrBmcys2a, BrBmcys2b, and BrBmcys2c) or larva (BrBmcys2d and BrBmcys2e). All qPCR amplifications were performed in duplicates and repeated twice, with the mean values considered for comparison. To check for genomic DNA contamination, test controls were performed with a template lacking reverse transcription.

Expression and purification of recombinant cystatins

Plasmids constructs pET-5a/BrBmcys2a and pET-5a/BrBmcys2b were transformed into *Escherichia coli* strain C41(DE3) while the plasmid pET-5a/BrBmcys2c was transformed into *E. coli* C43(DE3) strain. Bacterial colonies were grown in LB medium, and recombinant protein expression was induced with 0.4 mM isopropyl-b-D-thiogalactopyranoside for 16 h at 25 °C, post induction. Cell cultures were harvested by spinning at 6000 × g for 30 min at 4 °C, and pellets were lysed through resuspension in BugBuster Protein Extraction Reagent (Novagen), following the manufacturer's protocol. The soluble and insoluble protein fractions were separated by centrifugation at 12,000 × g for 20 min at 4 °C. Recombinant cystatins were purified from the soluble fractions by immobilized metal affinity chromatography using TALON Metal Affinity Resin (Clontech Laboratories) following the

manufacturer's instructions. Eluted fractions were further concentrated (Centricon 10,000 MW cut-off, Millipore) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and gels stained with Coomassie blue G-250. Protein concentrations were determined by SDS-PAGE using bovine serum albumin standards.

Mice immunizations

Six mice divided in groups of 2 were inoculated with BrBmcys2b, BrBmcys2c, or PBS. Immunizations were performed intraperitoneally consisting of 7 doses (1st/2nd/3rd/4th/5th dose = 50 μg each; 6th/7th dose = 100 μg each) at 14-day intervals with the recombinant proteins emulsified in Freund's incomplete adjuvant. This dosing regime was used to induce a high antibody response. Blood was collected 14 days after the last booster, and serum was separated by spinning the samples at 10,000 × g for 5 min at 4 °C. Sera aliquots were preserved at −20 °C until use.

SDS-PAGE and Western blotting

The recombinant proteins were resolved by 16% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Billerica). The PVDF membranes were incubated for 1 h at room temperature in 5% non-fat dried milk diluted in 0.05% tween phosphate buffered saline (Blotto-PBS-T) and further probed with mice sera diluted 1:100 in Blotto-PBS-T for 2 h. After the sera incubations, anti-mouse IgG alkaline peroxidase conjugate was used as secondary antibody, and blots were developed with 0.05% 3,3'-diaminobenzidine tetrahydrochloride, 0.2% H_2O_2, and 0.03% $CoCl_2$ in PBS-T.
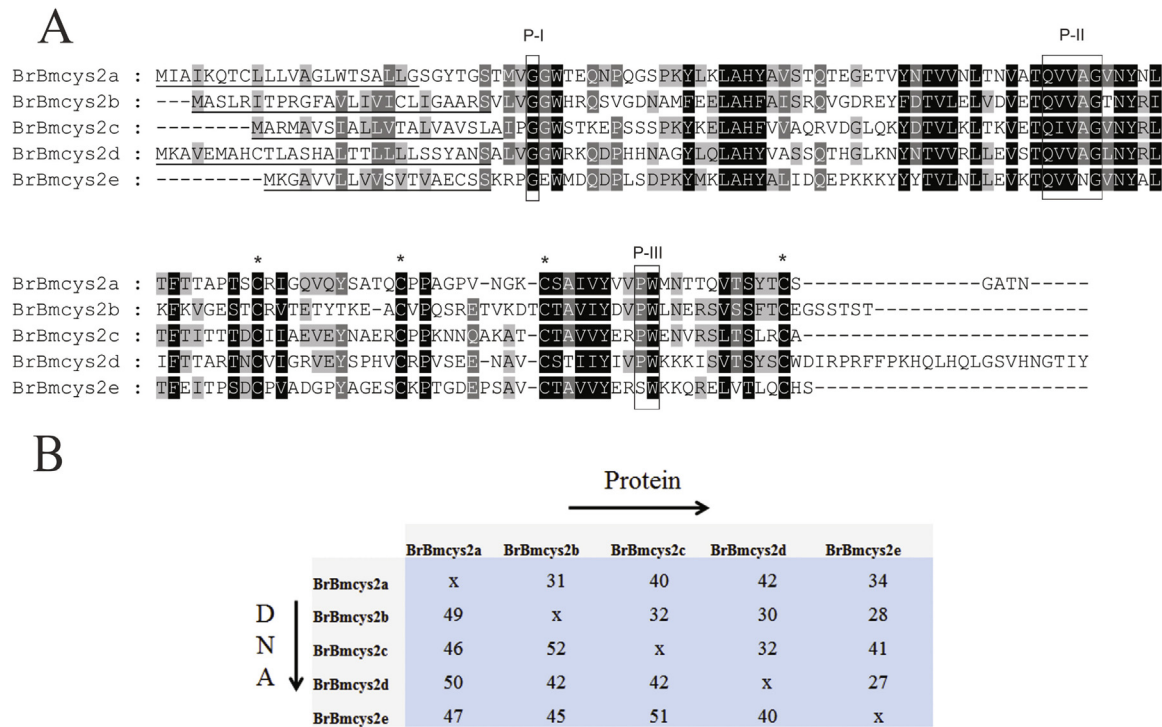
Fig. 1. (A) Sequence alignment of BrBmcys2a, BrBmcys2b, BrBmcys2c, BrBmcys2d, and BrBmcys2e. Dark, dark gray, and light gray shading shows 100, 80, and 60% conserved residues between cystatin sequences, respectively. Asterisks indicate the cysteine residues of 2 putative disulfide bridges. The cystatin motifs P-I (G), P-II (QxVxG), and P-III (PW) are boxed. Predicted cleavage signal peptides are underlined. (B) Similarity of DNA and putative protein sequences of BrBmcys2a, BrBmcys2b, BrBmcys2c, BrBmcys2d, and BrBmcys2e. Values are expressed as percentage of similarity.

Results

Identification and sequence analyses of cystatin homologs

Blast searches in the BmGI at DFCI repository yielded sequences with high similarity to those of published tick cystatin genes. Primers were designed and used to amplify the cystatins from *R. microplus* isolates from Brazil. Five putative cystatin genes were cloned from the Brazilian *R. microplus* isolate. These sequences encode polypeptides of 129 (BrBmcys2c and BrBmcys2e), 140 (BrBmcys2a and BrBmcys2b), and 157 (BrBmcys2d) amino acids (Fig. 1A). The cystatin BrBmcys2d was perhaps partial, since it was not possible to determine the stop codon for this particular sequence. The deduced amino acid sequences contained the highly conserved motif QxVxG characteristic of cystatins, a glycine in the N-terminal region and the PW motif in the second hairpin loop in the C-terminal region. However, the consensus PW motif is lacking in BrBmcys2e, in which a proline residue is replaced by a serine. In addition, all the cystatin sequences contained signal peptides, and 4 cysteine residues in the C terminus responsible for the formation of 2 disulfide bonds, which is characteristic of type 2 cystatins. The nucleotide and amino acid similarity for these cystatins ranged between 42 and 52% and 27 and 42%, respectively (Fig. 1B).

To determine whether polymorphisms occur in cystatins of different stocks of *R. microplus*, 6 BrBmcys2c were isolated from Uruguayan *R. microplus* and compared at sequence level with those from Brazil and the DFCI database (TC21643) (Fig. 2). The Uruguayan sequences were called UrBmcys2c 1 (Mozo population), UrBmcys2c 2, 3, 4, and 6 (Rivera populations), and UrBmcys2c 5 (Cerro Largo population). The similarity at amino acid and nucleotide level between the Brazilian BrBmcys2c, Uruguayan BrBmcys2c, and the corresponding cystatin in the DFCI database sequences was 97–100%. Amino acid substitutions were found in signal peptides between the cystatin deposited in DFCI database,

BrBmcys2c, and UrBmcys2c1–4, in which leucines and isoleucines are replaced by valines, at positions 10 and 16, respectively. Furthermore, in BrBmcys2c and UrBmcys2c1–3, the glutamine acid at position 50 is replaced by aspartic acid, while in BrBmcys2c phenylalanine at position 78 is replaced by serine.

Gene expression

qPCR quantification of *R. microplus* cystatin mRNAs showed varying expression patterns between tick tissues and also developmental stages (Fig. 3). BrBmcys2a transcripts were detected in all tissues analyzed and were more abundant in the gut, salivary glands, ovary, and fat body. Moreover, BrBmcys2a expression level was higher in salivary glands and ovary of fully engorged over that in partially engorged ticks. Both BrBmcys2b and BrBmcys2c were expressed at similar levels in egg, larva, and the gut, while smaller amounts were found in the salivary glands, ovary, and fat body. BrBmcys2d and BrBmcys2e showed a higher expression level in the larva, while in other tissues these cystatin transcripts were absent, at low levels, or below the assay detection limit.

Phylogenetic analysis and in silico antigenicity

A phylogenetic tree of cystatins from *R. microplus* and other *Rhipicephalus* spp. was constructed from the deduced amino acid sequences (Fig. 4). The phylogeny yielded 2 clades bearing *R. microplus* cystatins, with similarity ranging between 54 and 91% to their respective orthologs (Fig. 4). Whereas BrBmcys2a, BrBmcys2b, and BrBmcys2d group with *Rhipicephalus* spp. cystatins, it was not possible to identify BrBmcys2c and BrBmcys2e orthologs. Antigenic index analysis of BrBmcys2a, BrBmcys2b and BrBmcys2d suggested high antigenicity in conserved regions of *Rhipicephalus* spp. cystatins (Fig. 5).
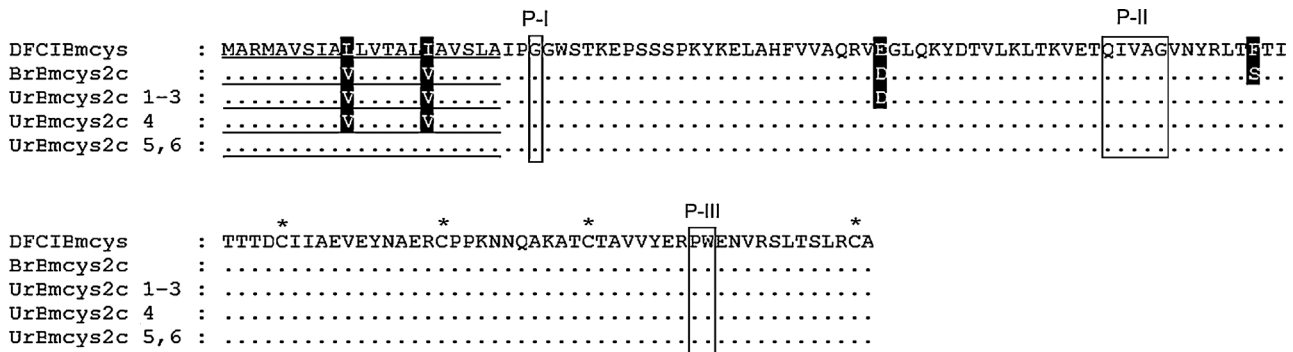
Fig. 2. Sequence alignment between BrBmcys2c, URBmcys2c (1–6), and DFCI database cystatins. Dots show conserved amino acids among cystatin sequences. Dark shading shows differential amino acid among cystatin sequences. Asterisks indicate the cysteine residues of 2 putative disulfide bridges. The cystatins motifs P-I (G), P-II (QxVxG), and P-III (PW) are boxed. Predicted cleavage signal peptides are underlined.
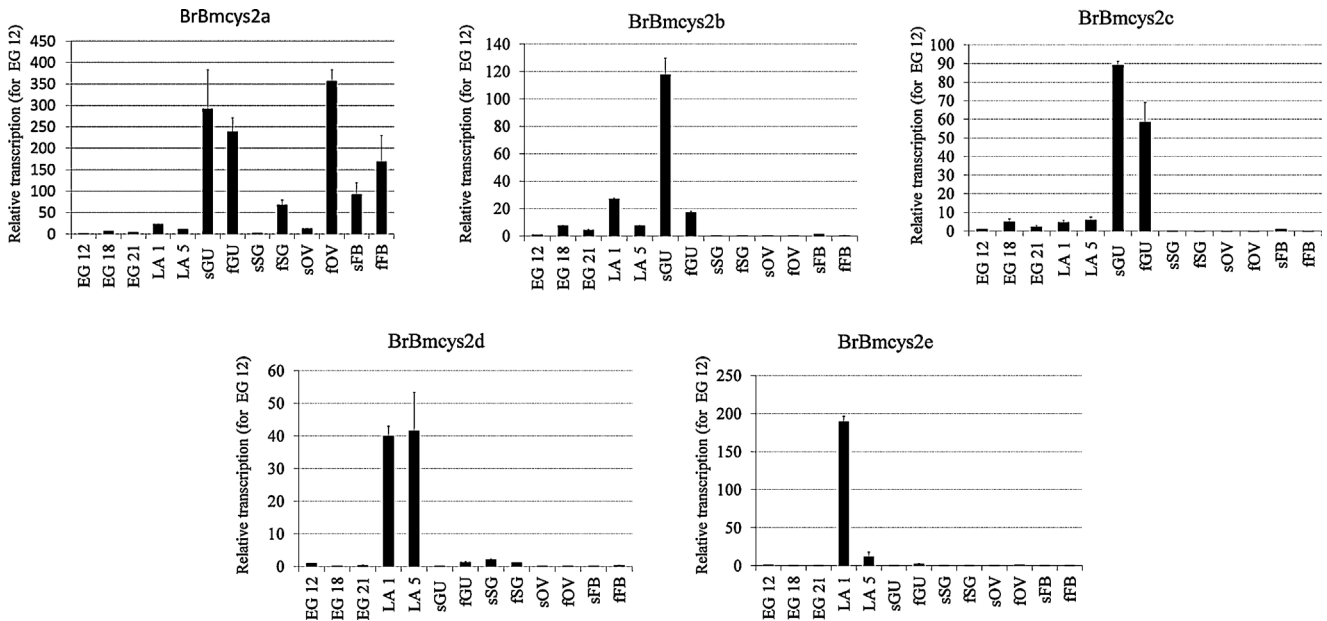
Fig. 3. Gene expression patterns in tissues and stages of *R. microplus* cystatins. The relative expression (%) refers to 12-day-old egg respective cystatin expression (1 unit). Each column represents the mean and standard deviation (error bars) of 2 analyses. EG, eggs; LA, larvae; GU, gut; SG, salivary gland; OV, ovary; FB, fat body; p, partially engorged adult female ticks; f, fully engorged adult female ticks. Numbers indicate the day of eggs and larvae collection.
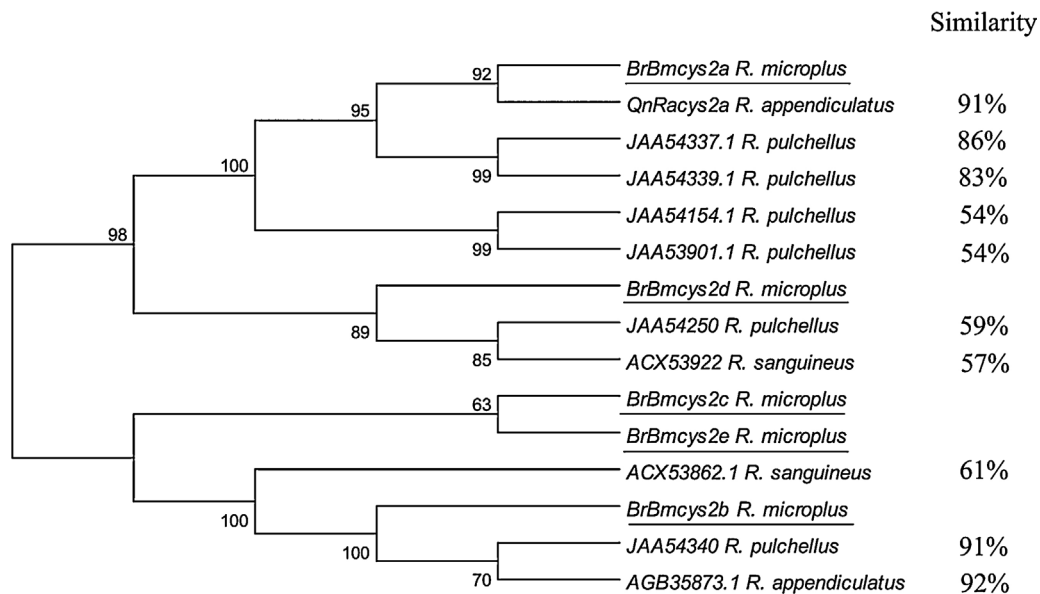
Fig. 4. Phylogenetic analysis of *R. microplus* and other *Rhipicephalus* spp. cystatins. The percent similarity among *R. microplus* and *Rhipicephalus* spp. cystatin orthologs is indicated. GenBank accession numbers sequences are described in "Materials and methods. Bootstrap values of 1000 simulations are shown at the branches.
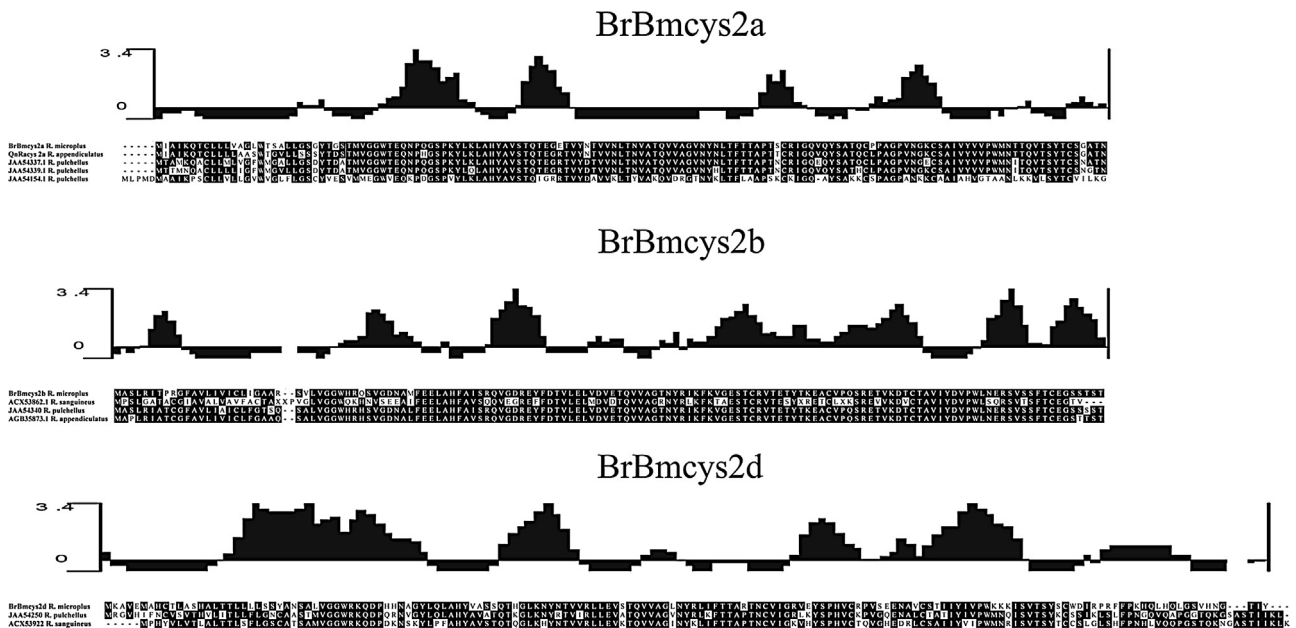
Fig. 5. Antigenicity analyses of 3 *Rhipicephalus (Boophilus) microplus* cystatins and sequence alignment of their *Rhipicephalus* spp. orthologs. Antigenic index plots predicted using the Jameson–Wolf algorithm, where an increased positivity is predictive of antigenic sites. Black boxes indicate conserved amino acid among sequences.

