UNIVERSIDADE FEDERAL DE MATO GROSSO DO SUL
PROGRAMA DE PÓS-GRADUAÇÃO EM DOENÇAS INFECCIOSAS E PARASITÁRIAS

FRANCISCO TOBÍAS BARRADAS PIÑA

Amblyomma spp.: ASPECTOS DA BIOLOGIA, RESISTÊNCIA AOS ACARICIDAS E O EFEITO DA IMUNOPROTEÇÃO DO ANTIGENO AQUAPORIN NO SEU CONTROLE

CAMPO GRANDE
2018

FRANCISCO TOBÍAS BARRADAS PIÑA

Amblyomma spp.: ASPECTOS DA BIOLOGIA, RESISTÊNCIA AOS ACARICIDAS E O EFEITO DA IMUNOPROTEÇÃO DO ANTIGENO AQUAPORIN NO SEU CONTROLE

Tese apresentada ao Programa de Pós-Graduação em Doenças Infecciosas e Parasitárias da Faculdade de Medicina da Universidade Federal de Mato Grosso do Sul, como requisito à obtenção do título de Doutor, sob orientação do Doutor Renato Andreotti e Silva, e coorientação do Doutor Marcos Valerio García.

CAMPO GRANDE, MS.
2018

Ministério da Educação
Fundação Universidade Federal de Mato Grosso do Sul
Coordenadoria de Pós-Graduação (CPG/PROPP)

Ata de Defesa de Tese
Programa de Pós-Graduação em Doenças Infecciosas e Parasitárias
Doutorado

Aos vinte e nove dias do mês de junho do ano de dois mil e dezoito, às treze horas, no Anfiteatro II-FAMED, da Fundação Universidade Federal de Mato Grosso do Sul, reuniu-se a Banca Examinadora composta pelos membros: Renato Andreotti e Silva (EMBRAPA), Ana Rachel Oliveira de Andrade (UFPI), Everton Falcao de Oliveira (UFMS), Sonia Maria Oliveira de Andrade (UFMS) e Wilson Werner Koller (EMBRAPA CNPGC), sob a presidência do primeiro, para julgar o trabalho do aluno: **FRANCISCO TOBIAS BARRADAS PIÑA**, CPF 70652094155, do Programa de Pós-Graduação em Doenças Infecciosas e Parasitárias, Curso de Doutorado, da Fundação Universidade Federal de Mato Grosso do Sul, apresentado sob o título **"Amblyomma spp.: aspectos da biologia, resistência aos acaricidas e o efeito da imunoproteção do antígeno Aquaporin no seu controle"** e orientação de Renato Andreotti e Silva. O presidente da Banca Examinadora declarou abertos os trabalhos e agradeceu a presença de todos os Membros. A seguir, concedeu a palavra ao aluno que expôs sua Tese. Terminada a exposição, os senhores membros da Banca Examinadora iniciaram as arguições. Terminadas as arguições, o presidente da Banca Examinadora fez suas considerações. A seguir, a Banca Examinadora reuniu-se para avaliação, e após, emitiu Parecer expresso conforme segue:

EXAMINADOR	ASSINATURA
Dr. Renato Andreotti e Silva (Interno)	
Ana Rachel Oliveira de Andrade (Externo)	
Dra. Doroty Mesquita Dourado (Interno) (Suplente)	
Dr. Everton Falcao de Oliveira (Interno)	
Dra. Sonia Maria Oliveira de Andrade (Interno)	
Dr. Wilson Werner Koller (Externo)	

RESULTADO FINAL:

☒ Aprovação ☐ Aprovação com revisão ☐ Reprovação

OBSERVAÇÕES:

A banca, após a exposição oral e avaliação do relatório impresso, considera o aluno apto a receber o título de doutor. Ajustes de formal foram sugeridos o que não minimiza o mérito e o valor da pesquisa.

Nada mais havendo a ser tratado, o Presidente declarou a sessão encerrada e agradeceu a todos pela presença.

Assinaturas:

Presidente da Banca Examinadora

Aluno

AGRADECIMENTOS

Principalmente agradeço a Deus por a força que me proporciona para avançar em minha vida.

Agradeço à minha esposa e filhos (Jenny, Luz Marie e Franco Emiliano) por estar perto de mim alem de estar no México ao longo de quatro anos, obrigado pela paciencia, peço desculpas a vocês por estar longe por muito tempo, amo vocês.

Mãe, ainda lembro o dia que me falou tchau para sempre no aeroporto, e pediu para mim não desistir deste novo desafio na vida. Hoje não tenho a oportunidade de te falar obrigado fisicamente, mas eu sei que agora nosso sonho esta feito, agradeço a você por me trazer ao mundo e me acompanhar até seu ultimo dia, a gente se encontra na frente beijos mãe.

Agradeço a meu Pai e irmãs (Tobias, Isabel e Hortencia) por me acompanhar na minha vida toda.

Agradeço infinitamente ao Dr. Renato, meu orientador, por me ajudar nestes quatro anos de pós graduação. Obrigado por aceitar ser meu orientador e amigo, eu gostaria de ser um amigo e companheiro por muitos anos mais.

Obrigado Jacqueline e Marcos, agradeço por sua ajuda e orientação .

Parceiro (Leandro), valeu pela ajuda e dicas durante minha estadia no Brasil, agradeço a sua mãe e irmão por me aceitar na sua casa, estamos juntos parceiro.

Agradeço aos companheiros por me ajudar no laboratório e pelos churrascos (Isabella Blecha, Pâmella Duarte, Leandra Oshiro, Barbara Guimares, Vinicius Rodrigues, André Aguirre, Jaqueline Matias).

Dr. Adalberto Pérez de León, muito obrigado por suas valiosas contribuções desde antes de começar meu doutorado e por me aceitar no laboratório em Texas.

Dra. Dora, obrigado por me acompanhar e escutar durante os quatro anos de doutorado.

Ao Ing. Aldo Fernando Varela Martínez por sua ajuda e amisade (Valeu Aldo).

Agradeço a Banca examinadora por aceitar formar parte de meu processo para obter o título de Doutor.

Agradeço a Marcelo Bahia Labruna por me receber um mês no seu laboratório e me mostrar parte de suas atividades.

Agradeço a Guilherme Klafke, Robert Miller, Jason Tidwell, Michel Mosse, por me receber e acompanhar no laboratório em Edinburgh, Tx (USDA).

Agradeço para o resto da minha vida a UFMS (DIP) pela oportunidade de fazer parte da instituição.

Ao órgão de fomento, CONACyT, Muito obrigado por me ajudar economicamente neste projeto de vida.

Ao INIFAP por me ajudar durante os quatro anos de estudos de posgrado.