Immunogenicity in mice

The 3 cystatins from *R. microplus* that showed higher transcription level in the gut (BrBmcys2a, BrBmcys2b, and BrBmcys2c) were selected for expression in recombinant forms (Fig. 6). Purified rBrBmcys2b and rBrBmcys2c were subsequently used to immunize mice. Due to poor expression and recovery of rBrBmcys2a from *E. coli*, it was not used for immunization. Sera of representative mice in the inoculated groups displayed mixed patterns between the 3 *R. microplus* cystatins on Western blots (Fig. 6). Sera from mice immunized with rBrBmcys2b recognized rBrBmcys2b, rBrBmcys2a, and rBrBmcys2c. Interestingly, sera from mice immunized with rBrBmcys2c recognized rBrBmcys2c and rBrBmcys2a, but not rBrBmcys2b. The Western blot (Fig. 6) results corroborate the in silico analysis (Fig. 7) showing differences in antigenicity of the conserved regions between the *R. microplus* recombinant cystatins.

Discussion

Recent studies indicate that cystatins play a role in tick blood feeding, hemoglobin digestion, immune system regulation at the host-parasite interface, and pathogen transmission (reviewed by Schwarz et al., 2012). Thus, the prospect of impairing tick functions by blocking cystatins-mediated pathways is attractive in the pursuit of improved anti-tick vaccine (Kotsyfakis et al., 2007, 2008; Salát et al., 2010). This has previously been demonstrated through RNAi-mediated gene silencing and anti-cystatin antibodies production through vaccination (Karim et al., 2005; Kotsyfakis et al., 2007, 2008; Salát et al., 2010). In both approaches, it was observed that the blockade of cystatins impairs tick survival on the hosts as well as their feeding capacity.

Except for BrBmcys2e, all cystatin sequences from *R. microplus* analyzed in the course of this work exhibit the triple cystatin motifs forming the domain that characterizes type 2 cystatins. Currently it is not known whether the observed variant in the motif PW of BrBmcys2e correspond to a change in the function of this protein, and further investigations are required to shed more light on this possibility. Moreover, the 5 *R. microplus* cystatin sequences contain signal peptides and 4 cysteine residues on the C terminus, placing them under type 2 cystatins. Protein sequence similarity among the cystatins identified from *R. microplus* is low, suggesting that their enzyme targets in ticks or vertebrate hosts are different, as observed with previously characterized tick cystatins (Grunclová et al., 2006; Kotsyfakis et al., 2007; Yamaji et al., 2010; Zhou
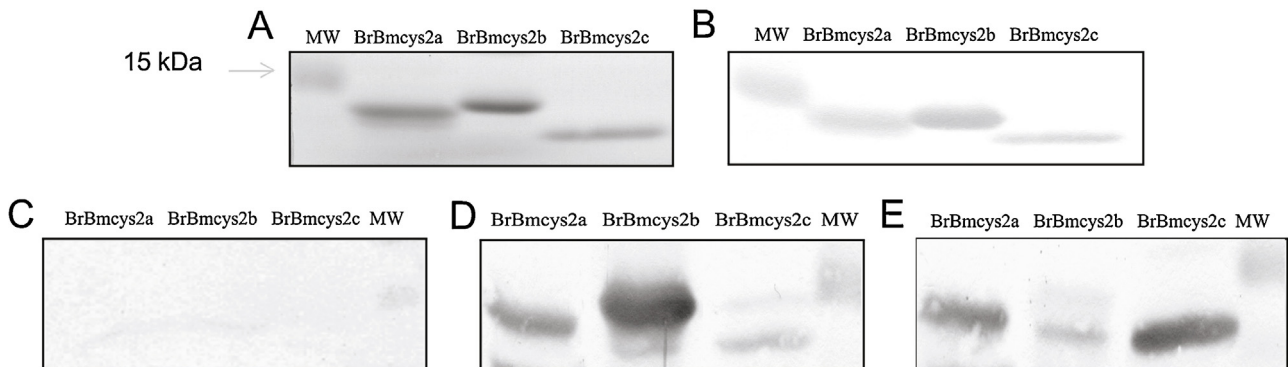

Fig. 6. Cross-immunogenicity between recombinant *R. microplus* cystatins in mice. (A) Recombinant *R. microplus* cystatins resolved by 16% SDS-PAGE stained with Coomassie blue. Western blotting of recombinant *R. microplus* cystatins probed with: (B) anti-histidine tag antibody; (C) negative control mice serum; (D) BrBmcys2b-immunized mice serum, and (E) BrBmcys2c-immunized mice serum. Molecular mass standards are expressed in kDa.
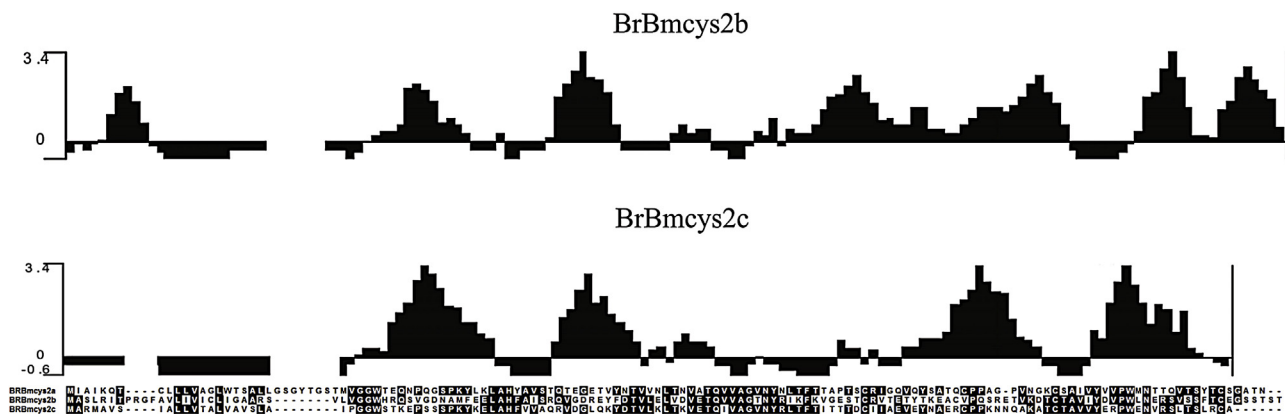
Fig. 7. Antigenicity analyses of BrBmcys2a, BrBmcys2b, and BrBmcys2c. Antigenic index plots predicted using the Jameson–Wolf algorithm, where an increased positivity is predictive of antigenic sites. Black boxes indicate conserved amino acid among sequences.

et al., 2009). Consistent with other non-orthologous cystatin genes (Schwarz et al., 2012), the degree of homology between *R. microplus* cystatins and cystatins from other tick species is highest around the common cystatin domain, but decreases in other regions.

Past studies indicated differential cystatin gene expression in hard as well as soft ticks. Hlcyst-2, a cystatin from *Haemaphysalis longicornis*, is abundant in the gut (Zhou et al., 2006), where it is involved in regulating hemoglobin degradation through inhibition of HlCPL-A, a cysteine protease present in the lysosomes of *H. longicornis* gut cells that plays a role in hemoglobin digestion (Yamaji et al., 2010). Like Hlcyst-2, Hlcyst-3 is largely expressed in the *H. longicornis* gut, but its function is yet to be identified (Zhou et al., 2009). HlSC-1, another *H. longicornis* cystatin, is associated with blood feeding processes, but exhibits high expression in salivary glands (Yamaji et al., 2009). In *I. scapularis*, the transcription pattern of cystatins was shown to fluctuate in response to blood feeding in the gut and in the salivary glands (Kotsyfakis et al., 2007; Ibelli et al., 2013). In the soft tick *O. moubata*, 2 cystatins (Om-cystatin 1 and Om-cystatin 2) are expressed at high levels in non-feeding ticks, indicating down-regulation following blood feeding (Grunclová et al., 2006). Om-cystatin 1 was found to be expressed mainly in the gut, while Om-cystatin 2 was found in the gut, in the salivary glands, in the ovary, and the Malpighian tubules. Similar to those studies, we observed variations in the gene expression of *R. microplus* cystatins between different developmental stages and also between tick organs, suggesting specific roles of these cystatins in tick physiology. Tissue/stage-specific expression patterns as well as the reported differential inhibition of cathepsins by tick cystatins (Grunclová et al., 2006; Lima et al., 2006; Kotsyfakis et al., 2007, 2008; Yamaji et al., 2009, 2010) suggest a non-redundant biological function for these inhibitors. The low redundancy in target-enzyme inhibition is an attractive consideration in employing tick cystatins as anti-tick vaccine components due to the possibility of interrupting multiple physiological processes, in addition to the lack of compensatory inhibitions between cystatin homologs.

A growing group of tick proteins have been evaluated as vaccine antigens against these ectoparasites (reviewed by Parizi et al., 2012; Seixas et al., 2012). Successful anti-tick vaccines based on recombinant proteins are thought to be correlated with a strong protective antibody response following inoculation with these antigens (de la Fuente et al., 1999; Hajdusek et al., 2010; Carreón et al., 2012; Moreno-Cid et al., 2013). Among the 3 cystatins that were highly expressed in the gut of adult *R. microplus*, BrBmcys2b and BrBmcys2c were found to be immunogenic in mouse in the recombinant form. Furthermore, mouse sera raised against these 2 proteins cross-reacted with BrBmcys2a. The difference in cross-reactivity among recombinant cystatins suggests the differential

presence of B cell epitopes in these proteins. Furthermore, bioinformatic analysis supports the presence of antigenic regions in the conserved regions of the cystatins.

The fact that *R. microplus* cystatins were found to be expressed in different tick organs and/or stages and the in silico analysis showed high antigenicity in conserved regions of *Rhipicephalus* spp. cystatin orthologs make them attractive candidates for inclusion in anti-tick vaccines. Since polymorphisms in the tick antigen Bm86 have been shown to make some populations of *R. microplus* resistant to vaccination (Garcia-Garcia et al., 1999, 2000; de la Fuente et al., 1999), the likelihood that these events occur with cystatins was evaluated. Since Brazil and Uruguay are South American livestock producers, BrBmcys2c from *R. microplus* populations from Uruguay was compared to the one obtained from the Brazilian ticks. At protein level, BrBmcys2c from ticks of the 2 countries are highly similar, suggesting that vaccine based on this antigen could be applicable to control ticks from these regions. However, laboratory and field vaccine studies are necessary to evaluate the potential of cystatins as protective antigens. In addition, there is the need to establish whether the observed transcription patterns of *R. microplus* cystatins are present in orthologous genes as well as the cross-reactivity level between cystatins of the *Rhipicephalus* spp. complex.

Acknowledgments

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4 CAPÍTULO 2: "Putative physiological roles of two *Rhipicephalus microplus* cystatins in partially and fully engorged tick"

Este capítulo apresenta os dados descritos no artigo em redação para o periódico *Veterinary Parasitology*: "Putative physiological roles of two *Rhipicephalus microplus* cystatins in partially and fully engorged tick*"*.

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Putative physiological roles of two *Rhipicephalus microplus* cystatins in partially and fully engorged tick

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ABSTRACT

Recent studies on tick physiology have demonstrated the importance of cystatins, specific cysteine protease inhibitors. Various cathepsins involved in tick endogenous metabolism as well as host-immune interactions have been characterized, clarifying the roles of cystatins in tick blood feeding, digestion and development. However, these functions remain unknown in *Rhipicephalus microplus*, a tick responsible for significant economic losses in livestock. Here we report the inhibition profile of cathepsins B, C, and L by two *R. microplus* cystatins, BrBmcys2b and BrBmcys2c. These cystatins showed distinct affinities for these cathepsins: BrBmcys2b inhibits all of them, showing higher affinity for cathepsin B, while BrBmcys2c does not inhibit cathepsin B, and shows higher affinity for cathepsin C than cathepsin L. Furthermore BrBmcys2b was detected in ovary, salivary glands and fat body of partial and fully engorged *R. microplus*. BrBmcys2c was detected in partially engorged *R. microplus* gut and in fully engorged *R. microplus* ovary, salivary glands and fat body. These results suggest that BrBmcys2b and BrBmcys2c play differential roles in tick physiology, modulating cathepsins involved in blood digestion, ovary development and host-immune system.

Keywords: cathepsin, inhibitor, cystatin, tick, *Rhipicephalus microplus*.