RESUMO

Revisões morfotaxonômicas recentes incluem *Amblyomma sculptum* e *Amblyomma mixtum* como membros do complexo de espécies *A*. *cajennense A. sculptum* e *A. mixtum* são vetores de patógenos que causam doenças de importância veterinária e de saúde pública. Juntamente com o *Rhipicephalus microplus*, esses carrapatos têm impacto significativo na saúde pública e na lucratividade dos sistemas de produção animal no Brasil e no México. O presente trabalho teve como objetivo desenvolver estudos sobre os aspectos da biología, resistência aos acaricidas e o efeito da imunoproteção do antígeno “aquaporin” no controle de carrapatos heteroxenos que efetam a cadeia produtiva e de impacto na saúde pública. Experimentos de laboratório mostraram que o ciclo de vida médio de *A. mixtum* foi de 88 e 79 dias quando alimentados em coelhos e bovinos, respectivamente. Ovinos mostraram-se inadequados como hospedeiros porque nenhum carrapato foi recuperado após infestação artificial. Os resultados dos bioensaios mostraram que os diferentes estágios de vida de *A. mixtum* foram sensíveis a misturas acaricidas comerciais (variando de 87,6% a 100%) e organofosforados (98,8% a 100%). *A. mixtum* foi menos sensível a amitraz (0 a 24,4%) e cipermetrina (2,2% a 40%). Ensaios acaricidas *in vivo* revelaram uma eficácia média de 35,1% e 95,8% durante a primeira e segunda avaliação, respectivamente, quando os bovinos da raça Brangus foram infestado naturalmente. Nos ensaios em que o gado Nelore foi infestado naturalmente, a eficácia média após o primeiro e segundo tratamento foi de 51% e 97,1%, respectivamente. A eficácia de uma vacina experimental baseada em peptídeos foi de 68.1%, a eficacia se menteve durante 82 dias após a imunização inicial, utilizando um modelo em que coelhos foram infestados artificialmente com *A. mixtum*. Os dados laboratoriais sobre o ciclo de vida de *A. mixtum* podem ser considerados como referência para pesquisas futuras. Os resultados do bioensaio revelaram que o Teste de Pacote de Larvas (TPL) é uma alternativa para o diagnóstico de resistência em carrapatos do ciclo de vida do trioxeno. Resultados de experimentos com bovinos da raça Brangus relatados aqui mostraram que esta raça é suscetível ao alto parasitismo com *R. microplus*, o que pode gerar grandes infestações em pastagens. Os resultados dos testes com a vacina peptídica sintética identificaram essa estrategia como uma alternativa para a imunoproteção do gado contra a infestação por *A. mixtum* e as doenças transmitidas por esse vetor de doença do carrapato.

Palavras-chave**:** Ciclo biologico. *Amblyomma mixtum. Amblyomma sculptum,* Complexo cajennense. Diagnóstico de resistência.

.

ABSTRACT

Recent tick morphotaxonomical revisions included *Amblyomma sculptum* and *Amblyomma mixtum* as members of the *Amblyomma cajennense* species complex. *A. sculptum* and *A. mixtum* are tick vectors of pathogens that cause diseases of veterinary and public health importance. Together with *Rhipicephalus microplus*, these ticks have a significant impact on animal health and the profitability of livestock production systems in Brazil and Mexico. The present work aimed to develop studies on the biology, resistance to acaricides and the effect of the immunoprotection of the aquaporin antigen on the control of heteroxene ticks that affect the production chain and impact on public health. Laboratory experiments showed that the average life cycle of *A. mixtum* was 88 and 79 days when fed on rabbits and cattle, respectively. Sheep were shown inadequate as hosts because *A. mixtum* were not recovered upon artificial infestation. Results from bioassays showed that the different life stages of *A. mixtum* were sensitive to a commercial acaricide mixtures (ranging from 87.6% to 100%), and organophosphates (98.8% to 100%). *A. mixtum* was less sensitive to amitraz (0 to 24.4%), and cypermethrin (2.2% to 40%). *In vivo* acaricide trials revealed a mean efficacy of 35.1% and 95.8% during the first and second evaluations, respectively, when Brangus cattle were naturally infested. In trials where Nelore cattle were naturally infested, the mean efficacy after the first and second treatments was 51% and 97.1%, respectively. The efficacy of an experimental vaccine based on peptides was 68.1%, effectiveness was maintained during 82 days after the initial immunization using a model where rabbits were infested artificially with *A. mixtum*. The laboratory data on the life cycle of *A. mixtum* can be considered as a reference for future research. Bioassay results revealed that the Larva Pack Test (LPT) is an alternative for the diagnosis of resistance in trioxene life cycle ticks. Results of experiments with Brangus cattle reported here showed that this breed is susceptible to high parasitism with *R. microplus*, which can generate large infestations in pastures. Test results with the synthetic peptide vaccine identified this approach as an alternative for immunoprotection of livestock against infestation with *A. mixtum* and the diseases transmitted this tick disease vector.

Keywords: Biological cycle. *Amblyomma mixtum. Amblyomma sculptum*. Cajennense complex, Diagnosis of resistance.

LISTA DE ABREVIATURAS E SIGLAS

µL	microlitros
®	marca comercial
B.O.D.	demanda bioquímica de oxigênio
CONCEA	Conselho Nacional de Controle de Experimentação Animal
CEUA	Comitê de Ética no Uso Animais
DNA	adenosina difosfato
FMB	febre maculosa brasileira
GFM	grupo da febre maculosa
IFN-γ	interferon gama
IgG	imunoglobulina G
Kda	kilodaltons
KLH	keyhole limpet hemocyanin
m	metros
Mg	miligramas
mm	milimetros
mL	mililitros
ng	nanogramas
nm	nanômetros
N-	amino-terminal
NK	Células *Natural Killer*
PBS	tampão fosfato-salino
PCR	reação em cadeia da polimerase (*polymerase chain reaction*)
rpm	rotações por minuto
SC	subcutâneo
S. l.	sensu latu
UR	umidade relativa
USA	Estados Unidos da América
S.s.	sensu estrictus
RMSF	rocky moutain spotted fever
TCD8+	linfócitos T citotóxicos
™	marca comercial

SUMÁRIO

1 INTRODUÇÃO

Encontram-se registradas mais de 900 espécies de carrapatos distribuidas em três famílias: Argasidae, considerados carrapatos moles (ausência de escudo) com 193 espécies; Ixodidae, os carrapatos duros (presença de escudo) com aproximadamente 700 espécies registradas. A família Nuttalliellidae é representada por apenas uma espécie (NAVA *et al*., 2009; SONESHINE, ROE, 2014). No México os argasideos estão representados por 32 espécies em 5 gêneros: *Argas* (6 espécies); *Antricola* (3); *Ornithodoros* (20); *Otobius* (2); *Nothoaspis* (1). Dentro da familia Ixodidae estão registrados 68 espécies em 5 gêneros: *Ixodes* (26 espécies); *Rhipicephalus* (3); *Amblyomma* (26); *Dermacentor* (10); *Haemaphysalis* (3) (PEREZ *et al*., 2014). No Brasil, até o momento, a fauna ixodídica é representada por 73 espécies divididas em 47 Ixodidae e 26 Argasidae (NAVA *et al*., 2014; KRAWCZAK *et al*., 2015; LABRUNA *et al*., 2016; MUÑOZ-LEAL *et al*., 2017).

A utilização do controle químico pode gerar uma pressão de seleção artificial nas populações de carrapatos em tratamento, provocando um fênomeno conhecido como resistência, o qual pode ser definida como capacidade de sobrevivência dos carrapatos à exposição de produtos químicos (acaricidas), segundo a Organização das Nações Unidas para Agricultura e Alimentação (FAO, 2004). Na década de 1990, foram lançadas ao mercado duas vacinas com antígenos específicos (Bm86) para *R. microplus*, com intuito de contribuir na redução do uso de acarícidas e diminuir as perdas econômicas geradas por os carrapatos. Atualmente existem algumas outras pesquisas com a finalidade de gerar vacinas contra diferentes espécies de carrapatos, já que atualmente as vacinas comercialmente utilizadas têm baixa eficacia (de VOS *et al*., 2001; ANDREOTTI *et al*., 2002; De La FUENTE, KOCAN, 2003; NUTTAL *et al*., 2006; GUERRERO *et al*., 2014).

Considerando esta problemática que envolve todos os aspectos anteriormente mencionados e que abrange diferentes áreas, no presente trabalho teve-se como objetivos desenvolver estudos sobre os aspectos da biologia, a resistência aos acaricidas e o efeito da imunoproteção do antigeno aquaporina no controle do carrapato *Amblyomma* spp. o qual afeta a cadeia produtiva e têm impacto na saúde pública no Brasil e no México.

2 REVISÃO DE LITERATURA

2.1 Classificação e distribuição dos carrapatos: aspectos gerais

Os carrapatos são ectoparasitas hematófagos obrigatórios que se alimentam de mamíferos, répteis, anfíbios, aves e seres humanos (VOLTZIT, 2007). Mundialmente são registradas aproximadamente 900 espécies de carrapatos, pertencentes ao filo Arthropoda, classe Arachnida, subclasse Acari e são classificados em três famílias: Ixodidae - carrapatos duros (presença de quitina no dorso), com aproximadamente 692 espécies (NAVA *et al*., 2009; SONENSHINE, ROE, 2014); Argasidae carrapatos moles (ausência de quitina no dorso), com registro de 183 espécies e a família Nuttaliallidae com registro de uma espécie, a qual é endêmica do continente africano (BARROS-BATTESTI *et al*., 2006; NAVA *et al*., 2009).

Os carrapatos apresentam ampla distribuição, sendo encontrados na região neotropical, a qual abrange desde o sul do Texas e Ilhas do Caribe, até a América do Sul. Os carrapatos argasídeos compreendem 74 espécies (14 *Antricola*; 11 *Argas*; 1 *Nothoaspis*; 47 *Ornithodoros* e 1 *Otobius*), das quais 59 espécies são endêmicas da região neotropical. A família Ixodidae envolve 114 espécies (57 *Amblyomma*; 2 *Haemaphysalis*; 45 *Ixodes*; 1 *Anocentor;* 1 *Boophilus*; 7 *Dermacentor*; 1 *Rhipicephalus*), das quais 87 espécies estão registradas na região em questão (GUGLIELMONE *et al*., 2006).

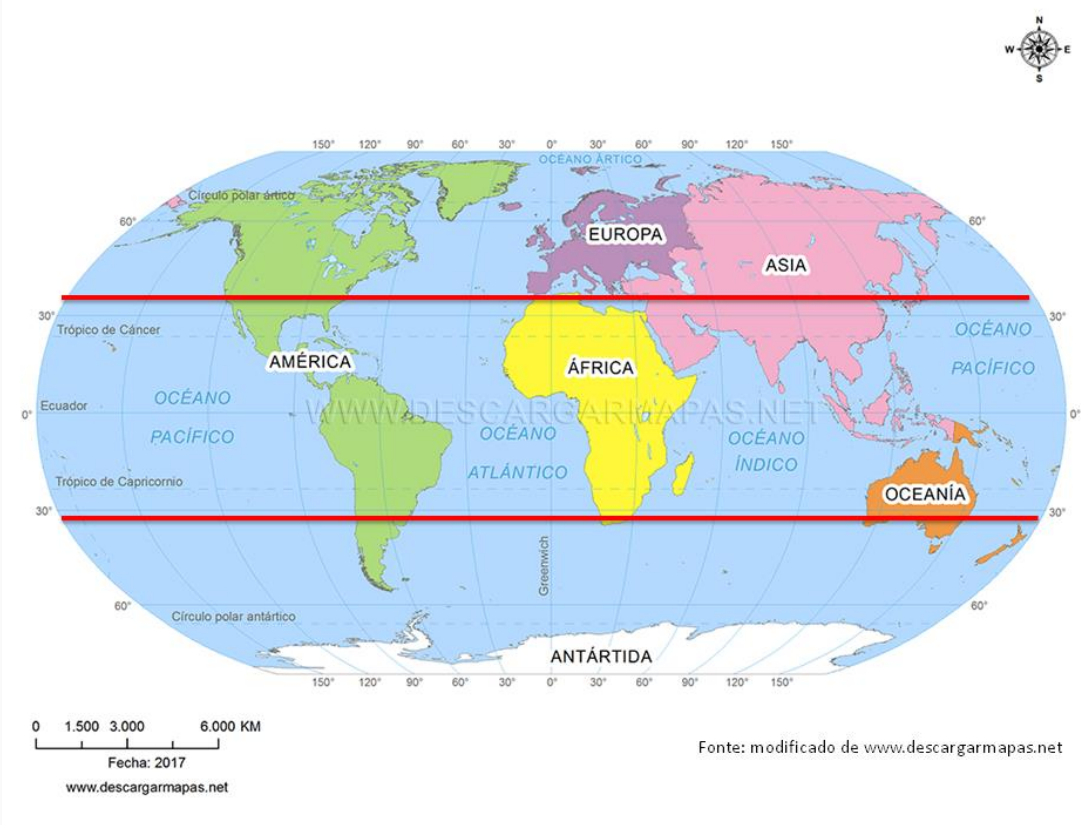
2.1.1 *Rhipicephalus microplus*: distribuição, importância e hospedeiro

Na figura 1 o carrapato *R. Microplus*, está distribuído entre os paralelos 32° N e 32° S (ALI *et al*. 2016), sendo o principal transmissor dos agentes patogénos da Tristeza Parasitária Bovina (TPB), causada pelos parasitas protozoários *Babesia bigemina*, *B. bovis* e *Anaplasma marginale*.

Dentre as 73 espécies de carrapatos registradas no Brasil, destaca-se o *Riphicephalus microplus*, também conhecido como carrapato-do-boi, sendo bovinos os principais hospedeiros e, como hospedeiros secundários outros herbívoros (WALL, SHEARER, 1997). Este ectoparasito apresenta ampla distribuição no Brasil, sendo

entre os parasitas o principal problema econômico na pecuária brasileira (GRISI *et al*., 2014 NAVA *et al*., 2014; KRAWCZAK *et al*., 2015; LABRUNA *et al*., 2016; WOLF *et al*., 2016; MUÑOZ-LEAL *et al*., 2017).

Figura 1 Distribuição do carrapato *Rhipicephalus microplus* no mundo.



Fonte: Ali *et al*., (2016).