Introduction

Cystatins are tightly binding inhibitors of cysteine proteases (Nicklin and Barrett, 1984). In some parasites, these inhibitors are involved in internal protective and regulatory biological processes (Zavasnik-Bergant, 2008) as well as in the modulation of the host's defense responses (Hewitson et al., 2009). Classically, cystatins are divided in three groups known as stefin (type 1), cystatin (type 2), and kininogen (type 3), although in ticks only stefins and cystatins have been reported (Schwarz et al., 2012). It was only in recent years that the physiological functions of tick cystatins (Schwarz et al., 2012) and their target cysteine cathepsins (Renard et al., 2002; Yamaji et al., 2009) during tick blood-feeding, digestion and development became the object of more consistent research. Previous studies have also demonstrated that tick cystatins modulate host cathepsins involved in inflammation, antigen processing and presentation, phagocytosis, and cytokine expression processes (Zavasnik-Bergant, 2008). The regulation of these physiological processes by tick cystatins promotes blood uptake and survival of parasite while it is attached to the host.

The first tick cystatin biochemically or molecularly characterized was isolated from *Amblyomma americanum* (Karim et al., 2005). It was suggested that this cystatin participates in host immunomodulation and tick protection to harmful ingested factors during blood feeding. In *Ixodes scapularis*, two cystatins were biochemically characterized, Sialostatin L and Sialostatin L2 (Kotsfaskis et al., 2006; 2007). Sialostatin L showed host immune system modulation and cathepsins L, V, C, X, B and papain inhibition, while Sialostatin L2 inhibited cathepsins L, V, S and C, showing higher expression rate in late feeding. The enzymatic inhibition profile of *Haemaphysalis longicornis* cystatins for papain, cathepsins L, B, H, as well as tick cathepsins have been

characterized in previous research (Zhou et al., 2006, 2009, 2010; Yamaji et al., 2009, 2010). Furthermore, it was demonstrated that some of the cystatins from *H. longicornis* play a role in innate immunity (Zhou et al., 2006) and blood feeding (Yamaji et al., 2009, 2010). Cystatins from *Ornithodoros moubata* were able to inhibit cathepsins B, L, S, H and C (Grunclová et al, 2006; Salát et al., 2010), showing proliferation and cytokine release modulation in T-cell and dendritic cells (Salát et al., 2010). Finally, it was suggested that one *Rhipicephalus appendiculatus* cystatin present in nymph, male, and female gut after feeding is involved in blood digestion process (Imamura et al., 2013). Taken together, these results suggest that cystatins play widespread and distinct regulatory roles in different tick species.

Rhipicephalus microplus is one of the most consistently studied cattle tick specie, mainly because of the vast economic losses to the livestock industry that it causes (Grisi et al., 2002). However, despite the great interest in understanding the physiology of this tick, few studies have analyzed *R. microplus* cystatins. Some *R. microplus* cysteine proteases were identified and characterized (Renard et al., 2000; Estrela et al., 2007; Seixas et al, 2010), demonstrating the importance of these enzymes in a variety of physiological processes and parasite stages. Nevertheless, few regulatory mechanisms of *R. microplus* cysteine proteases have been characterized (Lima et al., 2006). Consequently, the control of *R. microplus* cysteine proteases activities by cystatins remains unknown. Against this background, in a previous work (Parizi et al., 2013) we analyzed the sequence properties and immunogenicity of putative cystatins from *R. microplus*. These cystatins showed a high degree of homology among *Rhipicephalus* spp., differential RNA expression patterns, as well as cross-reactivity between them, suggesting shared epitopes.

In order to improve the understanding of the physiological roles of these inhibitors, we characterized the inhibitory profile and tissue expression of two *Rhipicephalus microplus* cystatins, BrBmcys2b and BrBmcys2c. The presence of these proteins in different tissues and their ability to inhibit cathepsins differently suggest distinct roles for rBrBmcys2b and rBrBmcys2c in blood digestion and modulation of host immune response in *R. microplus* physiology.

Experimental procedures

Animals and ticks

Partially and fully engorged female ticks (Porto Alegre *R. microplus* strain) were collected from Hereford (*Bos taurus taurus*) cattle housed at Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Brazil for tissue dissection. Partially engorged *R. microplus* females weighing between 25 and 60 mg were recovered manually from calves (Gonsioroski et al., 2012). Cattle care was in accordance with institutional guidelines.

Hamsters were maintained in a P3 animal facility at Graduate School of Veterinary Medicine, Hokkaido University in accordance with the Institutional Animal Care and Use Committee guidelines.

Expression and purification of recombinant cystatins

Escherichia coli C41(DE3) and C43(DE3) strains were transformed with plasmids containing the BrBmcys2b and BrBmcys2c ORFs (Parizi et al., 2013). The recombinant proteins were expressed in SOB medium with 0.4 mM isopropyl-β-D-thiogalactopyrano for 16 h at 25 °C. Cells were further harvested by centrifugation at 10,000 x g for 10 min at 4 °C and resuspended in phosphate buffer containing 100 mM of Imidazol. For cell lysis, the suspension was sonicated five times for 30 s at 40 MHz on ice. The soluble and insoluble fractions were separated by centrifugation at 10,000 x g for 10 min at 4 °C.

The soluble fractions containing the rBrBmcys2b and rBrBmcys2c were purified by a nickel-chelating Sepharose chromatography (GE Healthcare). Briefly, the soluble fractions were filtered in 0.45-µm filters (Millipore) and then applied onto the columns

previously equilibrated with phosphate lysis buffer. Proteins of interest were eluted with phosphate buffer containing 150 mM of Imidazol at room temperature. Eluted fractions were purified by filtration (Centricon YM10 - 50,000 MW cut-off, Millipore), lyophilized, and dialyzed against PBS. Protein concentrations were determined by BCA Protein Assay kit following manufacturer's instructions (Thermo Scientific Pierce).

Enzymatic assays

Apparent inhibition constants (KIs) were estimated to measure the inhibitory profile of rBrBmcys2b and rBrBmcys2c. Enzymes were preincubated with rBrBmcys2b or rBrBmcys2c in concentrations ranging from 10 to 500 nM in the corresponding assay buffer for 15 min, and protease-specific substrates were added to estimate residual enzyme activity. Enzymes and substrates (all purchased from Sigma) concentrations are presented in Table 1. Assay buffer and enzymes were used as follows: 100 mM sodium acetate, pH 5.5, 100 mM NaCl, 1 mM EDTA, and 0.005% TritonX-100 for bovine cathepsin C and human cathepsin L; 100 mM sodium acetate, pH 5.5, 60 mM NaCl, 1 mM EDTA for bovine cathepsin B; 100 mM HEPES, pH 7.5, 1 mM EDTA for human cathepsin G. Substrates were as follows: Z-Phe-Arg-MCA for cathepsin L; Z-Arg-Arg-pNA for cathepsin B; Gly-Phe-pNA for cathepsin C; N-Succinyl-Ala-Ala-Pro-Phe-pNA for cathepsin G. Chromogenic assays were monitored by Spectramax Microplate Reader (Molecular Devices Corporation) at 405 nm of absorption. Fluorescence intensity was monitored at 370 and 460 nm for emission and excitation, respectively. Data were fit for appropriate tight-binding inhibitors using a nonlinear regression analysis equation (Morrison, 1969) performed using GraphPad Prism version 5.00 for Windows (GraphPad Software).

Tick tissues extraction

Salivary glands, ovary, gut and fat body were disrupted and homogenized using a mortar and pestle in an ice bath with 10 mM phosphate buffer, pH 7.2. The homogenate was centrifuged at 16,000 × g for 15 min at 4 ºC to remove the insoluble material and the soluble supernatant fraction was collected. The protein extracts were prepared according to the method previously described (da Silva Vaz et al., 1998).

Immunization of hamsters and sera collection

Two hamsters were subcutaneously inoculated with PBS or 50 µg of rBrBmcys2c previously produced (Parizi et al., 2013). Immunizations consisted of four doses at 14-day intervals with the recombinant proteins emulsified in Freund's incomplete adjuvant. Blood was collected 14 days after the last booster and serum was separated by spinning the samples at 10,000 × g for 5 min at 4° C. Sera aliquots were preserved at −20° C until use. Mice sera used in serological analysis were produced in a previous study (Parizi et al., 2013).

SDS-PAGE and Western blotting

The production of recombinant cystatins and the presence of native cystatin in tissues were analyzed by SDS–PAGE and Western blot. SDS-PAGE analysis was performed as previously described (Laemmli, 1970). For Western blot analyses, recombinant cystatins (1 µg protein/lane) and tissue extracts (150 µg protein/lane) were resolved in 16% gel for SDS-PAGE, followed by transference to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in PBS. For native cystatins recognition, the membranes were further incubated with mice and hamster sera diluted to 1:50. For recombinant cystatins recognition, anti-histidine tag antibodies

(1:2,000) were used. After sera incubations, anti-IgG species specific alkaline phosphatase (mouse sera) and peroxidase (hamster sera) conjugates (1:5,000) were used as secondary antibodies. Alkaline phosphatase revelations were performed with NBT (nitro blue tetrazolium) and BCIP (5-bromo-4- chloro-3-indolyl phosphate, Sigma) in PBS. Peroxidase revelations were performed with 3,3'-diaminobenzidine tetrahydrochloride, H_2O_2, and $CoCl_2$ in PBS.

Results

Production of recombinant cystatins

Soluble recombinant cystatins were expressed in *E. coli* (100 μg / L of culture medium) and purified by affinity chromatography (Supplementary Fig. 1). SDS-PAGE shows proteins of approximately 15 kDa that fit *in silico* rBrBmcys2b and rBrBmcys2c molecular size estimation. These recombinant proteins were recognized by anti-histidine tag antibodies in the Western blot assay. The purified recombinant BrBmcys2b and BrBmcys2c were subsequently used in inhibitory and vaccination assays.
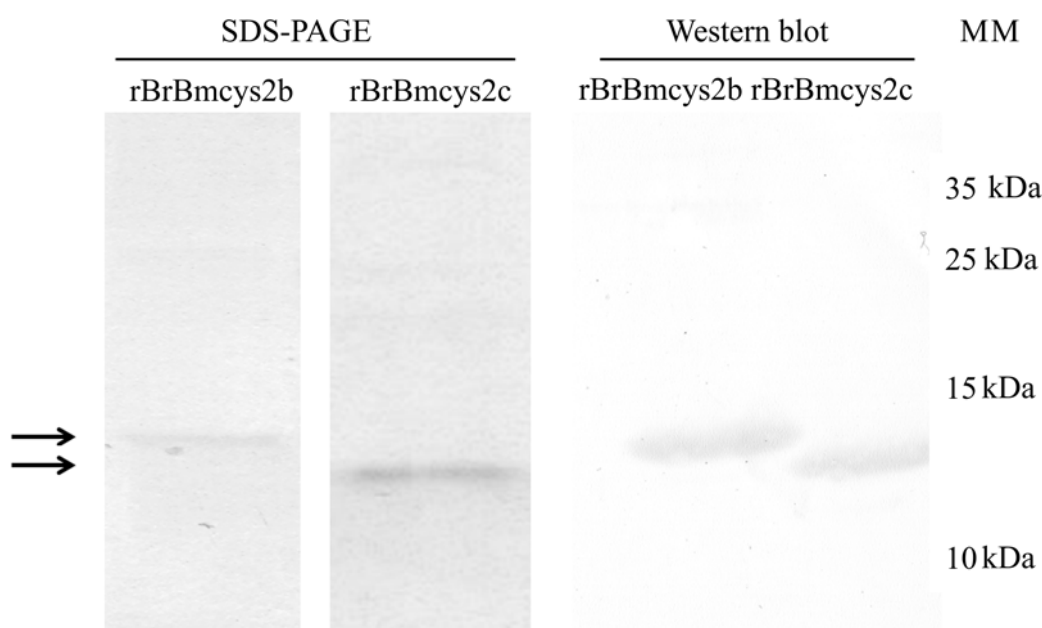
Supplementary Figure 1. Purity of recombinant BrBmcys2b and BrBmcys2c (indicated by arrows) analyzed by SDS-PAGE and Western blot. SDS-PAGE (16%) was stained with Coomassie blue G-250. Western blot was probed with anti-histidine tag antibodies alkaline phosphatase conjugate. MM - Molecular mass standards.

rBrBmcys2b and rBrBmcys2c inhibitory specificity

Inhibitory assays were performed to characterize the specificity of rBrBmcys2b and rBrBmcys2c for target enzymes. rBrBmcys2b and rBrBmcys2c modulated the activity of mammal cathepsins B, C and L at different levels. rBrBmcys2b inhibited all cysteine cathepsins, showing higher affinity for cathepsin B. In contrast, rBrBmcys2c did not inhibit cathepsin B, showing higher affinity for cathepsin C. Both cystatins were able to inhibit cathepsin L. Also, rBrBmcys2b and rBrBmcys2c inhibited these peptidases with apparent inhibition constants ranging from approximately 0.6 to 16.3 nM and 0.8 to 32.0 nM, respectively. Cathepsin G, a serine protease, was not inhibited by these recombinant cystatins (Table 1).

Table 1

Dissociation constants (Ki) values from cystatins for different proteinases

Enzyme	Family	Enzyme concentration	Substrate concentration	Ki (nM)	
				rBrBmcys2b	rBrBmcys2c
Cathepsin B [1]	Cysteine protease	0.500 μM	0.125 mM	0.6	n.i.
Cathepsin C [1]	Cysteine protease	0.160 μM	1.875 mM	16.3	0.8
Cathepsin L [2]	Cysteine protease	0.043 μM	0.020 μM	3.5	32.0
Cathepsin G [2]	Serine protease	0.100 μM	0.670 mM	n.i.	n.i.

n.i., not inhibited using 0.5 μM recombinant cystatin;
[1]Bovine cathepsins;
[2]Human cathepsins.

Recognition of native cystatins by mice and hamster sera

Sera against rBrBmcys2b and rBrBmcys2c were used to determine the presence of cystatins in *R. microplus* tissues as well as the cross-antigenicity between native and recombinant cystatins (Fig. 1). Native ovary, gut, salivary gland and fat body cystatins (apparent molecular mass of 14 kDa) were differentially recognized by these sera. In partially engorged female tissues, native cystatins were recognized by anti-rBrBmcys2b (in ovary, salivary glands and fat body) and rBrBmcys2c (in gut) sera. In fully engorged female tissues (in ovary, salivary glands and fat body), native cystatins were recognized by anti-rBrBmcys2b and rBrBmcys2c sera. Gut native cystatin in fully engorged female tissues was not analyzed due to the high background present in control sera (data not shown). Since these sera were raised against recombinant cystatins, this recognition shows the cross-antigenicity between native and recombinant cystatins. The mouse and hamster sera inoculated with PBS did not recognize native cystatins (data not shown).
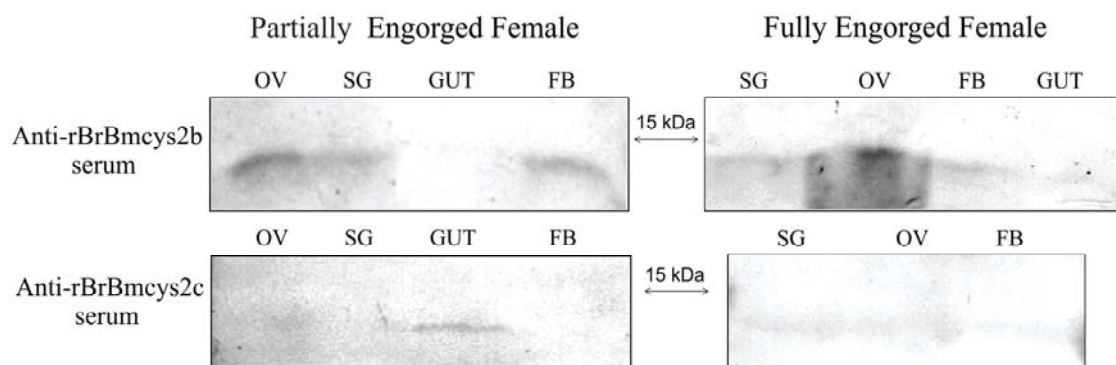
Figure 1. The presence of native cystatin was analyzed by Western blot in partially and fully engorged female extracts. Primary antibodies: anti-rBrBmcys2b and anti-rBrBmcys2c sera raised in mice and hamster, respectively. Secondary antibodies: anti-species specific alkaline phosphatase or peroxidase conjugates, respectively. OV, ovary; SG, salivary glands; FB, fat body.