No México, os carrapatos que afetam a cadeia produtiva da pecúaria pertencem a três espécies: *Rhipicephalus annulatus*; *R. microplus* e *A. Mixtum*. Estes carrapatos encontram-se distribuídos da seguinte forma: *R. annulatus* pode ser encontrado nos estados do Norte do México; *R. microplus* está distribuído na região tropical e *A. mixtum* pode ser encontrado em quase todo o país. Este ultimo parasita, principalmente, os bovinos, os cavalos, os animais da fauna silvestre e, também, os humanos (GUZMAN-CORNEJO *et al*., 2011).

No Brasil o carrapato que afeta a cadeia produtiva pecúaria é *Rhipicephalus microplus* gerando um impacto econômico negativo de 3,236,35 bilhões de dólares por ano e estão representados por: 922,36 bilhões de dólares no gado produtor de leite, 1587,68 *Bos turus*, 376,67 gado cruzado (*Bos taurus indicus* X *Bos taurus taurus*) e 349,64 bilhões de dólares em *Bos indicus* (GRISI *et al*., 2014).

No México as percas econômicas estimadas por causa do carrapato do boi são de 0.573,608,076 bilhões de dólares por ano aproximadamente, divididos da seguinte forma: produção leiteira 0.68,878,694 bilhões de dólares por ano, gado produtor de carne 0.295,459,145 bilhões de dólares por ano, gado *Bos taurus taurus* X *Bos taurus indicos* 0.29,365,226 bilhões de dólares por ano e no gado *Bos taurus indicus* são de 0.179,905,011 bilhões de dólares por ano segundo dados estimados por (RODRIGUEZ-VIVAS *et al*., 2017).

2.1.2 *Rhipicephalus microplus*: ciclo biológico

O ciclo biológico do carrapato monoxeno *R*. *microplus* apresenta duas fases: parasitária (alimentação) e não parasitária (pastagem). A fase parasitaria é de 21 dias no hospedeiro. A larva começa a se alimentar, sofre a metamorfose no hospedeiro (muda para ninfa: apresenta um par a mais de pernas; uma fileira de dentes a mais, e um novo tegumento).

Depois que a ninfa muda para adulto, o carrapato pode se diferenciar sexualmente. Após um periodo de 17 dias ocorre à maturação e os machos já estão aptos para a reprodução sexual. Nos últimos três dias da fase parasitária, o carrapato mostra-se parcialmente ingurgitado e no último dia as fêmeas ingurgitam ao máximo, alcançando um tamanho de até 300 vezes a mais que as larvas. Na fase não parasitária as teleóginas se despendem do hospedeiro e procuram uma área adequada para postura. Cada teleogina pruduz, em média, de 2000 a 3000 ovos.

2.1.3 Complexo *Amblyomma cajenennse*: distribuição

O complexo *Amblyomma cajenne nse*, pertence à subclasse Acari, família Ixodidae e subfamília *Amblyomminae*. A. *cajennense* é mais comumente conhecido como carrapato estrela, carrapato do cavalo e, também, rodoleiro. Ele foi identificado pela primeira vez na Guiana Francesa, e descrito por Fabricius em 1787.

Até o ano 2013, A. *cajennense* estava distribuído em toda a América Latina, bem como, em todas as unidades federativas do Brasil. Estudo realizado por Nava *et al*. (2014) identificaram que, de acordo com análises taxonômicas e filogenéticas, a espécie supracitada deu origem ao complexo *A*. *cajennense*, envolvendo seis espécies: *A. mixtum, A. sculptum, A. tonelliae, A. interandinum, A. patinoi* e *A*. *cajennense* (s.s.). As espécies do complexo *Amblyomma cajennense* estão distribuídas desde o sul do Texas até o norte de Argentina (CASTAGNOLLI *et al*.,

2003). *A. interandinum* pode ser encontrado mais especificamente no vale do Peru, *A. patinoi* na cordilheira da Colômbia, *A. tonelliae* se encontra em zonas áridas no Norte da Argentina, Bolívia e Paraguai e *A. cajennense* se encontra na região da Amazônia (Figura 2).

Figura 2. Distribuição das 6 espécies do complexo *Amblyomma cajennense*.

Fonte: Nava *et al*. (2014).

Amblyomma cajennese s.s.; † *Amblyomma tonelliae* n. sp.; ¥ *Amblyomma interandinum* n. sp.; # *Amblyomma patinoi* n. sp.; ■ *Amblyomma mixtum* n. sp.; 0 *Amblyomma sculptum* n. sp.; X, ficha de *A. sculptum* no departamento de Beni, Bolívia, localidade desconhecida.

2.1.3.1 *Amblyomma sculptum*: distribuição, importância e hospedeiros

Amblyomma sculptum encontra-se distribuído desde a região norte da Argentina, regiões contíguas da Bolívia e Paraguai até a costa e centro ocidente do Brasil (bem

estabelecido em biomas cerrado e áreas desmatadas) (BEATI *et al*., 2013; PIRES *et al*., 2013; NAVA *et al*., 2014; RAMOS *et al*., 2014). As diferentes espécies de carrapatos são reservatórios naturais de uma grande diversidade de patógenos como bactérias, vírus e protozoários, que comprometem a saúde animal desenvolvendo doenças: (babesiose, anaplasmose, erliquiose e borreliose) e, támbem, a saúde pública, principalmente como transmissor da Febre Maculosa Brasileira (*Rickettsia rickettsi*). *A. aureolatum* e *A. sculptum* podem transmitir *R. rickettsi* de forma vertical para as larvas, fato que aumenta o risco em doenças de importância na saúde pública (PINTER, LABRUNA, 2006; ABDU, BEARD, 1998; KRAWCZAK *et al*., 2015). *R. rickettsi* é endêmica em algumas regiões do país, com maior prevalência em Minas Gerais, que de acordo com Monteiro *et al*. (2006) e Freitas *et al*. *(*2011), reportaram taxa de mortalidade de 26% desde 1930 a 2006. A doença também é endêmica no Rio de Janeiro e São Paulo, tendo o primeiro registro da doença datada em 1929 (GUEDES *et al*., 2005).

O carrapato *A. sculptum* parasita principalmente os cavalos, capivaras e algumas outras espécies de mamíferos da fauna silvestre (BEATI *et al*., 2013; PIRES *et al*., 2013; NAVA *et al*., 2014; RAMOS *et al*., 2014). Durante a fase de larvas e ninfas parasitam animais de pequeno porte e na fase adulta, animais de grande porte (CASTAGNOLLI *et al*., 2003). Uma vez no hospedeiro, este carrapato apresenta predileção principalmente onde a pele é mais fina (região inguinal, axila, tábua do pescoço e a região vulvar). Pinna *et al*. (2004) reportaram que de 1434 carrapatos coletados em cavalos, 55,2% ocuparam a parte anterior e 44,8% a região posterior do hospedeiro.

2.1.3.2 *A*. *mixtum*: distribuição, importância e hospedeiros.

A. mixtum (antigo *A. cajennense*) encontra-se distribuído em diferentes regiões do México, tais como, manguezais e pântanos da costa do México com altitudes baixas onde a temperatura média situa-se em torno de 13 a 16 ^{0}C (ESTRADA-PEÑA *et al*., 2014).