Discussion

The function of cystatins in parasite physiological processes is a subject of growing interest among many research groups (Hartmann et al., 2003; Hewitson et al., 2009). In ticks, however, only recently the characterization of cystatin effector mechanisms began to be elucited (Schwarz et al., 2012). The main goals of these works were the characterization of cathepsin targets, tissue localization and immune system modulation by tick cystatins. To date, only one *R. microplus* cystatin was biochemically characterized (Lima et al., 2006). This cystatin is expressed in tick fat body, ovary and salivary glands, and inhibited cathepsin L and VTDCE, an egg *R. microplus* cysteine endopeptidase (Seixas et al., 2003), suggesting its role during tick embryogenesis.

In the present work, we characterized the inhibition profile and tissue localization of two *R. microplus* cystatins. To uncover the physiological role of these cystatins, we selected three cysteine proteases involved in host hemoglobin proteolytic degradation cascade by ticks (Horn et al., 2009; Franta et al., 2011). Interestingly, despite the capacity of rBrBmcys2b to inhibit cathepsins B, C and L, this inhibitor was not detected in female gut during rapid engorgement, a stage in which major blood degradation takes place (Franta et al., 2010). Among the tissues from partially engorged females analyzed, only BrBmcys2c was detected in the gut, suggesting its role in blood metabolism. A potential target for this cystatin would be BmCL1, a cathepsin L from *R. microplus* (Renard et al., 2000) that was only detected in tick gut during feeding stages. The pattern of native BrBmcys2c recognition corroborates previous qPCR results, which showed a higher mRNA transcripts expression in gut, as compared to other tick tissues (Parizi et al., 2013). However, BrBmcys2b transcripts also were shown to be higher in female gut, contrasting with the absence of BrBmcys2b protein detection in present work. One hypothesis to

explain this contradiction is that BrBmcys2b is synthesized in gut and further exported to other tissue, similarly to other tick proteins (Pohl et al., 2008; Galay et al., 2013).

In vitro and *in vivo* experiments have demonstrated the role of tick cystatins in the modulation of host immune system components responsible for anti-parasite infestation (Kotsyfakis et al., 2007, 2010; Salát et al., 2010). Similarly to Sialostatin L2 (Kotsyfakis et al., 2008), rBrBmcys2b and rBrBmcys2c were not detected for tick infested host sera (data not shown), indicating that the *R. microplus* cystatins are not immunogenic for bovines when inoculated through tick bite. The presence of BrBmcys2b and BrBmcys2c in *R. microplus* salivary glands as well as its ability to inhibit cathepsin L suggests the participation of these cystatins in mechanisms to avoid host immune system. Cathepsin L is involved in mammal immune system process by MHC class II-presentation pathway regulation (Hsing et al., 2005), as well as extracellular matrix breakdown during inflammation (Serveau-Avesque et al., 2006). Cathepsin L secreted by macrophages inside tick feeding cavity would destroy host tissue elasticity, which is required for the effective parasite attachment (Kotsfaskis et al., 2007). Inhibition of cathepsin L by secreted tick cystatins would help tick feeding, whereas the absence of these compounds would result in inflammation and parasite rejection as result of host immune response. This outcome was observed in tick cystatin knock down and vaccination experiments (Karim et al., 2005; Kotsfaskis et al., 2007; 2008; Salát et al., 2010). Consequently, host cathepsin L inhibition would result in a weaker immune response against tick infestation. Besides, the detection of BrBmcys2b in partially and fully engorged female salivary glands, suggests its importance throughout the engorgement period. The presence of BrBmcys2b was higher in partially and fully engorged female ovary, indicating that this cystatin is relevant in egg development. In ovary, a vitellin degrading cysteine endopeptidase (VTDCE) that plays a

crucial physiological role in egg maturation through vitellin mobilization (Seixas et al., 2003), is a potential enzyme target for BrBmcys2b.

In short, this work showed the differential presence of BrBmcys2b and BrBmcys2c in *R. microplus* tick tissues, and demonstrates that cathepsins B, C and L are inhibited by these cystatins in different degrees. These results suggest distinct tick physiological roles of these inhibitors: BrBmcys2b would act during egg development and in host immune system modulation, while BrBmcys2c would act during blood meal processing. Future RNAi and immunomodulatory experiments will better clarify the importance of BrBmcys2b and BrBmcys2c in tick physiology.

Acknowledgments

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5 DISCUSSÃO

As funções dos inibidores de cisteíno-peptidases dos parasitos durante a hematofagia e sobrevivência nos hospedeiros vêm sendo reveladas nas últimas duas décadas (HARTMANN *et al.*, 2003; HEWITSON *et al.*, 2009), embora somente nos últimos anos as cistatinas dos carrapatos venham despertando interesse no seu estudo (SCHWARZ *et al.*, 2012). Dados recentes indicam que as cistatinas dos carrapatos participam do processo de alimentação sanguínea através da regulação das peptidases responsáveis pela digestão do conteúdo ingerido, interferência na resposta do sistema imune dos hospedeiros, além de estarem envolvidas na imunidade inata desses parasitos (SCHWARZ *et al.*, 2012). Assim sendo, esses inibidores constituem alvos em potencial para o desenvolvimento de novas formas de controle dos carrapatos, pois interferências nas rotas fisiológicas controladas pelas cistatinas poderiam impossibilitar o correto desenvolvimento desse parasito e/ou expô-los a infecções por patógenos (KOTSYFAKIS *et al.*, 2007, 2008; SALÁT *et al.*, 2010). O uso das cistatinas como forma de controle contra a infestação por carrapatos evidenciou-se através de trabalhos de vacinação ou interferência por RNA (RNAi), nos quais carrapatos tiveram o seu desenvolvimento prejudicado durante a alimentação sanguínea (KARIM *et al.*, 2005; KOTSYFAKIS *et al.*, 2007, 2008; SALÁT *et al.*, 2010). Através desses estudos, foi observado que o bloqueio da ação das cistatinas afeta o ingurgitamento, assim como a sobrevivência dos carrapatos sobre o hospedeiro.

Até o momento, apenas uma cistatina do carrapato *R. microplus* havia sido caracterizada molecularmente e bioquimicamente, a Bmcystatin, pertencente ao tipo 1 (LIMA *et al.*, 2006). Assim sendo, o papel fisiológico das cistatinas de *R. microplus* ainda não está plenamente entendido, assim como o uso potencial desses inibidores no controle

imunológico do carrapato. No intuito de contribuir para a elucidação dessas questões, cinco cistatinas de *R. microplus* foram selecionadas para caracterização a partir de sequências identificadas em um transcritoma de glândulas salivares (FRANCISCHETTI *et al*., 2009). A escolha das cistatinas estudadas baseou-se no grau de conservação das sequências nos motivos responsáveis pela formação do domínio que caracterizam essa família de inibidores. O sequenciamento das ORFs dessas cistatinas confirmou a conservação total desses motivos, exceto para a BrBmcys2e, que apresentou uma alteração no motivo P3. Maiores estudos *in silico* e *in vitro* são necessários para esclarecer os efeitos acarretados por essa diferença na estrutura do domínio dessa proteína, e assim avaliar a capacidade inibitória frente às catepsinas. Além disso, as cinco sequências de cistatinas codificam um peptídeo-sinal na porção N-terminal, assim como quatro aminoácidos de cisteína na porção C-terminal, caracterizando essas proteínas como pertencentes ao grupo do tipo 2 das cistatinas. A similaridade entre as proteínas parálogas codificadas por essas sequências é baixa, variando de 28-42%, uma vez que análises filogenéticas identificaram possíveis ortólogas para essas cistatinas pertencentes a *Rhipicephalus* spp. com similaridades chegando a 91%. Similar ao observado em outras sequências de cistatinas de carrapatos (SCHWARZ *et al*., 2012), o grau de similaridade entre as cistatinas de *R. microplus* e de outros gêneros de carrapato é alta próximo dos motivos, mas baixa em outras regiões desses inibidores.

Após as análises da conservação e similaridades entre as sequências das cistatinas, a etapa seguinte para a caracterização das cistatinas de *R. microplus* foi a análise do grau de transcrição desses inibidores. Estudos de diversos gêneros de carrapatos duros e moles demonstraram uma expressão diferencial de transcritos de mRNA entre as cistatinas de diferentes estádios e tecidos. Hlcyst-2, uma cistatina de *H. longicornis,* apresentou uma

transcrição abundante no intestino desse carrapato (ZHOU *et al.*, 2006). Essa cistatina estaria envolvida na regulação da degradação do conteúdo sanguíneo ingerido pelo carrapato através da inibição da HlCPL-A, uma cisteíno-endopeptidase presente nos lisossomos das células do intestino desse carrapato e que está envolvida na digestão de hemoglobina (YAMAJI *et al.*, 2010). Semelhante à Hlcyst-2, a cistatina Hlcyst-3 possui uma transcrição majoritária no intestino do *H. longicornis*, mas sua função fisiológica ainda está para ser caracterizada (ZHOU *et al*, 2009). HlSC-1, outra cistatina de *H. longicornis*, exibe alta transcrição em glândulas salivares do carrapato e está associada ao processo de alimentação sanguínea (YAMAJI *et al*, 2009b). Em *I. scapularis*, o padrão de transcrição de duas cistatinas (sialostatina L e sialostatina L2) no intestino e nas glândulas salivares mostrou ser regulado positivamente em resposta a alimentação sanguínea (KOTSYFAKIS *et al.*, 2007, IBELLI *et al.*, 2013). No carrapato mole *O. moubata*, duas cistatinas (Om-cystatin 1 and Om-cystatin 2) são transcritas em altos níveis em carrapatos não alimentados, indicando a regulação negativa da sua expressão após a alimentação sanguínea (GRUNCLOVÁ *et al*, 2006). A transcrição de Om-cystatin 1 foi encontrada principalmente no intestino, enquanto transcritos de Om-cystatin 2 estavam presentes no intestino, nas glândulas salivares, no ovário e nos túbulos de Malpighi (GRUNCLOVÁ *et al*, 2006).

Semelhante a esses estudos, foram observadas variações na expressão de transcritos de cistatinas de *R. microplus* entre diferente estádios e tecidos, sugerindo papéis fisiológicos distintos dessas cistatinas na fisiologia desse carrapato. A BrBmcys2a foi a cistatina com os níveis de transcrição mais diversificados entre os tecidos, sendo detectada em ovos, larvas, intestino, glândulas salivares, ovário e corpo gorduroso de partenógina e teleóginas, o que sugere estar desempenhando o controle de cisteíno-peptidases endógenas

do carrapato. Os transcritos de BrBmcys2b e BrBmcys2c mostraram estar presentes majoritariamente no intestino do carrapato, sugerindo uma regulação do metabolismo da degradação dos componentes do sangue ingerido. Finalmente, a BrBmcys2d e BrBmcys2e mostraram maiores níveis de transcrição em larva, o que sugere a importância dessas proteínas em fases iniciais da vida do carrapato.

Outro ponto crucial para o entendimento do papel fisiológico das cistatinas é a caracterização do perfil de inibição enzimática. Dessa forma, grande parte dos trabalhos com cistatinas envolve a mensuração das afinidades de ligação frente a catepsinas comerciais. Em carrapatos, outra análise importante seria a possível modulação do sistema digestório do parasito ou imunológico dos hospedeiros por esses inibidores. Trabalhos com *I. scapularis* mostraram que a sialostatina L modula o sistema imune do hospedeiro e inibe catepsinas L, V, C, e B, além de papaína, enquanto a sialostatina L2 inibe catepsinas L, V, S e C (KOTSFASKIS *et al.*, 2006; 2007). Cistatinas de *H. longicornis* inibem catepsinas L, B, H, papaína, assim como catepsinas do próprio carrapato (ZHOU *et al.*, 2006, 2009, 2010; YAMAJI *et al.*, 2009b, 2010). Além disso, foi demonstrado que algumas dessas cistatinas desempenham papéis na imunidade inata do hospedeiro contra o protozoário *B. bovis* (ZHOU *et al.*, 2006) e durante a alimentação sanguínea (YAMAJI *et al.*, 2009b, 2010). Cistatinas de *O. moubata* inibem as catepsinas B, L, S, H e C (GRUNCLOVÁ *et al.*, 2006; SALÁT *et al.*, 2010), mostrando modular a proliferação e secreção de citocinas de células T e de células dendríticas (SALÁT *et al.*, 2010). Finalmente, foi sugerido que uma cistatina de *R. appendiculatus* presente em ninfa, macho e intestino de fêmea após alimentação está envolvida no processo de digestão sanguínea (IMAMURA *et al.*, 2013). Esses trabalhos sugerem a participação das cistatinas tanto em processos endógenos durante a digestão sanguínea no intestino, como a secreção desses inibidores no local de

fixação no hospedeiro para contrapor a resposta imune gerada contra a infestação pelo carrapato. Além disso, a alta especificidade de inibição das catepsinas-alvo seria um fator desejável para o emprego das cistatinas em uma vacina anti-carrapato, pois diminuiria a possibilidade da ação compensatória de uma cistatina paráloga.

Em *R. microplus*, a Bmcystatin nativa foi detectada nas glândulas salivares, no ovário e no corpo gorduroso, inibindo, na sua forma recombinante, uma catepsina L e a VTDCE, uma cisteíno-endopeptidase importante na formação do ovo (SEIXAS *et al.*, 2003), sugerindo a participação desse inibidor durante a embriogênese do carrapato. No presente trabalho, duas cistatinas foram produzidas e purificadas em níveis que possibilitaram a análise do seu perfil inibitório e de expressão tecidual. Para estabelecer o papel dessas cistatinas na fisiologia do carrapato, foram selecionadas cisteíno-peptidases envolvidas na rota metabólica de degradação proteolítica das hemoglobinas ingeridas do hospedeiro (HORN *et al.*, 2009; FRANTA *et al.*, 2011). rBrBmcys2b e rBrBmcys2c mostraram constantes de dissociação semelhantes a outras cistatinas de carrapatos descritas na literatura, variando de aproximadamente 0,6 a 32 nM. Interessantemente, embora a rBrBmcys2b tenha mostrado inibir as catepsinas B, C e L, essa cistatina não foi detectada em intestino de fêmeas durante a fase de ingurgitamento, estágio no qual a maior parte da degradação do sangue é realizada (FRANTA *et al.*, 2010). Diferentemente, entre os tecidos de partenóginas analisados, a BrBmcys2c foi detectada somente no intestino, sugerindo a sua participação do metabolismo do sangue ingerido. Um alvo potencial para essa cistatina seria a BmCL1, uma catepsina L de *R. microplus* presente apenas no intestino de partenóginas (RENARD *et al.*, 2000). O padrão de reconhecimento tecidual da BrBmcys2c nativa corrobora os resultados de qPCR, que mostraram uma maior expressão de transcritos de mRNA dessas cistatinas no intestino, comparado aos outros tecidos.

Curiosamente, a expressão dos transcritos de mRNA para BrBmcys2b também foi maior no intestino das fêmeas, contrastando com a falta de detecção da BrBmcys2b nativa nesse tecido. Uma hipótese para explicar essa contradição seria que a BrBmcys2b é sintetizada no intestino e exportada para outros tecidos, processo já descrito para outras proteínas do carrapato (POHL *et al.*, 2008; GALAY *et al.*, 2013).