Medina-Sánchez *et al*. (2005) reportaram que, no México de 89 pessoas doentes com suspeita de dengue, por meio da técnica da PCR foram identificadas as bactérias *R*. *prowazekii* (*R. tiphi*) 25% e 16% para *R. parkeri*. Os carrapatos identificados como

vetores dos patógenos foram *Amblyomma imitator* e *A. mixtum*, sendo carrapatos que parasitam animais da fauna silvestre e domésticos (GUZMÁN-CORNEJO *et al*., 2011).

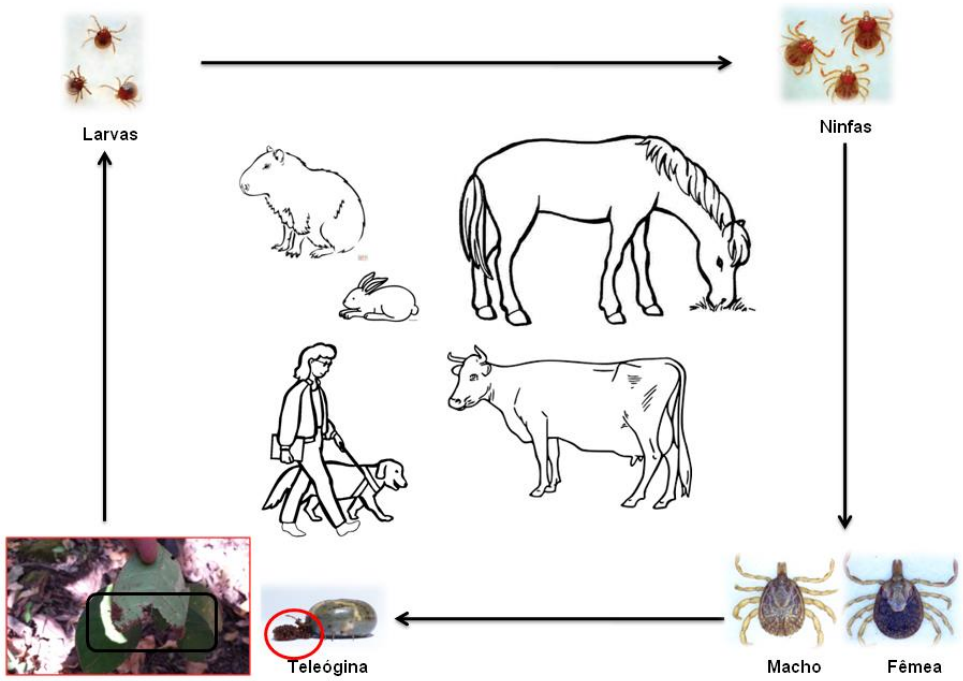
2.2 *Amblyomma* spp. ciclo biológico

O ciclo biológico dos carrapatos heteroxenos (*Amblyomma* spp.) caracteriza-se por ter uma fase parasitária e uma fase não parasitária. A quantidade de ovos pode variar para cada espécie e está relacionada com o peso da teleógina (GUGLIELMONE *et al*., 1991). Depois da eclosão das larvas, estas apresentam três pares de pernas e ficam nas folhas dos arbustos a espera do primeiro hospedeiro (LABRUNA *et al*., 2003; ALMAZÁN *et al*., 2016).

O ciclo de vida inicia-se com a queda da teleógina (fêmea ingurgitada), a qual ocorre preferencialmente no final da tarde ou início da manhã (menor temperatura ambiental). A teleógina procura um microambiente protegido dos inimigos naturais e realiza a oviposição, e então a mesma morre. As larvas eclodem e mantêm uma reserva de energia proveniente do vitelo para sobrevivência no meio ambiente.

A partir do momento em que encontram um hospedeiro e se fixam no mesmo, inicia-se a fase parasitária do ciclo biológico. As larvas parasitam o hospedeiro e se alimentam até ingurgitar, desprendendo-se e voltando ao meio ambiente. Depois do processo de ecdise surgem as ninfas, realizando o mesmo processo descrito anteriormente. Do processo de metamorfose das ninfas surgem os adultos, podendo finalmente seram diferenciados em machos e fêmeas (dimorfismo sexual). Os adultos parasitam outro hospedeiro, sobre o qual alimentam-se e se reproduzem e, posteriormente, começam a ovipostura no ambiente.

Figura 3 - Ciclo biologico *Amblyomma* spp.



Fonte: Proprio Autor

2.3 Métodos de controle dos carrapatos

Os acaricidas são aplicados por diferentes vias de contato (imersão, pour-on) em sistêmica (injetáveis e pour-on). O desenvolvimento da resistência pode ser acelerada por fatores genéticos do parasita (dominância de alelos, número de genes envolvidos, diversidade genética da população), fatores operacionais (modo de aplicação, frequência e forma de tratamento, sub dosagem, e fatores biológicos (fatores bióticos, diversidade de hospedeiros, relação parasita-hospedeiro) (ABBAS *et al*., 2014). Outras formas de controle do carrapato incluem o uso de vacinas, rotação de pastagens, fungos entomopatogênicos e uso de raças naturalmente resistentes (*Bos indicus*) (BASSO *et al*., 2005; XAVIER, AVILA, 2006; OLIVO *et al*., 2008, ABBAS *et al*., 2014; GUERRERO *et al*., 2014).

No Brasil, segundo Pires *et al*. (2013), o método de controle de *A. sculptum* em cavalos é realizado com acaricidas, utilizando o método de aspersão (CUNHA *et al*.,

2007; FREITAS *et al*., 2011). Segundo Freitas *et al*. (2011), as famílias utilizadas são organosfosforados, piretroides e associações, em 32 (52,6%) das fazendas monitoradas. No estado de Goiás, *A. sculptum* (antigo *A. cajennense*) tem uma sensibilidade à deltametrina de 76% em adultos e de 86% de sensibilidade em larvas.

2.4 Diagnóstico de resistência

Os métodos utilizados para diagnóstico da resistência aos acaricidas constituem uma ferramenta que contribui para selecionar acaricidas potencialmente eficazes e estabelecer programas de controle estratégicos de carrapatos. Mundialmente os métodos de diagnóstico são utilizados para carrapatos com ciclo biológico, tanto monoxeno como trioxenos (GUERRERO *et al*., 2002; MILLER *et al*., 2002; NATALA *et al*., 2005; RODRIGUEZ-VIVAS *et al*., 2006; KLAFKE *et al*., 2017).

O teste de imersão de adultos (TIA) é usado para diagnóstico de resistência utilizando 10 teleóginas em triplicata para cada família de acaricida avaliada. A taxa de mortalidade das teleóginas oferece resultados preliminares, já que nesta metodologia é preciso avaliar os parâmetros reprodutivos das teleóginas (DRUMMOND *et al*., 1973).

A metódologia do teste de pacote de larvas (TPL), preconizada pela Organização das Nações Unidas para Agricultura e Alimentação (FAO), é utilizada para diagnóstico de resistência, utilizando 100 larvas com idade de 14 a 21 dias por pacote com triplicata para cada tratamento (acaricida avaliado). Esta técnica é utilizada principalmente para organofosforados, piretróides e amidinas (MILLER *et al*., 2002). As larvas são expostas a papéis filtro impregnado com acaricidas e sua mortalidade é quantificada após 24 horas do desafio, sendo possível a determinação das respectivas doses discriminantes.