Outra ação das cistatinas dos carrapatos seria a modulação dos componentes do sistema imune do hospedeiro responsáveis pela resposta antiparasitária, como demonstrado por experimentos *in vivo* e *in vitro* (KOTSYFAKIS *et al.*, 2007, 2010; SALÁT *et al.*, 2010). Similar a sialostatina L2 (KOTSYFAKIS *et al.*, 2008), rBrBmcys2b e rBrBmcys2c não foram detectadas pelo soro de hospedeiro infestados pelo carrapato (dados não mostrados), indicando que essas cistatinas de *R. microplus* não são imunogênicas para bovinos quando inoculadas durante o processo de alimentação do parasito. A presença de BrBmcys2b e BrBmcys2c nas glândulas salivares de *R. microplus*, assim como a capacidade dessas proteínas em inibirem catepsina L, sugere a participação dessa cistatina nos mecanismos de evasão do sistema imune do hospedeiro. As catepsinas L estão envolvidas na resposta imune de mamíferos pela regulação da via de apresentação de antígenos por MHC de classe II (HSING *et al.*, 2005), assim como no remodelamento da matriz extracelular durante o processo de inflamação (SERVEAU-AVESQUE *et al.*, 2006). As catepsinas L são secretadas por macrófagos na cavidade na qual o carrapato esta se alimentando, diminuindo a elasticidade tecidual necessária para a correta fixação do parasito (KOTSFASKIS *et al.*, 2007). Portanto, a inibição da catepsina L por cistatinas secretadas pelo carrapato poderia ajudar na fixação e alimentação desse parasito, enquanto a ausência da secreção desse inibidor resultaria em um processo inflamatório com a rejeição do parasito pelo hospedeiro. Experimentos em que o bloqueio da ação das

cistatinas dos carrapatos foram realizados por RNAi ou vacinação resultaram em aumento da inflamação, rejeição e diminuição da alimentação e sobrevivência dos carrapatos (KARIM *et al*., 2005; KOTSFASKIS *et al*., 2007; 2008; SALÁT *et al*., 2010). Consequentemente, a inibição de catepsinas L dos hospedeiros poderia resultar em uma resposta imune deficiente contra a infestação pelo carrapato. Interessantemente, coelhos infestados primeiramente por carrapatos silenciados para cistatinas e, após, por carrapatos não tratados, desenvolveram resistência contra esses parasitos, demonstrando a importância das cistatinas na modulação do sistema imune desse hospedeiro (KARIM *et al*., 2005; KOTSFASKIS *et al*., 2008). A detecção de BrBmcys2b em glândulas salivares tanto de partenóginas com de teleóginas indica a ação desse inibidor mesmo após o término do período de alimentação. Finalmente, dentre os tecidos analisados, a presença de BrBmcys2b foi maior nos ovários de partenóginas e teleóginas, sugerindo a participação dessa cistatina também durante o desenvolvimento do ovo do carrapato. No ovário, a VTDCE desempenha uma função essencial na maturação do ovo através do processamento da vitelina (SEIXAS *et al*., 2003), constituindo um potencial alvo para a BrBmcys2b.

Outro ponto importante caracterizado foi o potencial vacinal das cistatinas de *R. microplus* contra infestações por esse ou outras espécies de carrapatos. Nos últimos anos, um crescente número de proteínas de carrapatos vem sendo avaliadas como antígenos vacinais contra a infestação por diferentes espécies de carrapatos (PARIZI *et al*., 2012; SEIXAS *et al*., 2012). Estudos demonstram que vacinas que conferem proteção contra carrapatos estão relacionadas com os níveis de anticorpos produzidos pelo hospedeiro contra os antígenos inoculados (DE LA FUENTE *et al*., 1999; HAJDUSEK *et al*., 2010; CARREÓN *et al*., 2012; MORENO-CID *et al*., 2013). As cistatinas recombinantes mostraram ser imunogênicas quando inoculadas em camundongos (rBrBmcys2b e

rBrBmcys2c) e hamsters (rBrBmcys2c), sendo que os anti-soros gerados contra essas proteínas reconheceram também a BrBmcys2a. Curiosamente, o soro anti-rBrBmcys2c não reconheceu a rBrBmcys2b, embora o soro anti-rBrBmcys2b tenha reconhecido a rBrBmcys2c. A distinta reatividade cruzada entre as cistatinas recombinantes sugere diferenças na presença de epitopos de células B nessas proteínas. Análises *in silico* demonstram alta antigenicidade das BrBmcys2a, BrBmcys2b e BrBmcys2d em regiões conservadas entre essas e outras cistatinas do gênero *Rhipicephalus* spp. A alta similaridade em regiões antigênicas entre as cistatinas de *Rhipicephalus* spp. fazem desses inibidores candidatos atraentes para comporem uma vacina multi-espécies, embora mais estudos avaliando os níveis de expressão das cistatinas de outras espécies de carrapatos, assim como o reconhecimento cruzado pelos anti-soros entre as proteínas ortólogas sejam necessários.

6 CONCLUSÕES

Esses resultados sustentam a importância das cistatinas durante a alimentação sanguínea dos carrapatos, além de gerar conhecimento para auxiliar o desenvolvimento de novas estratégias de controle contra esses parasitos. Com base nos resultados obtidos, conclui-se que:

1) As cistatinas de *R. microplus* possuem motivos e estruturas que as caracterizam como pertencentes a família do tipo 2;

2) BrBmcys2a, BrBmcys2b e BrBmcys2e apresentam similaridade para com cistatinas de carrapatos do gênero *Rhipicephalus*;

3) As cistatinas de *R. microplus* possuem distintos perfis de transcrição nos tecidos de intestino, glândulas salivares, ovário, corpo gorduroso, ovo e larva desse carrapato;

4) BrBmcys2b foi detectada em ovário, glândulas salivares e corpo gorduroso de fêmeas parcialmente e totalmente ingurgitadas;

5) BrBmcys2c foi detectada principalmente no intestino de fêmeas parcialmente ingurgitadas;

6) rBrBmcys2b e rBrBmcys2c apresentaram distintos perfis de inibição para catepsinas B, C e L, sugerindo estarem envolvidas na regulação da digestão sanguínea e modulação do sistema imunológico do hospedeiro;

7) As rBrBmcys2b e rBrBmcys2c são imunogênicas, sendo que os anticorpos gerados contra essas proteínas reconhecem cruzadamente as cistatinas recombinantes e nativas de *R. microplus*, possibilitando o uso dessas proteínas em uma vacina recombinante.

7 PERSPECTIVAS

Os resultados obtidos nesse trabalho sustentam futuros experimentos de vacinação utilizando-se uma ou mais cistatinas de *R. microplus* contra infestações por esse carrapato. Os efeitos de anticorpos anti-cistatinas administrados através de alimentação artificial de *R. microplus* poderá direcionar as cistatinas mais promissoras para comporem um teste de vacinação. Além disso, análises da presença de cistatinas parálogos nos tecidos de *Rhipicephalus* spp possibilitariam testes de proteção cruzada.

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ANEXOS

Anexos A e B: artigos publicados como primeiro autor no período do doutorado, mas que não estão vinculados diretamente ao tema da tese.

Anexo C: Curriculum Vitae resumido.

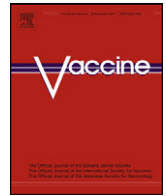**ANEXO A: Multi-antigenic vaccine against the cattle tick *Rhipicephalus (Boophilus) microplus*: a field evaluation**

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Participação dos autores no desenvolvimento do trabalho:

Luís F. Parizi, José Reck Jr., Daiane P. Oldiges, Melina G. Guizzo, Adriana Seixas: delineamento, realização e análise dos experimentos; redação do manuscrito.

Carlos Logullo, Pedro L. de Oliveira, Carlos Termignoni, João R. Martins, Itabajara da Silva Vaz Jr.: delineamento e análise dos experimentos; redação do manuscrito.

Multi-antigenic vaccine against the cattle tick *Rhipicephalus* (*Boophilus*) *microplus*: A field evaluation

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ABSTRACT

The tick *Rhipicephalus* (*Boophilus*) *microplus* is a blood-sucking ectoparasite of cattle that severely impairs livestock production. Studies on tick immunological control address mostly single-antigen vaccines. However, from the commercial standpoint, so far no single-antigen vaccine has afforded appropriate protection against all R. microplus populations. In this context, multi-antigen cocktails have emerged as a way to enhance vaccine efficacy. In this work, a multi-antigenic vaccine against *R. microplus* was analyzed under field conditions in naturally infested cattle. The vaccine was composed by three tick recombinant proteins from two tick species that in previous single-vaccination reports provided partial protection of confined cattle against R. microplus infestations: vitellin-degrading cysteine endopeptidase (VTDCE) and boophilus yolk pro-cathepsin (BYC) from *R. microplus*, and glutathione S-transferase from *Haemaphysalis longicornis* (GST-Hl). Increased antibody levels against three proteins were recorded after immunizations, with a distinct humoral immune response dynamics for each protein. Compared to the control group, a statistically significant lower number of semi-engorged female ticks were observed in vaccinated cattle after two inoculations. This reduction persisted for 3 months, ranging from 35.3 to 61.6%. Furthermore, cattle body weight gain was significantly higher in vaccinated animals when compared to control cattle. Compared to the single-antigen vaccines composed by VTDCE, BYC or GST-Hl, this three-antigen vaccine afforded higher protection levels against *R. microplus* infestations.

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1. Introduction

The tick *Rhipicephalus* (*Boophilus*) *microplus* has a significant economic impact on cattle breeding industry worldwide, estimated at billions of dollars annually [1,2]. This parasite causes a variety of deleterious effects in cattle, mainly as result of bodyweight reduction, blood loss and the transmission of disease-causing agents [1,2]. The intensive use of acaricides in order to control tick infestation raises concerns as to the potential presence of pesticide residues in milk, meat, and the environment [3]. For these reasons, a tick vaccine, as an alternative control method, is a major economic issue [4,5].

It has been repeatedly demonstrated that the stimulation of bovine immune system by tick proteins vaccination induces a

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protective immune response against *R. microplus* [6]. In 1986, a protective protein from *R. microplus* named Bm86 was discovered, when this antigen became the first tick antigen to compose a commercial vaccine against an ectoparasite [7]. Although vaccine formulations based on Bm86 in most cases elicit protective immune responses against *R. microplus*, they vary considerably in terms of protection level depending, among other things, on the genetic variability of tick and bovine populations [8–13]. Therefore, the discovery of new tick antigens focusing on those displaying minimal genetic variability among *R. microplus* populations could improve vaccination efficacy and reduce variation in the protection level afforded by the Bm86-based vaccines. However, except for a few studies [14], data regarding cross-reactivity between tick proteins are scarce, although some tick antigens have been shown to induce cross-protective immunity against some tick species [14,15]. Another strategy to enhance anti-tick vaccine efficacy is to combine two or more antigens [16]. The initial proof of concept supporting this approach came from vaccination experiments, in which mixtures of antigens were more efficacious than single components, including Bm86 [4]. Some experimental studies used this approach against tick infestations [16–23]; however, in most cases, this strategy resulted in a statistical significant but slightly improvement in protection level.

Although tick infestation experiments using bovines in confined indoors can indicate vaccine efficacy, field trials are necessary to evaluate vaccine performance under real husbandry conditions [24]. However, most of the protocols used in experiments to evaluate bovine vaccination against ticks employ confined bovines, a more practical and cost-saving approach, compared to field experiments which demand laborious handling of cattle and the availability of a large area [16,25]. Our research group has been studying several *R. microplus* molecules in order to find antigens that could be used in an anti-tick vaccine. In previous studies, immunizations of cattle with native or recombinant forms of an aspartic protease named *Boophilus*Yolk pro-cathepsin (BYC) induced overall protections (measured by the reproductive potential, including reduction in number and weight of engorging ticks and in egg weight and hatchability) around 30% [26,27]. Also, immunization with a *R. microplus* cysteine endopeptidase (VTDCE), involved in vitellin digestion [28,29], elicited an immunoprotection of 21% in vaccinated cattle [30]. More recently, an overall protective efficacy of 57% against *R. microplus* was achieved using a recombinant *Haemaphysalis longicornis* GST (rGST-Hl) [31]. In this work, we evaluated a multi-antigenic vaccine composed by BYC, VTDCE and GST-Hl recombinant proteins against *R. microplus* infestation in cattle. Vaccine efficiency was evaluated under field conditions, based on semi-engorged female tick numbers and weight gain differences between vaccinated and control cattle groups.

2. Materials and methods

2.1. Expression of the recombinant proteins

rGST-Hl, rBYC, and rVTDCE were expressed and purified as previously described [32–34]. Briefly, rBYC and rGST-Hl were expressed in *Escherichia coli* strain AD494 (DE3) pLysS. Recombinant VTDCE was expressed in *E. coli* strain BL21 (DE3) Star. The insoluble forms of rBYC and rVTDCE were solubilized with 6 M guanidine hydrochloride (GuHCl) and purified using a nickel-chelating Sepharose column (GE Healthcare, Uppsala, Sweden). The soluble form of recombinant GST-Hl was purified through affinity chromatography using GSTrap FF column (GE Healthcare, Uppsala, Sweden). Protein concentrations were determined by the Bradford method [35] and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using bovine serum albumin as standard.

2.2. Animals

From September 2009 to January 2010 (spring to summer), a total of 38 Aberdeen Angus and Devon cattle heads (15 ± 1-month-old) the beginning of the trial were maintained under field conditions in a experimental farm (Estação FEPAGRO São Gabriel, São Gabriel, RS, Brazil; 30°20′S, 54°15′W). Cattle were allowed to graze freely on natural pastures, characterized by annual grass species, and supplemented with mineral salt, receiving water *ad libitum*. All animals were treated with levamisole (600 mg/100 kg body weight) three times (days 22, 43 and 64) to avoid endoparasite infestations along the vaccine trial, and managed under identical conditions in the same paddock during the whole trial. Cattle were managed in accordance with local institutional guidelines and all procedures were in accordance with international guidelines [36].

2.3. Immunization protocols

Vaccinated and control groups were formed by 18 and 20 animals, respectively. Antigens were administered subcutaneously. Each dose consisted of a mixture of recombinant proteins rBYC, rGST-Hl and rVTDCE (200 μg each, 0.5 mL) mixed with 0.5 mL of adjuvant (Montanide 888 and Marcol 52), emulsified according to the vortex method [37]. The control group received an emulsion of PBS (0.5 mL) plus adjuvant (0.5 mL). Both groups received three booster injections at 21-day intervals (days 22, 43, and 64).

2.4. Cattle sera collections and body weight

Blood samples (10 mL) were collected via caudal vein from pre-immunized and post-immunized cattle (days 1, 78 and 127), and used for sera recovery. Blood samples were centrifuged at $5000 \times g$ for 10 min and sera were stored at $-20\,°C$. At days 1 and 127, all bovines were weighted.

2.5. SDS-PAGE and Western blot

SDS-PAGE and Western blot analysis were performed as previously described [31]. Purified recombinant proteins (1 μg protein/lane) were applied to SDS-PAGE (14% gel). For Western Blot, the nitrocellulose membranes were incubated with cattle sera (diluted 1:100) collected on days 1 and 78.

2.6. Antigen-specific IgG detection in sera by dot-blot

Levels of antigen-specific antibodies in the serum samples were assessed by dot-blot. Nitrocellulose membrane circles of 0.5 cm of diameter were coated with 1 μg of each antigen in PBS. The membranes were dried and incubated for 1 h at 37 °C with blotto [38], followed by a second incubation with cattle sera diluted in blotto (1:100) for 16 h at 37 °C. Washing times with blotto for 10 min ensued, and the peroxidase conjugated antibody diluted in blotto (1:5000) was added and incubated for 1 h at 37 °C. After three washes with PBS for 10 min, the membranes were incubated with 2.5 mg 3,3′-diaminobenzidine tetrahydrochloride, 10 μL H_2O_2, and 150 μL $CoCl_2$ in 5 mL of PBS. The recognition levels were quantified by gel scanning, and were analyzed using the software Image J [39].

2.7. Tick analysis

Along the vaccination trial, bovines were continuously exposed to tick infestation (since the beginning of the immunization process) because they were under natural conditions in a tick-infested pasture. Attached adult female ticks (sized between 4.5 mm and 8.0 mm) were counted on the left side of vaccinated and control groups, to follow the tick infestation rate [40]. Animals were
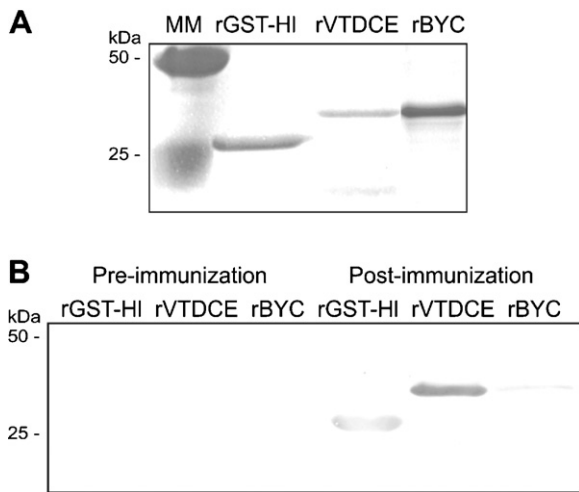
Fig. 1. Purity and antigenicity of recombinant GST-Hl, VTDCE and BYC proteins, analyzed by 14% SDS-PAGE stained with Coomassie blue G-250 (A) and Western blot (B). Western blot was probed with the pre-immunized and post-immunized sera from one bovine belonging to vaccinated group. Molecular mass standards are expressed as kDa.

immobilized and ticks were counted by the same investigator. All examinations were carried out at the same period of the day (morning/afternoon). Tick count was a blind-procedure: the investigator did not know which bovines belonged to control or vaccinated groups.

2.8. Statistical analysis

Statistical significance differences among the experimental groups concerning level of antigen-specific antibodies, tick count and cattle body weight gain was analyzed by Student's t test. Data were expressed as mean ± S.E.M. of each group. A p value of less than 0.05 was considered significant. Statistical analysis was performed using GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, USA) software.

3. Results

3.1. Production of recombinant proteins

The recombinant proteins BYC, GST-Hl and VTDCE were expressed in *E. coli* strains and purified by affinity chromatography. The purity of the three recombinant proteins was analyzed by a 14% SDS-PAGE (Fig. 1A). All preparations showed a major protein band for rBYC, rGST-Hl, and rVTDCE in the gel, and these bands matched the predicted molecular masses for respective proteins.

3.2. Development of humoral immune response in cattle

Dot blot analysis revealed an increased antibody recognition level of vaccinated bovine sera (collected at day 78) to the three recombinant proteins, compared to the vaccinated bovine pre-immune sera (day 1) (Fig. 2). Compared to day 1, the level of recognition from vaccinated cattle sera on day 78 for rGST-Hl, rVTDCE and rBYC increased by more than 6, 10, and 2 times, respectively. The level of recognition remained constant at the end of the experiment (day 127) for rGST-Hl, reducing by half for rVT-DCE, and returning to pre-immunization level for rBYC. Also, the level of recognition measured from vaccinated cattle sera was approximately 8, 4, and 2.5 times higher for rGST-Hl, rVTDCE, and rBYC respectively, than those recorded from animals injected with placebo on day 78.
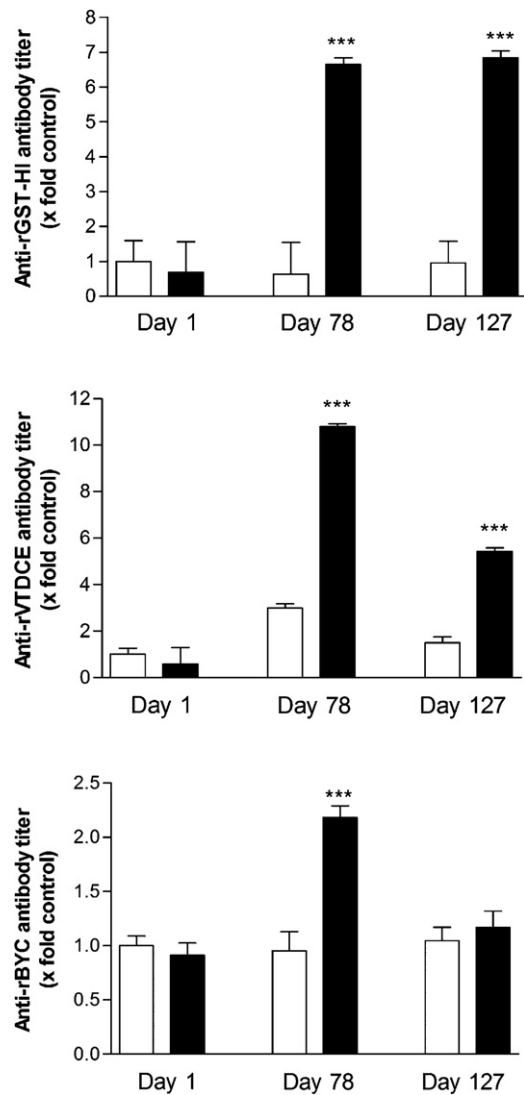

Fig. 2. Antigens-specific IgG levels from cattle sera analyzed by dot-blot. The levels of recognition of pre-immunized (day 1) and post-immunized (days 78 and 127) cattle sera from vaccinated and control groups were analyzed for recombinant proteins GST-Hl (A), VTDCE (B) and BYC (C). ***$p < 0.001$ (Student's t-test).

Western blot revealed that sera from one representative bovine of the vaccinated group recognize all recombinant proteins (Fig. 1B). The proteins rBYC, rGST-Hl and rVTDCE were not recognized by pre-immune serum of this animal.

3.3. Vaccination effect on tick infestation and body weight

The reduction in the number of ticks attached to bovines conferred by immunization with rBYC, rGST-Hl and rVTDCE is shown in Fig. 3 and Table 1. In the first three counts, tick number means from both groups were similar. From the fourth count on (days 36–127), means in the two groups were statistically different, except for day 57. During this period, bovines vaccinated with recombinant proteins showed statistical reductions that ranged from 35.3 to 61.6% (Table 1) in the number of semi-engorged ticks, as compared with the control group. Interestingly, even before the immunization period had ended it was already possible to detect a drop in tick infestation (Fig. 3, day 36). Also, there was an increase in cattle body weight in both groups between days 1 and 127, although the gain was statistically higher in the vaccinated group (Fig. 4). In
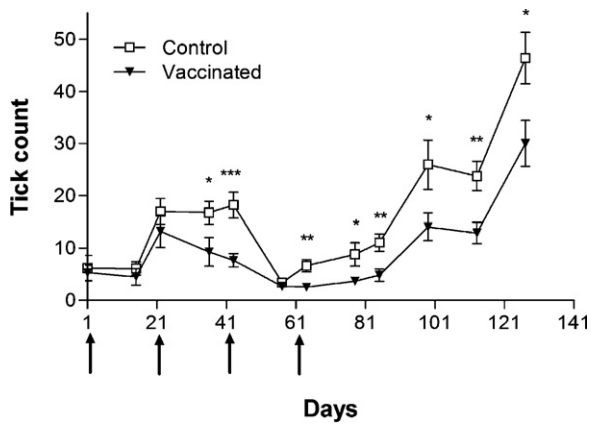
Fig. 3. Kinetics of the average numbers of semi-engorged ticks in the vaccinated and control groups. Arrows indicate the days of immunization. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (Student's t-test).
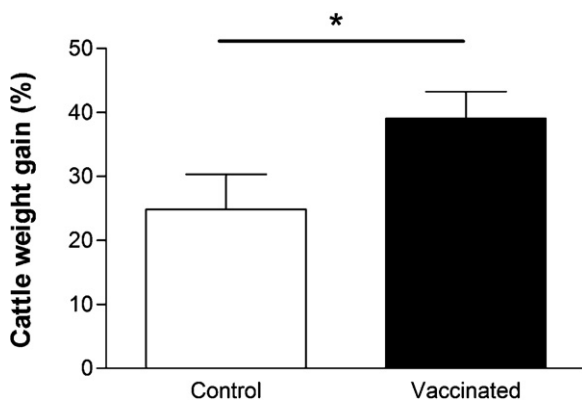

Fig. 4. Body weight gain in cattle along the vaccination trial. Cattle body weight gain in vaccinated group and control groups is expressed as percentage of mean body weight gain from day 1 to day 127. $*p < 0.05$ (Student's t-test).

the vaccinated and control cattle groups, body weight gain was 39% and 25%, respectively.

4. Discussion

Tick vaccines derived from the gut antigen Bm86 have been extensively investigated in the quest for a suitable tick control

Table 1
Effect of cattle vaccination on the number of semi-engorged *R. microplus* females.

Day	Tick count [a]		
	Control group	Vaccinated group	Difference (%) [b]
1	6.1 ± 2.4	5.3 ± 1.4	14.2
15	6.1 ± 1.3	4.5 ± 1.6	26.2
22	17.0 ± 2.4	13.2 ± 3.1	22.2
36	16.7 ± 2.3	9.3 ± 2.7	44.6[*]
43	18.2 ± 2.5	7.7 ± 1.3	57.9[***]
57	3.4 ± 0.6	2.7 ± 0.6	21.6
64	6.6 ± 1.2	2.6 ± 0.5	61.6[**]
78	8.8 ± 2.2	3.7 ± 0.6	58.3[*]
85	11.0 ± 1.7	4.8 ± 1.2	56.3[**]
99	25.9 ± 4.7	14.1 ± 2.6	45.8[*]
113	23.7 ± 2.8	12.9 ± 2.0	45.7[**]
127	46.4 ± 4.9	30.0 ± 4.4	35.3[*]

[a] Measured by counting the number of semi-engorged female ticks on the animals (average values ± S.D.).
[b] Difference (%) = 100 × [1 − (vaccine/control)].
[*] $p < 0.05$ (Student's t-test).
[**] $p < 0.01$ (Student's t-test).
[***] $p < <0.001$ (Student's t-test).

method. This antigen was shown to be partially protective against *R. microplus* field infestations in Australia, Cuba, and in some regions of Argentina, Brazil, Mexico and other countries [12,13,24,41,42]. These and other studies provide proof of concept for anti-arthropod vaccines. Nevertheless, following the commercialization of Bm86-based vaccines, a considerable body of results challenged the initial optimism that Bm86 would be effective against all *R. microplus* populations [24,43,44]. Consequently, there is a need to enhance the efficacy of the available tick vaccines as well as to develop new ones against other tick species, especially of medical and veterinary importance. Several antigens are currently under field investigation [14,45,46], though so far no single antigen has been found to achieve the desired protection threshold against all tick populations under field conditions [14,45]. To increase the field performance of anti-tick vaccine candidates, it is theoretically possible to design a multi-component vaccine, a concept that has already been shown to work against other parasites [16,47,48]. Theoretically, vaccines composed of synergistic antigens could elicit more effective responses against ticks [16]. However, limited studies reporting comprehensive evaluation of the performance of tick antigens cocktails against tick infestation have been published [16–23].

The proteins selected as antigens in this study play crucial physiological roles in ticks, such as vitellin mobilization (BYC and VTDCE) [28,29,49] and detoxification (GST) [50,51]. Indeed, previous studies demonstrated that these antigens, when administered in a mono vaccine, induce partial protective immune responses [27,30,31]. In these studies, the biological parameters evaluated to analyze tick control were the number of fully engorged ticks, egg laying capacity, and egg fertility, while the main parameter affected in ticks fed on vaccinated cattle was the number of fully engorged ticks, although the other parameters investigated were also affected, improving overall protection. These studies also demonstrated the immunogenicity of rGST-Hl, rBYC, and VTDCE and confirmed that specific IgG were elicited in vaccinated cattle for these proteins.

The present work demonstrated that these three recombinant proteins are immunogenic in cattle when administered simultaneously, although differences in immune response dynamics occur between antigens. In agreement with previous studies [27,30,31], we found that rGST-Hl elicited a more persistent humoral response than rBYC and rVTDCE. Immunization with the three recombinant proteins together induced a partial protective immune response in the experimental animals, evidenced by a decrease in the number of female ticks feeding on the vaccinated animals, in comparison with the control group. The number of females feeding on the hosts was statistically different between the two groups 14 days after the second immunization, and remained lower in the vaccinated group until the last day of the experiment (days 36–127). During days 43–85, vaccination conferred a statistically significant protection against tick infestation, ranging from 56.3 to 61.6%. However, the protection decreased to 35.3% two months after the last booster, along a decrease in antibody levels to rBYC and rVTDCE, suggesting the importance of these antibodies in protection rates obtained in previous counts.

The reduction in tick infestation following immunization with the three proteins is directly correlated with cattle body weight gain. Actually, body weight signals cattle fitness, a major productive parameter that is used as an indicator of vaccine effectiveness in field trials [1,41,42]. Under experimental conditions, body weight gain was significantly higher in vaccinated animals than in the control group. This effect seems to be a result of reduction in cattle damage by parasitism due to blood loss caused by the attaching ticks, and consequently, an improving in the overall health of the cattle.

In sum, the immune response generated by simultaneous vaccination with rGST-Hl, rBYC, and VTDCE affects tick physiology,

decreasing the number of females feeding in the host, resulting in an improved body weight gain of cattle. When compared to rGST-Hl, rBYC, or VTDCE single-antigenic vaccination in confined cattle, the multi-antigenic vaccine produced higher protection against R. microplus infestation. In spite of the differences between the vaccination protocols, these results demonstrate the possibility of developing a cattle multi-antigenic vaccine against *R. microplus* that seems to be more effective than a single antigenic vaccine against tick infestation under natural field conditions. More work is necessary to evaluate the economic benefits of a multi-antigen or a single-antigen vaccine to control ticks. However, the use of such vaccine, associated with existent and/or available control methods could result in a more efficient control of *R. microplus*.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.08.078.

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ANEXO B: The quest for a universal vaccine against ticks: Cross-immunity insights

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Review

The quest for a universal vaccine against ticks: Cross-immunity insights

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ABSTRACT

As blood-sucking parasites, ticks inflict great damage to animals and humans in many parts of the world. The continued use of chemical acaricides is not sustainable due to increasing tick resistance, growing public concern over drug residues in food and in the environment, and the high cost of developing new acaricides. Therefore, an alternative control strategy is urgently needed. Vaccines against ticks have been shown to be functionally feasible, as highlighted by the success of Bm86 vaccines against *Rhipicephalus (Boophilus) microplus* and closely related tick species. However, a limited number of tick antigens with cross-protective epitopes have been characterized so far, limiting widespread deployment of the available vaccines, including those derived from Bm86. Therefore, identifying tick antigens with potential broad-spectrum protection against multiple tick species is subject of vigorous research at present. In this paper, progress towards effective anti-tick vaccines is reviewed in the light of emerging data from studies including heterologous tick challenge. Taken together, these studies indicate that the decades-long search for a universal tick vaccine is making progress, with such a vaccine likely to be based on multiple cross-reactive antigens.

Introduction

Ticks are exclusively haematophagous ectoparasites with a worldwide distribution (de la Fuente et al., 2008a). In addition to inflicting direct damage by their voracious blood feeding, ticks rank second only to mosquitoes as vectors of disease-causing pathogens in humans, domestic and wild animals (de la Fuente et al., 2008a), leading to direct and indirect economic losses estimated at billions of dollars worldwide (Guerrero et al., 2006). The current methods of controlling ticks and tick-borne diseases (TBDs) rely heavily on topical application of chemical acaricides (Ghosh et al., 2007). However, the continued overreliance on these pesticides is unsustainable as there is widespread cross-species resistance (Rodriguez-Vivas et al., 2011), growing public health concern over chemical residues in animal food products and the environment, and of course the cost of developing and registering them continues to increase. Alternative strategies for controlling ticks and TBDs are needed (Ghosh et al., 2007).

Immunological control of ticks using vaccines is a feasible cost-effective and environment-friendly alternative to the use of chemical acaricides (Jongejan, 1998; Sonenshine et al., 2006; Willadsen, 2006; de la Fuente et al., 2007a). The use of vaccines, unlike chemical pesticides, poses minimal risks of selecting acaricide-resistant ticks, since point mutations that render acaricides ineffective are less likely to alter epitopes on target proteins (Willadsen, 2004), and, although difficult to evaluate, existing evidence suggests concomitant use of vaccines and acaricides can reduce the selection of resistant ticks in field populations (Willadsen, 2004). However, the mechanisms that might lead to resistance against tick vaccines are poorly understood. Furthermore, although the evolutionary rate and long-term immunological stability of a given tick antigen cannot be predicted with precision (Pepper, 2008), rapid sympatric speciation driven by host immune specificity has been shown to occur in ixodid ticks (De Meeûs et al., 2010).

Although the potential for vaccines to reduce tick infestations is a well-established concept, the lack of highly efficacious antigens (except for Bm86) remains a major limitation to the development of such vaccines. Bm86-based vaccines are highly effective against *Rhipicephalus (Boophilus) microplus* and their discovery and commercialization has spurred great interest in the field of tick vaccinology, leading to numerous vaccine trials (de la Fuente et al.,

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2007a). In addition to Bm86, a growing list of tick proteins has been identified and evaluated as candidate participants in new vaccines, which could complement and overcome the limitations of the existing anti-tick vaccines (Almazán et al., 2012; Seixas et al., 2010, 2012).