O teste de imersão larval (TIL) proporciona resultados em seis semanas aproximadamente, no entanto não é amplamente utilizado para o diagnóstico da resistência. Atualmente não se encontra preconizado pela FAO, sendo empregado principalmente para diagnóstico da resistência as lactonas macrocíclicas e fipronil (KLAFKE *et al*., 2006).

2.5 Uso de acaricidas

Desde os anos de 1930 têm-se utilizado os acaricidas na Austrália, no continente Africano e Americano, para o controle do *Rhipicephalus microplus* e *Boophilus decoloratus*. A falta de conhecimento aliada ao mau uso dos acaricidas levou ao surgimento da resistência aos organoclorados entre a década de 1940 e 50, tais como DDT e ciclodienos (lindanos, dieldrin, toxafeno). Em 1960, aproximadamente, nestes mesmos continentes foi reportada resistência a compostos organofosforados e carbamatos, e às demais bases ao longo dos anos.

Tabela 1 - Primeiros relatos de resistência as principais bases acaricidas

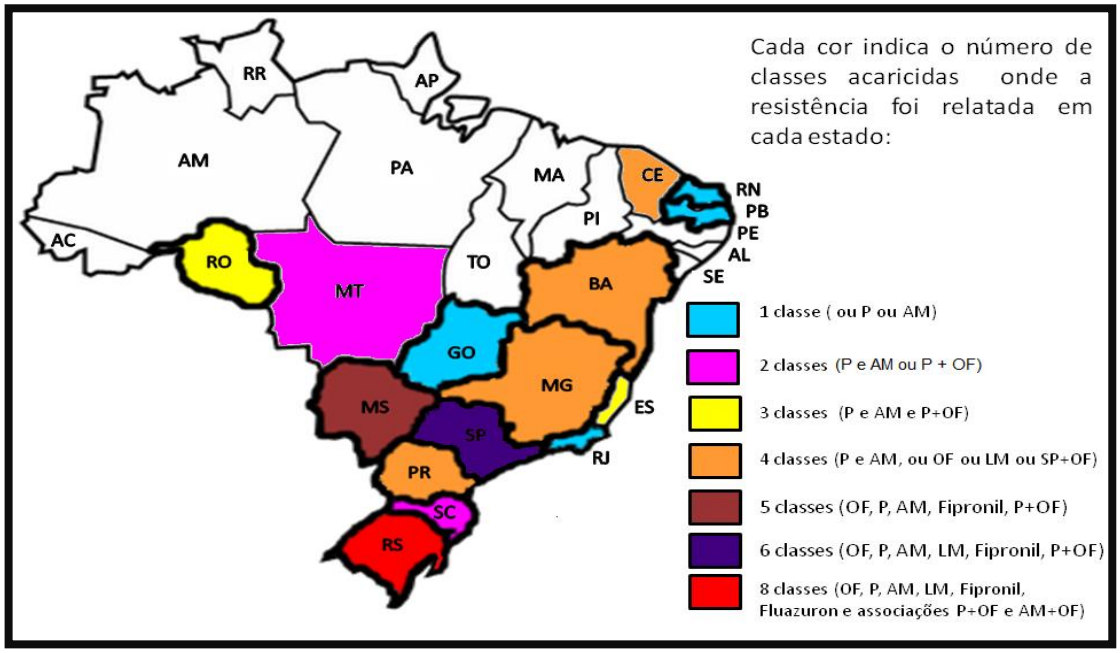
Acaricida (ano de introdução)	Espécies	Paises
Arsenicais	*Rhipicephalus microplus*	Australia e Argentina 1936; Brasil e Colombia 1948; Uruguay 1953; Venezuela 1966
	Rhipicephalus decoloratus	sul de Africa 1937; Kenya 1953; Zimbawe 1963; Malawi 1969;
	Amblyomma hebraeum	Sul de Africa 1975
	Amblyomma variegatum	Zambia 1975
	Hyalomma rufipes, H. truncantum	Sul de Africa 1975
	Rhipicephalus apendiculatus, R. Evertsi	Sul de Africa 1975
DDT (1946)	*Rhipicephalus microplus*	Argentina, Australia e Brasil 1953; Venezuela 1966; Sul de Africa 1979
	Rhipicephalus decoloratus	Sul de Africa 1954
Cliclodienes e Toxaphene (1947)	*Rhipicephalus microplus*	Argentina, Australia e Brasil 1953; Venezuela e Colombia 1966; Sul de Africa 1979
	Rhipicephalus decoloratus	Sul de Africa 1948; Kenya 1964; Zimbawe 1969; Uganda 1970
	Amblyomma hebraeum	Sul de Africa 1975
	Amblyomma variegatum	Kenya 1979
	H. marginatum	Espanha 1967
	Hyalomma rufipes, H. truncantum	Sul de Africa 1975
	Rhipicephalus apendiculatus	Sul de Africa 1964; Zimbawe 1966; Kenya 1968; Tanzania 1971
	R. evertsi	Sul de Africa 1959; Kenya 1964; Zimbawe 1966; Tanzania 1970

Organofosforados - Grupo dos carbamatos (1955)	*Rhipicephalus microplus*	Australia e Brasil 1963; Argentina 1964; Colombia 1967; Sul de Africa 1979; Uruguay 1983; México 1986
	Amblyomma hebraeum	Africa do Sul 1975
	Amblyomma variegatum	Tanzania 1973; Kenya 1979
	Rhipicephalus decoloratus	Sul de Africa 1966; Zambia 1976
	Rhipicephalus apendiculatus	Sul de Africa 1975
	R. evertsi	Sul de Africa 1975
	Amblyomma mixtum	México 2013
Formamidinas (1975)	*R. microplus*	Australia 1981; Brasil 1995; Colombia 2000; México 2002
	Rhipicephalus spp	Sul de Africa 1997
	Amblyomma mixtum	México 2013
Lactonas Macrociclicas	*Rhipicephalus decoloratus*	Sul de Africa 1987
	Rhipicephalus microplus	Brasil 2001; México 2010
Fipronil	*Rhipicephalus microplus*	México 2013
	Amblyomma mixtum	México 2013

Fonte: adaptado de George *et al.* (2004) e Graf *et al.* (2004).

Alonso-Díaz *et al*. (2013) reportaram que, em A. *Mixtum*, a resistência aos acaricidas foi de 100; 91,7 e 12,5% a diazinon, coumafós e clorpirifós (organofosforados), respectivamente. *A. mixtum* menifestou-se resistente, também, para a família de amitraz com 12.5% de mortalidade, sendo susceptivel para flumethril e fipronil. Assim mesmo foi identificada uma taxa de 86% de fazendas infestadas por *A. mixtum* em 43 fazendas avaliadas.

Figura 4 - Distribuição da resitencia aos acaricidas em *Rhiphicephalus microplus* no Brasil



Fonte Higa *et al*. 2015 atualizado.

2.6 Uso de vacinas

O desenvolvimento de vacinas contra carrapatos é uma ferramenta que está sendo avaliada para controlar o problema do carrapato e diminuir a situações da resistência aos acaricidas. As vacinas surgiram na década de 1990 com o intuito de reduzir o uso de acaricidas, e diminuir as perdas econômicas na agropecuária (NUTTAL *et al*., 2006). No mercado estão disponíveis a vacina Gavac^{TM} produzida em Cuba e a TickGard^{TM}, produzida na Austrália. Elas foram desenvolvidas com a caracterização do genoma do carrapato, com base na proteína Bm86, mediante a expressão na levedura *Pichia pastoris* dirigida para *Rhipicephalus microplus* (De La FUENTE *et al*., 1998).

Os antígenos Bm86 e Bm95 foram identificados a partir do intestino do carrapato e testados com o intuito de intervenir nos parâmetros reprodutivos dos carrapatos (diminuir o número de teleóginas, e a taxa de eclosão) e, de forma indireta, ter uma redução de doenças como a TPB (WILLADSEN, 2006; DOMÍNGUEZ-GARCÍA *et al*., 2010; LAMBERTZ *et al*., 2012).

Rodríguez *et al*. (2004) relataram que a vacina GAVAC™ reduziu o uso de acaricidas em 87%, prolongando o tempo do uso de produtos químicos até por 71 dias. Na Austrália a vacina TickGard ™ contribuiu no rendimento produtivo (18.6 kg em bovinos) (JOHNSON *et al*., 2000; de la FUENTE *et al*., 2007). Na Colômbia o uso da vacina a base de Bm86 reduziu o número de tratamentos de carrapaticidas e, tambem diminuiu a população de carrapatos. O uso da vacina contribuiu, para a diminuição de surtos de babesioses y ercliquiose em bovinos (De La FUENTE *et al*., 1998). Andreotti *et al*. (2002) reportaram no Brasil uma eficácia 72.8% da vacina a base de BmTI contra *Rhipicephalus microplus*. Fragoso *et al*. (1998), mencionaram que a vacina é especifica para *R. microplus* sem ter efeito em *A. mixtum*.

2.7 Desenvolvimento de vacinas

A Bm86 é uma proteína ligada a membrana celular nas microvilosidades do intestino dos carrapatos adultos *R. microplus*, (WILLADSEN, JONGEJAN, 1999). A purificação realizada da proteína Bm86 pela primeira vez por cientistas australianos se baseou em algumas propriedades físicas anteriormente conhecidas tais como peso molecular, motilidade em SDS-PAGE, ponto isoelétrico de 5,1 a 5,6 e afinidade para lectina (WILLADSEN *et al*., 1989).

Torna-se necessario desenvolver uma vacina que proporcione controle eficaz de carrapatos do gênero *Amblyomma*. Esses carrapatos ingerem grandes quantidades de sangue, por tal motivo, mecanismos de transporte de água são eficientes com a finalidade de concentrar os componentes do sangue para uma digestão satisfatória. Atualmente se tem utilizado a combinação de aminoácidos (aquaporinas) que podem ativar e desativar por diferentes mecanismos de regulação de água (GUERRERO *et al*., 2014).

Estas proteínas aquaporinas se estendem por toda a membrana celular e podem localizar-se nas células dos rins, eritrócitos, glândulas salivares, trato digestivo e pulmonar em humanos (LÓPEZ-DOMÍNGUEZ, PASANTES, 2009). As aquaporinas regulam o transporte de água formando uma linha de dez moléculas através de seus compartimentos celulares. A importância das aquaporinas também pode ser pelo fato

de poderem participar de processos fisiológicos e alterações patológicas (GUERRERO *et al.,* 2014).

2.8 Vacinologia reversa

A bioinformática disponibiliza ferramentas que contribuem com o desenho de vacinas sintéticas, envolvendo áreas da biologia, estatística e imunologia, com o objetivo de utilizar dados de genômica, como sequências estabelecidas em bancos de dados, para identificar antígenos específicos contra doenças causadas por vírus, bactérias ou parasitos. O propósito da vacinologia reversa é encontrar proteínas ou determinantes antigênicos presentes na região extracelular do parasita, que sejam reconhecidos pelo sistema imune do hospedeiro tendo a presença de epítopos lineares de células B e/ou T (MARITZ-OLIVER *et al*., 2012; AGUIRRE *et al*., 2018).

3 OBJETIVOS

3.1 Objetivo geral

Desenvolver estudos sobre os aspectos da biologia, resistência aos acaricidas e o efeito da imunoproteção do antigeno aquaporin no controle do carrapato *Amblyomma* spp.

3.2 Objetivos específicos

a) Caracterizar o ciclo biológico do carrapato *Amblyomma mixtum* (Acari: Ixodidae) parasitando bovinos, coelhos e ovelhas em condições de laboratório.
b) Analisar o perfil de eficácia dos acaricidas à base da cipermetrina e clorpirifós no controle do carrapato *Rhipicephalus microplus* em bovinos em fase de recria, naturalmente infestados e expostos a agentes da TPB no Brasil Central.
c) Determinar a resistência de *Amblyomma mixtum* proveniente do México às diferentes familias de acaricidas comerciais em diferentes estágios da vida, alimentados e não alimentados.
d) Avaliar a eficácia da imunoproteção de um peptídeo sintético baseado na proteína aquaporina no carrapato *Amblyomma sculptum*.

4 METODOLOGIA

O trabalho realizado que atende o primeiro objetivo especifico do presente estudo é de tipo experimental e foi desenvolvido no laboratorio de Embrapa Gado de Corte, no periodo de novembro de 2014 até Março 2016. Foram avaliados parâmetros produtivos e repodutivos de carrapatos da especie *A. Mixtum* parasitando diferentes espécies de hospedeiros (Coelhos, Ovelhas e Bovinos) estabelecido pela metodologia de (SZABÓ et al 1995). Os procedimentos foram considerados de acordo com os cuidados e uso de animais, de acordo com as regras do Conselho Nacional de Experimentação Animal (CONCEA). A pesquisa em animais foi aprovada pelo Comitê de Ética em Uso Animal /CEUA da Universidade Federal do Mato Grosso do Sul, Campo Grande, MS, Brasil, protocolo número 699/2015.

A metodologia utilizada para analisar o perfil de eficácia aos acaricidas à base da cipermetrina e clorpirifos sobre o controle de *Rhipicephalus microplus* em bovinos em fase de recria, naturalmente infestados e expostos a agentes da TPB no Brasil central. Foi realizado durante o periodo de Junho ao Novembro de 2016 na Fazenda agropecuaria, Sanyo, ubicada no Municipio de Agua Clara, Mato Grosso do Sul. A eficacia dos acaricidas utilizados foi mediante a formula estabelecida por Corrêa *et al*., (2015) Cruz *et al*., 2015) tipo de estudo foi experimental, o manejo dos animais foi em base aos procedimentos do uso de animais que obedeceram às normas publicadas pelo Conselho Nacional de Controle de Experimentação Animal / CONCEA e foram aprovados pela Comissão de Ética do Uso de Animais / CEUA da Embrapa Gado de Corte, protocolo no. 01/2016.