A highly desirable feature for the ideal anti-tick vaccine component-antigen is the ability to elicit a protective immune response across more than one tick species or genus. This would have practical application in the control of multiple tick vectors and, quite importantly, boost tick control in countries with limited capacity to develop vaccines against local tick species. Understandably, the majority of past studies addressing anti-tick vaccines have addressed specific tick species. Hence, in this paper, we review a number of recent studies reporting protective immunity against heterologous tick feeding. We conclude by discussing the possible directions that tick vaccinology could take in the future in the light of emerging laboratory and field trials data.

Over the past three decades or so, an increasing number of tick proteins have been evaluated as components of tick control vaccines (de la Fuente et al., 1999, 2011; de la Fuente and Kocan, 2006; Willadsen, 2006; Parizi et al., 2009a). However, given that close to 900 tick species have been documented (Guglielmone et al., 2010), without considering intra-specific genetic heterogeneity observed in many ixodid species (Labruna et al., 2009; Odongo et al., 2009), it follows that animals bred in different geographic areas are exposed to different tick species/stocks. To control such a diverse group of vectors with existing vaccines requires formulating different immunization regimens for every region and/or tick species, a costly and technically challenging approach.

Developing a universal anti-tick vaccine consisting of one or more common tick antigens capable of triggering protective immune responses against heterologous tick challenges would be both economically and technically attractive (Fragoso et al., 1998). Moreover, the finding of an antigenic protein conserved between mosquitoes and ticks (Canales et al., 2009a; Prudencio et al., 2010a) has boosted the prospects of a pan-arthropod vaccine. The development of a universal vaccine could therefore afford to rely on highly conserved tick proteins with limited and manageable antigenic variations, which are capable of inducing protective cross-reactive immunity against different tick species. In this regard, the availability of gene sequences for vaccine candidates from different vectors allows sequence comparison and evaluation of possible cross-reactivity (Parizi et al., 2009b; Canales et al., 2009a).

Bm86 and its homologues

Currently, two vaccines that confer significant protection against R. microplus infestation and reduce or replace the use of acaricides have been commercialized in Cuba, Australia, Mexico, Colombia and Brazil (de la Fuente et al., 2007a). The efficacy of these vaccines, prepared using recombinant Bm86 produced in bacteria or yeast, ranges from 51–91% in the field. This difference in protection reflects the vaccine susceptibility of different R. microplus strains, as well as host factors such as nutritional status and breeds (Rodriguez et al., 1995; Patarroyo et al., 2002).

It has been shown that the observed variation in susceptibility to Bm86 vaccines in different regions of the world is caused by sequence heterogeneity in the Bm86 gene within R. microplus strains (Garcia-Garcia et al., 2000). Tick population studies in Argentina revealed polymorphisms in the Bm86 gene that result in a soluble rather than a membrane-borne protein, as is the case in ticks from Australia and Cuba, making these ticks resistant to vaccination with the original Bm86 (de la Fuente et al., 1999; Garcia-Garcia et al., 2000). To overcome this resistance, a new recombinant vaccine based on Bm95, a Bm86 homologue, was developed. This new vaccine protected cattle against R. microplus infestations both in Argentina and Cuba (Garcia-Garcia et al., 2000). In a different vaccination strategy, synthetic peptides were designed from Bm86 by considering biochemical properties of the Bm86 glycoprotein, such as hydrophobicity and hydrophilicity potential. These peptides demonstrated an efficiency of 81% and 36% when used as a vaccine in cattle (Patarroyo et al., 2002). In a related study, Sales-Junior et al. (2005) reconstituted one of these peptides with saponin prior to vaccinating cattle. The authors observed that R. microplus infestation was reduced by 81%. Although attempts have been made to develop a DNA vaccine based on the Bm86 gene (de Rose et al., 1999; Ruiz et al., 2007), DNA vaccination against ticks of veterinary or medical importance is still in its infancy (Ghosh et al., 2007).

In several regions of the world, notably Africa and Latin America, R. microplus coexists with other Boophilus species, including Rhipicephalus (Boophilus) annulatus and Rhipicephalus (Boophilus) decoloratus, both of which are vectors of economically important livestock diseases (Walker, 1994; Estrada-Peñã et al., 2006). Consequently, there is a need to deploy vaccines capable of protecting against multiple Boophilus species in these regions. A number of studies are being undertaken to evaluate cross-immunity against heterologous tick infestations using recombinant Bm86. Fragoso et al. (1998) demonstrated, for the first time, the potential of using the Bm86 antigen in controlling R. annulatus infestations. Challenging cattle with R. annulatus larvae following vaccination with Bm86 resulted in a protection level of above 99%, in comparison with the control group. Vaccination affected tick viability, as determined by the number of ticks reaching maturity and diminished fecundity, which was indicated by a significant decrease in egg laying.

In another successful vaccination experiment, no R. annulatus nymph and adult stages were recovered from cattle vaccinated with Bm86 (Pipano et al., 2003). In contrast, in the animals in the control group, tick infestations ranged from 1380 to 4653 per animal. In that study, it was also observed that cattle infested with the progeny of females of R. annulatus infected with Babesia bigemina were not infected by the haemoparasite. Since B. bigemina is transmitted exclusively by nymphal stages of R. annulatus, these results support the observation that immunity induced by vaccination with Bm86 affects the physiology of larvae and/or nymphs (Pipano et al., 2003). Recently, a Bm86 vaccination trial in cattle involving R. microplus and R. annulatus infestations, reported overall vaccine efficacy of 60% and 100%, respectively (Hajdusek et al., 2010), indicating the potential of Bm86 as a component of a multi-species anti-tick vaccine.

Canales et al. (2009b) demonstrated that Ba86 protein (Bm86 homologue from R. annulatus) is effective in controlling infestation with R. annulatus and R. microplus. In the case of cattle infested with R. microplus, based on evaluating fecundity in the engorged females, including oviposition and fertility of eggs, the overall effect of vaccination with Ba86 was 71%. This supports a previous study that found sera cross-reactivity and amino acid similarity of over 90% between Bm86 and Ba86 proteins (Canales et al., 2008).

The protection provided by Bm86 vaccination against tick infestation by R. microplus, R. decoloratus, Hyalomma anatolicum anatolicum and Hyalomma dromedarii was tested in cattle by de Vos et al. (2001). Vaccination reduced reproductive capacity by 74% and 70% for R. microplus and R. decoloratus, respectively. For H. a. anatolicum ticks, there was an overall 50% reduction in the total weight of nymphs engorging on Bm86 vaccinated calves. In the case of H. dromedarii, there was a 95% reduction in the number of nymphs engorging and a further 55% reduction in weight of the surviving ticks. These observations represent significant cross-species and cross-genus cross-protection with a single antigen, suggesting conservation of protective epitopes in these ticks.

Odongo et al. (2007) demonstrated that sera from cattle immunized with Bm86 cross reacted with native Bd86, the Bm86

homologue from *R. decoloratus*. Subsequently, two conserved linear peptides were identified in the Bd86 protein through epitope mapping, whereby amino acid sequence similarity of 86% was observed between Bd86 and Bm86. When *R. decoloratus* ticks were fed on Bm86-vaccinated cattle, mean weight of engorged females (56%) and egg production (61%) were shown to decrease. In another study, a cross-reactive antibody was induced following immunization with a synthetic peptide derived from Bd86 (Kopp et al., 2009). Monoclonal antibody raised against this peptide recognized Bm86 homologues in immunohistochemical analyses of guts from *R. microplus, R. decoloratus, H. a. anatolicum* and *R. appendiculatus*.

A vaccine formulation consisting of recombinant Bm95 antigen was found to overcome resistance associated with low susceptibility to Bm86 vaccination by some *R. microplus* strains (Garcia-Garcia et al., 2000). Moreover, the ability of this antigen to provide cross-protection against other tick species has also been evaluated recently. Among these experiments, recombinant Bm95 protein was recently used in the formulation of a vaccine against infestation by the tick *Rhipicephalus haemaphysaloides* in cattle (Sugumar et al., 2011). This vaccine reduced burden of larval, nymph, and adult stages of the tick, with an efficacy of 98.7%, 84.6%, and 78.9%, respectively.

A different strategy used to design enhanced anti-tick vaccines was has also been reported recently (Almazán et al., 2012). In that study, three BM95 immunogenic peptides were fused with *Anaplasma marginale* major surface protein 1a N-terminal (MSP1a) region to form a peptide chimera that was subsequently tested in cattle against infestation with *R. annulatus* and *R. microplus* ticks. This BM95-MSP1a chimera had vaccine efficacy of 74% against *R. annulatus*, significantly higher than that observed against *R. microplus* (64%). The high protection against *R. annulatus* ticks is comparable to that observed by Hajdusek et al. (2010), suggesting that *R. annulatus* ticks are highly susceptible to Bm86 vaccination, and can be controlled together in areas where they overlap. This is significant since *R. annulatus* is the main vector of *Babesia bovis* and *B. bigemina* in the region extending over northern Africa, the Middle East and some parts of southern Europe (Bock et al., 2004).

Following successful vaccinations with Bm86 against multiple tick species in cattle, data from vaccine trials in other animal hosts are beginning to emerge. In a recent study, domestic dogs vaccinated with Bm86 were challenged with *R. sanguineus* (Perez-Perez et al., 2010). This led to a reduction of 38%, 29%, and 31% in attaching larvae, nymphs, and adult females, respectively. Furthermore, adult female tick fecundity was impaired by vaccination, as indicated by a reduction in full engorgement bodyweight, a decrease in both egg mass and efficiency rate of conversion to eggs. De la Fuente et al. (2006a) evaluated the physiological functions of two Bm86 homologues in *R. sanguineus* by RNA interference (RNAi). Knocking down these two genes resulted in considerable reduction in the number of ticks feeding to completion, as well as those attaining reproductive maturity.

Experimental vaccination with recombinant Bm86 has also been conducted in both white-tailed and red-tailed deer, with both hosts mounting strong antibody responses (Carreón et al., 2012). Furthermore, the antibody titre corrected positively with protection, ranging from 18 to 40% against *R. microplus* in red deer. More interestingly, when white-tailed deer were vaccinated with either Bm86 or tick subolesin, vaccine efficacy against *R. microplus* infestation was found to be comparable, at 76% and 83%, respectively.

Other tick proteins

Ticks possess two forms of ferritins (the iron-storage proteins) namely, an intracellular form (FER1), and a secreted form (FER2), with the latter, perhaps, being unique to ticks (Hajdusek et al.,

2009). In addition, FER2, an iron transporter in tick haemolymph, is expressed in all tick stages, and silencing it by RNAi adversely affects tick physiological processes such as blood acquisition and reproduction (Hajdusek et al., 2009). When recombinant ferritin 2 from *Ixodes ricinus* (IrFER2) was used to vaccinate rabbits, the animals were protected from *I. ricinus*, with an overall vaccine efficacy of 98%, attributed to a reduction in the number and weight of engorged ticks and egg fertility (Hajdusek et al., 2010). Similarly, when recombinant ferritin 2 from *R. microplus* (RmFER2) was used to immunize cattle, it had a vaccine efficacy of 64% and 72% against *R. microplus* and *R. annulatus*, respectively, with the protection level against *R. microplus* being comparable with that obtained elsewhere with Bm86.

The protein 64TRP is expressed in tick salivary glands (Havlikova et al., 2009) and seems to form part of the cement cone that anchors tick mouthparts to the host skin, preventing leakage of fluids and allowing ticks to remain firmly attached for several days (Trimnell et al., 2002). One antigenic cross-reactivity study has shown the potential of 64TRP constructs as anti-tick vaccines (Trimnell et al., 2005). In that experiment, guinea pigs were immunized with recombinant form of 64TRP protein from *R. appendiculatus* ticks, and challenged with nymphs and adults of *R. sanguineus* and *I. ricinus*. The immunized animals developed a protective immune response of humoral and cell type, apparently against the gut and salivary glands of these ticks. In another study, mice immunized with 64TRP constructs had reduced tick-borne encephalitis virus transmission by *I. ricinus* and high survival rate when exposed to lethal virus challenge (Labuda et al., 2006). Moreover, antigenic cross-reactivity of 64TRP from *R. appendiculatus* with its homologues in *R. sanguineus, I. ricinus, Amblyomma variegatum*, and *R. microplus* was confirmed by Western blot (Trimnell et al., 2005).

Tick subolesin and mosquito akirin are orthologous proteins which have been demonstrated to be involved in gene-expression regulation (Goto et al., 2008; Galindo et al., 2009; de la Fuente et al., 2008b). There is high amino acid similarity between these proteins corresponding to conservation of antigenic epitopes (Canales et al., 2009a). Moreover, linear and conformational protective epitopes from recombinant tick subolesin and mosquito akirin have been predicted using overlapping oligopeptides scan and phage display libraries (Prudencio et al., 2010a), enabling the possibility of development of a universal vaccine directed against ticks and mosquitoes.

Almazán et al. (2005) reported data showing that immunizing rabbits with recombinant subolesin from *Ixodes scapularis* could suppress infestations from nymphal stage of *Amblyomma americanum* and *Dermacentor variabilis* ticks. A similar experiment was performed by vaccinating cattle with subolesin derived from *R. microplus* and challenging the animals with *R. annulatus* and *R. microplus* (Almazán et al., 2010). In that test, the vaccine efficacy in vaccinated animals was 60% for *R. annulatus* and 51% for *R. microplus*, in comparison with the control group. Also, a chimera protein containing *R. microplus* subolesin fused to MSP1 was used in a vaccination trial in cattle against *R. annulatus* (Almazán et al., 2012). This SUB-MSP1a vaccine reduced *R. annulatus* infestations by 64%. A trial was set up to evaluate protection against tick infestations in red and white-tailed deer using the *Aedes albopictus*-derived akirin or *R. microplus* subolesin, respectively (Carreón et al., 2012). These vaccinations produced a 25–33% reduction in the number of *Hyalomma* spp. and *Rhipicephalus* spp. ticks attaching on the red deer, and protection of 83% in white-tailed deer against *R. microplus*, based on tick infestation, tick oviposition, and egg fertility.

Glutathione S-transferases (GSTs) constitute a family of biotransformation enzymes involved in metabolic detoxification of xenobiotics and other endogenous compounds (Agianian et al.,

2003). GSTs can be found in most animals (Agianian et al., 2003), and several tick GSTs have also been characterized (He et al., 1999; Rosa de Lima et al., 2002; da Silva Vaz et al., 2004a,b). We have recently reported a cross-protection trial using recombinant GST from the tick *Haemaphysalis longicornis*. Firstly, we evaluated the antigenicity of recombinant tick GSTs in mice, where we recorded a heightened humoral response in animals immunized with recombinant *H. longicornis* GST (rGST-Hl), compared to those immunized with recombinant *R. microplus* GST (rGST-Bm), perhaps as a result of differing levels of antigenicity between these proteins (unpublished data). In a further vaccination experiment, we vaccinated cattle with rGST-Hl and challenged the vaccinated animals with adult *R. microplus* ticks to determine cross-protection. In the vaccinated animals, a reduction of 53% and 52% in number and weight of engorged ticks was observed, respectively, while egg laying capacity and egg fertility of ticks feeding in vaccinated bovines were 0.6% and 8% lower than the control group, respectively. The overall efficacy ratio against *R. microplus* was 57%, when compared to the control group (Parizi et al., 2011).

Calreticulin (CRT) is a calcium-binding protein secreted by ticks into their hosts. The presence of CRT in tick saliva (Jaworski et al., 1995; Ferreira et al., 2002) and other parasite secretions (Kasper et al., 2001; Suchitra and Joshi, 2005; Cabezon et al., 2008) suggests a role for this protein in tick feeding, as well as immunosuppression and anti-haemostasis activity in the host. For an immunogenic comparative analysis, recombinant CRTs of *R. microplus* (rBmCRT) and *H. longicornis* (rHlCRT) were used to immunize cattle (Parizi et al., 2009b). Further, antigenic index analysis of HlCRT and BmCRT using the Jameson–Wolf algorithm indicated that both proteins are very similar antigenically, although six different epitopes were found between the two tick CRTs sequences. These data were corroborated by competitive ELISA analyses using anti-CRT antibodies in sera from vaccinated cattle, which suggested the presence of different epitopes within the proteins. Western blot analyses showed that anti-rBmCRT and anti-rHlCRT bovine sera could also recognized the native proteins in *R. microplus* larvae extracts. In addition, sera of cattle immunized with *R. microplus* saliva and extract of salivary glands could recognize both recombinant CRTs (Parizi et al., 2009b).

Several other tick proteins have potential cross-reactive epitopes but these proteins have not been studied in full detail, and their capacity to protect against tick infestation has not been properly evaluated. For instance, rabbit sera against immunogenic glycoproteins (GLPs) from the tick *H. dromedarii* recognizes GLPs from other tick species such as *R. sanguineus*, but not *Ornithodoros moubata* (El Hakim et al., 2011). Sterba et al. (2011) analyzed the antigenic similarities of *Dermacentor marginatus*, *R. appendiculatus*, *Rhipicephalus pulchellus*, and *R. sanguineus* lectins/fibrinogen-related haemolymph proteins. These proteins were all recognized by sera directed against the tick lectin Dorin M and the haemagglutination activity of the tick species *R. appendiculatus* and *D. marginatus*.

Discussion

The capacity of an antigen to induce cross-reactive immunity in the host is essential when considering candidate vaccines to control infestation by multiple tick species. Differences in tick physiological processes and host specificity may alter individual tick species susceptibility to the host immune response following vaccination. In addition, several other factors influence the outcome of a vaccine regimen, including the genetic variability of the target tick population, breed and nutritional status of the animal as well as the kind of adjuvants used. Further, a combination of proteins expressed in different tissues or at different stages of the vector's

life cycle could yield more potent vaccines since different stages and/or tick tissues could be targeted (Havlikova et al., 2009; Almazán et al., 2012). Thus, the use of proteins such as GST, which is expressed in different tissues in the tick, could lead to a multi-target vaccine.

Bm86, a 'concealed' gut antigen was the first antigen used in a commercial anti-tick vaccine. The expression of this protein on gut cells shields it from the host immunity (Willadsen et al., 1988), and as a result, it has not been subjected to adverse selective pressure that would render it less immunogenic (Willadsen et al., 1988; Imamura et al., 2007). The findings above correlate well with the observed level of immunity against both homologous and heterologous tick challenges. On the other hand, 'exposed antigens', which come into contact with the vertebrate-host immune system during blood-feeding, are likely to be less immunogenic as a result of prolonged exposure to the host immune defences (Bishop et al., 2002). However, it has been argued that these exposed antigens could potentially provide long-term protection following vaccination through repeated 'boosting' arising from tick feeding in the field (Bishop et al., 2002; Nuttall et al., 2006). A future anti-vaccine could have dual action by incorporating both concealed and exposed antigens, a vaccination strategy that has been shown to reduce the transmission of a tick-borne virus (Nuttall et al., 2006).

The remarkable success with Bm86 vaccination against *R. microplus* infestations has led great impetus to the search of more immunogenic proteins, leading to the discovery of several Bm86 homologues in other tick species, as well as entirely different classes of proteins (Trimnell et al., 2005; Liao et al., 2007; Parizi et al., 2009a, 2011; Jeyabal et al., 2010; Perez-Perez et al., 2010; Carreón et al., 2012). In the present review, we have come across evidence suggesting that Bm86-based vaccines could be used to control ticks of other species successfully, thus expanding their range beyond Australia, Cuba and South America.

In silico analysis of Bm86 from different tick species is necessary to predict potential cross-reactivity among these vectors, since an inverse correlation exists between Bm86 vaccine efficacy and sequence variations within its gene locus (Garcia-Garcia et al., 1999). In a molecular study conducted by Peconick et al. (2008), in which sequenced fragments of Bm86 of *R. microplus* were analyzed, the gene sequences of Rs86 from *R. sanguineus*, and HA98 from *H. a. anatolicum* indicated they were highly similar. In other studies, a high degree of identity was described between the Bm86 gene and its homologues Rs86 in *R. sanguineus* (Fang and Xu, 2007) and Ba86 in *R. annulatus* (Canales et al., 2008). Novel proteins that are structurally homologous to Bm86 were recently reported (Nijhof et al., 2010). These proteins, named ATAQ, which are structurally related to Bm86, were discovered through a combination of bioinformatics and RACE strategies with varying levels of amino acid sequence homology being detected between them and Bm86.

In other studies, a lower degree of homology has been observed between Bm86 and two of its homologues, Hd86 in *Hyalomma scupense* (Ben Said et al., 2012) and Hl86 in *H. longicornis* (Liao et al., 2007). For Haa86, the Bm86 homologue of *H. a. anatolicum*, in silico analysis predicted that there were seven epidermal growth factor-like domains which were structurally similar to those of Bm86 (Azhahianambi et al., 2009); the identity between these domains of Bm86 and Haa86 ranged from 51.3% to 78.3%. The recombinant forms of Haa86 induced an immune response in cattle that was able to reduce *H. a. anatolicum* infestation (Azhahianambi et al., 2009; Jeyabal et al., 2010) and to reduce *Theileria annulata* transmission to host (Jeyabal et al., 2010). So, Bm86 homologues could be useful not only for tick control but also in a transmission-blocking vaccine strategy.

Sequence polymorphism among Bm86 homologues could explain, at least for some antigens, the high efficacy of Bm86-derived vaccines against *R. annulatus* (Canales et al., 2009b). These findings

support the hypothesis that proteins conserved among tick species, including Bm86, could be useful in the development of immunological control against infestations with multiple tick species. Furthermore, as a concealed antigen, Bm86 is under minimal selection pressure; thus, the antigen epitopes have been retained in many closely-related species. Elucidating the biological function of Bm86 could perhaps reveal the functional and structural basis behind the retention of highly immunogenic regions on this protein and its homologues.

Despite these promising observations, completely ineffective vaccine trials have also been reported with Bm86 in *R. appendiculatus* and *A. variegatum* ticks (de Vos et al., 2001), indicating that Bm86 may not protect against all species despite taxonomic proximity to *R. microplus*. This observation is attributed to the fact that other factors besides sequence homology influence the efficacy of Bm86 vaccines against heterologous challenge (Willadsen, 2008; Kamau et al., 2011). Nevertheless, the use of Bm86 may extend to other vectors, as demonstrated with the mite *Dermanyssus gallinae* (Harrington et al., 2009), an important ectoparasite in the poultry sector. Vaccinating birds with Bm86 was found to induce a highly protective immune response against this mite. In addition, characterizing new vaccine targets is on-going. For instance, a novel strategy for identifying tick immunogens was recently reported. Vaccinating mice and cattle using phages bearing *R. microplus* mimotopes elicited a humoral response that was deleterious to *R. microplus* (Prudencio et al., 2010b). Moreover, sera from the vaccinated animals recognized specific tick proteins in *R. microplus*.

Ferritins are an example of a new class of vaccine targets that could overcome limitations associated with Bm86 (Hajdusek et al., 2009, 2010), specifically ferritin 2, an intracellular iron-transporter without orthologues in vertebrates. Vaccine studies with recombinant forms of this antigen showed vaccine efficacy comparable with that commonly obtained with Bm86 (Hajdusek et al., 2010). Since all ticks must contend with large amounts of iron during blood-feeding, ferritin 2, like ferritin 1, is perhaps conserved across tick species making it a suitable target for cross-reactive universal vaccine.

Cross-reactive anti-vector vaccines targeting arthropods sugar moieties have also been proposed (Dinglasan et al., 2005; Mejia et al., 2006). N-linked and O-linked glycans are extensively conserved across arthropods, (Mejia et al., 2006), designating them as suitable vaccine targets. N-glycans are particularly of interest as vaccine targets due to their high immunogenicity coupled by limited evolutionary diversity (Mejia et al., 2006). The role of glycosylated proteins in ticks, like many others, is likely well conserved, potentially allowing cross-reactivity. Interestingly, recombinant forms of Bm86 and Bm95, which are effective against *R. microplus* and related tick species, are significantly glycosylated both in native form and when expressed in *Pichia pastoris* (Gonzalez et al., 2004). It is plausible that some of the epitopes in these antigens are located along glycosylation sites, although the role of sugar epitopes in the immune response against tick antigens is poorly understood (de la Fuente et al., 2006b). To the present, no vaccine trial specifically targeting tick glycans has been reported, indicating that this concept is still at infancy.

Overall, the success of Bm86 in the field, the conservation of its antigenic epitopes across genera and the world-wide distribution of *R. microplus* suggest that this antigen continues to be a compelling candidate as a component of future universal vaccines. Extensive genetic variability in field tick populations exists and, as a result, field strains display varying susceptibility to vaccination. Hence, data from genetic studies on local tick populations should be considered in assessing the performance of potential broad-spectrum tick vaccines. Furthermore, a multi-species anti-tick vaccine could attract greater interest from pharmaceutical companies, which often require high vaccine efficacy before they can commit to developing and marketing a final product. Similarly, synthetic vaccines constituting multiple epitopes from different strains or species could provide a viable option in developing a universal tick vaccine (Peconick et al., 2008). A number of experiments have demonstrated the viability of inducing cross-protection in different hosts, although field trials are needed to confirm the preliminary results. A particularly interesting research for use of synthetic peptides is developing anti-tick/anti-pathogen combination vaccines (de la Fuente et al., 2011; Carreón et al., 2012). This is of great interest since the economic importance of ticks is largely as a result of the pathogens they transmit.

Broad advances in genomics are facilitating global characterization of tick proteins, including those with potential for inclusion in a universal anti-vector vaccine. The application of RNAi has hastened the characterization of protein functions (de la Fuente et al., 2006a,b; Kotsyfakis et al., 2007; Hajdusek et al., 2009; Fabres et al., 2010; Almazán et al., 2010). Moreover, another use of this technique was suggested by combining subolesin vaccination and release of ticks after subolesin knockdown by RNAi for the control of *R. microplus* tick infestations in cattle (Merino et al., 2011). Coupled with high-throughput sequencing technologies, RNAi will enable global profiling of promising anti-tick vaccine candidates across multiple species, leading to faster identification and characterization of antigens that potentially confer sufficient cross-immunity for use as universal vaccines.

However, approaching antigen identification from this direction may not be straightforward after all; although Bm86 and its homologues confer significant immunity against tick infestation, silencing Bm86 genes by RNAi leads to weak phenotypic effects, perhaps reflecting a more complex biological function of Bm86 (Liao et al., 2007; Nijhof et al., 2007, 2010). Genome sequencing is increasingly affordable and will, perhaps, supplement existing sequence EST libraries in the near future (Bellgard et al., 2011). These and other breakthroughs in functional genomics could aid in identifying and characterizing new vaccine candidates more quickly and at lower cost (Ghosh et al., 2007). In addition, computer models capable of simulating host-vector-pathogen interaction have been developed, enabling in silico evaluation of candidate universal vaccines prior to testing under field conditions (Tsao et al., 2012).

Taken together, the studies reviewed in this paper indicate that the decades-long search for a universal tick vaccine is making progress with such a vaccine likely to consist of multiple cross-reactive antigens. The number and categories of tick antigens with potential for protecting against tick infestation is rising, with a good number of these molecules demonstrating strong cross-reactivity. However, whether combining multiple antigens into a cocktail vaccine can lead to cumulative vaccine efficacy remains to be properly determined (Limbach et al., 2011), since immunodominant and immunosuppressive effects have been observed with DNA (Nascimento et al., 2002; Sedegah et al., 2004) and protein (Ruth et al., 2008) vaccines.

Conclusions

Tick infestation remains a serious impediment to profitable livestock production. Problems associated with widespread use of chemical acaricides call for alternative interventions, particularly, vaccines. Whereas the rate of tick antigens identification has accelerated over the past 20 years, an effective universal ant-tick vaccine is far from reaching the drug shelves. Protein antigens largely conserved across tick genera are candidates for the envisaged future vaccines. Vaccination with one or more of these could confer cross-protection against several tick species or strains. It is possible that combining tick antigens with pathogen-derived anti-

gens to form multivalent combination vaccines could offset poor protection resulting from vaccinating with tick antigens alone. This approach is conceivable, since reducing TBD transmission is equally important as reducing tick infestation. Meanwhile, integrated control of disease vectors using anti-tick vaccines to reduce the amounts and frequency of acaricides application remains a practical and sustainable approach to TBD management as we inch towards a fully effective universal anti-tick vaccine.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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ANEXO C: CURRICULUM VITÆ resumido

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Fevereiro/2014

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2. FORMAÇÃO:

Graduação em Medicina Veterinária, Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

Período: 2001 a 2007.

Mestrado no Programa de Pós-Graduação em Biologia Celular e Molecular da Universidade Federal do Rio Grande do Sul (UFRGS), Brasil.

Título: Proteção cruzada contra a infestação de *Rhipicephalus (Boophilus) microplus* em bovinos vacinados com a glutationa S-transferase recombinante de *Haemaphysalis longicornis*.

Orientador: Dr. Itabajara da Silva Vaz Junior.

Co-orientadora: Dra. Aoi Masuda.

Bolsista da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasil.

Período: 2008-2010.

Doutorado em andamento no Programa de Pós-Graduação em Biologia Celular e Molecular da Universidade Federal do Rio Grande do Sul (UFRGS), Brasil.

Título: Caracterização de cistatinas e possíveis funções na fisiologia do carrapato *Rhipicephalus (Boophilus) microplus*.

Orientador: Dr. Itabajara da Silva Vaz Junior.

Co-orientadores: Dra. Aoi Masuda, Dr. Carlos Logullo.

Bolsista do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brasil.

Período: 2010-2014.

3. ESTÁGIOS:

Estágio de iniciação científica, Laboratório de Imunologia Aplicada a Sanidade Animal, Centro de Biotecnologia, Universidade Federal do rio Grande do Sul (UFRGS), Brasil.

Orientador: Dr. Itabajara da Silva Vaz Junior. Sem Bolsa. Período: 04/2004 a 03/2005.

Orientadora: Dra. Aoi Masuda. Bolsista do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brasil. Período: 04/2005 a 08/2006.

Orientador: Dr. Itabajara da Silva Vaz Junior. Bolsista do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brasil. Período: 10/2006 a 07/2007.

4. ARTIGOS COMPLETOS:

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GUIZZO, M. G., PARIZI, LUÍS FERNANDO, FABRES, A., LOGULLO, CARLOS, MASUDA, AOI, DA SILVA VAZ JR., ITABAJARA. Cloning and Characterization of a Metalloprotease of the tick *Rhipicephalus (Boophilus) microplus.* In: XL Reunião Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular - SBBq, 2011, Foz do Iguaçu.

RODRIGUES, F., FERREIRA, V., PARIZI, LUÍS FERNANDO, POHL, P. C., MASUDA, AOI, DA SILVA VAZ JR., ITABAJARA. Cloning and expression of an ABC transporter from *Rhipicephalus (Boophilus) microplus*. In: XL Reunião Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular - SBBq, 2011, Foz do Iguaçu.

FERREIRA, V., RODRIGUES, F., PARIZI, LUÍS FERNANDO, ACEVEDO, C., BENAVIDES, U., MASUDA, AOI, DA SILVA VAZ JUNIOR, ITABAJARA. Cloning of cystatin gene of an Uruguayan *Rhipicephalus microplus* isolate. In: XL Reunião Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular - SBBq, 2011, Foz do Iguaçu.

BENAVIDES, U., ACEVEDO, C., PARIZI, LUÍS FERNANDO, MASUDA, AOI, DA SILVA VAZ, I. DETERMINACIÓN DEL POLIMORFISMO DEL GEN BM86 DE LA GARRAPATA DEL GANADO BOVINO *Riphicephalus microplus* EN POBLACIONES DE URUGUAY. In: VII Jornadas Técnicas Veterinária, 2011, Montevideo.

SCHULER, A.D, PARIZI, LUÍS FERNANDO, LOGULLO, CARLOS, MASUDA, AOI, DA SILVA VAZ JR, ITABAJARA. Expressão e Purificação de dois fragmentos da Glicogênio Sintase Quinase de *Rhipicephalus microplus*. In: XIII Salão de Iniciação Científica da UFRGS, 2011, Porto Alegre.

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