Os bioensaios utilizados para determinar a eficácia dos acaricidas em larvas e ninfas (alimentadas e não alimentadas) e adultos sem alimentar de *A. Mixtum*, foi realizada em EMBRAPA Gado de Corte, no periodo de Enero de 2016 até Março de 2016, A metodologia foi adaptada em base a (FAO 2004). O uso de coelhos foi importante para a reprodução e fornecimento dos carrapatos utilizados na metodologia estabelecida por Szabó et al. (1995). Os procedimentos utilizando animais estão de acordo com as normas do Animal Experimentação Conselho Nacional Brasileiro (Conselho Nacional Brasileiro de Experimentação Animal

CONCEA). O estudo foi aprovado pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Federal de Mato Grosso do Sul, protocolo nº 699/2015.

O trabalho realizado que atende o quarto objetivo especifico é um estudo de tipo experimental. Foi realizado no periodo de Julho 2016 até Janeiro 2017. Para avaliar os parametros reprodutivos e produtivos foi utilizada a metodologia estabelecida por Szabó et al. (1995). A eficacia do peptideo sintetico foi avaliada por a formula proposta por Aguirre et al. (2016). A metodologia foi aprovada pelo Comitê de Ética (CEUA nº 699/2015 da EMBRAPA Gado de Corte).

5 RESULTADOS

Os Resultados da pesquisa são apresentados sob a forma de artigos correspondentes aos objetivos especificos.

Artigo 1

Life cycle of *Amblyomma mixtum* (Acari:Ixodidae) parasitizing different hosts under laboratory conditions. Experimental Applied Acarology, v. 73. n. 2, p. 257-267, Sept. 2017, referente a caracterização do ciclo biológico de vida de *Amblyomma mixtum* (Acari: Ixodidae) parasitando bovinos, coelhos e ovelhas em condições de laboratório) os dados foram publicados na revista Journal of Experimental Applied Acarology (Qualis B1)

Exp Appl Acarol
DOI 10.1007/s10493-017-0178-y

Life cycle of *Amblyomma mixtum* (Acari: Ixodidae) parasitizing different hosts under laboratory conditions

Francisco Tobias Barradas Piña[1,2,7] · Vinicius da Silva Rodrigues[1,2] · Leandro de Oliveira Souza Higa[1,2] · Marcos Valério Garcia[3,4] · Jacqueline Cavalcante Barros[5] · Adalberto Angel Pérez de León[6] · Renato Andreotti[5]

Received: 29 June 2017 / Accepted: 5 September 2017

Abstract *Amblyomma mixtum* is a tick species in the *Amblyomma cajennense* complex. The known geographic range of *A. mixtum* extends from Texas in the USA to western Ecuador and some islands in the Caribbean. *Amblyomma mixtum* is a vector of disease agents of veterinary and public health importance. The objective of this study was to describe the life cycle of *A. mixtum* under laboratory conditions. Bovines, rabbits and sheep were infested with larvae, nymphs, and adult ticks under controlled conditions to assess several biological parameters. Eggs, larvae, nymphs and adults were kept in an incubator (27 °C temperature

✉ Renato Andreotti
renato.andreotti@embrapa.br

Francisco Tobias Barradas Piña
fcobarradast@gmail.com

Vinicius da Silva Rodrigues
vinicius_srod@hotmail.com

Leandro de Oliveira Souza Higa
leandro-higa@hotmail.com

Marcos Valério Garcia
marcosvagar@gmail.com

Jacqueline Cavalcante Barros
jacqueline.barros@embrapa.br

Adalberto Angel Pérez de León
beto.perezdeleon@ars.usda.gov

[1] Pós-Graduação em Doenças Infecciosas e Parasitárias - Faculdade de Medicina, UFMS - Universidade Federal Mato Grosso do Sul, Campo Grande, MS, Brazil

[2] Laboratório de Biologia do Carrapato, Embrapa Gado de Corte, Campo Grande, MS, Brazil

[3] Laboratório de Biologia do Carrapato, Empresa Brasileira de Pesquisa Agropecuária, Embrapa Gado de Corte, Campo Grande, MS, Brazil

[4] Bolsista DCR Fundect, MS – Governo do estado de Mato Grosso do Sul, Campo Grande, Brazil

[5] Empresa Brasileira de Pesquisa Agropecuária, Embrapa Gado de Corte, Avenida Rádio Maia, 830, Campo Grande, MS CEP 79106-550, Brazil

Published online: 09 September 2017

and 80% relative humidity) when they were off the host. The average life cycle of *A. mixtum* was 88 and 79 days when fed on rabbits and cattle, respectively. Sheep were found to be unsuitable because no ticks attached. The rabbit is a more practical host to maintain a colony of *A. mixtum* under laboratory conditions. The data from this study can be considered as an example for the life cycle of *A. mixtum*. However, caution must be exercised when making comparisons to the biology of *A. mixtum* in its natural habitat.

Keywords Bovine · Rabbit · Laboratory colony · Host suitability · Life cycle

Introduction

Ticks and tick-borne diseases affect humans and animals. Of the approximately 900 extant tick species described worldwide, those in the Ixodidae are the most diverse, comprising approximately 692 species (Nava et al. 2009; Sonenshine and Roe 2014).

Several species in the genus *Amblyomma*, one of 12 genera in the Ixodidae, are of veterinary and public health importance (Labruna et al. 2009; Guzman-Cornejo et al. 2011), including *Amblyomma mixtum* (Nava et al. 2014), which has a three-host life cycle (parasitizing humans, livestock, and wildlife) and is a vector of disease agents (Parola et al. 2005; Castro et al. 2015).

Following the original description by Koch (1844) based on specimens from Mexico, *A. mixtum* was reinstated as a species in the *A. cajennense* complex that includes *A. cajennense* sensu stricto (s.s.), *A. mixtum*, *Amblyomma tonelliae*, *Amblyomma interandinum*, *Amblyomma patinoi* and *Amblyomma sculptum* (Nava et al. 2014). The geographic range of *A. mixtum* extends from Texas in the USA to western Ecuador and some islands in the Caribbean, including Cuba and Trinidad and Tobago (Estrada-Pena et al. 2014; Nava et al. 2014; Beck and Orozco 2015). In some parts of Mexico, *A. mixtum* is an economically important ectoparasite of cattle, second only to *Rhipicephalus microplus* (Guzman-Cornejo et al. 2011; Rodriguez-Vivas et al. 2014). Controlling *A. mixtum* infestations in livestock can be challenging because of its euryxenous parasitic habit (Rodriguez-Vivas et al. 2016).

The biology of *A. mixtum* is not yet fully understood. The availability of laboratory tick colonies facilitates the study of tick biology, vector competence, and control technologies (Bouchard and Wikel 2005). However, some of the challenges to establishing and maintaining tick colonies in the laboratory setting include a shortage of adequate facilities, difficulties in maintaining experimental conditions, and high costs (Allan 2014; Thangamani and Bente 2014).

The results of our study on the colonization and life cycle of *A. mixtum* under laboratory conditions are presented here. Because of the complex interactions at the tick-host interface and their influence on tick rearing (Bouchard and Wikel 2005; Gerardi et al. 2013; Allan 2014), three animal species were tested for host suitability under laboratory conditions. The findings are discussed in the context of available data for other laboratory colonies of species in the *A. cajennense* complex.

[6] USDA-ARS, Knipling-Bushland U.S. Livestock Insects Research Laboratory, and Veterinary Pest Genomics Center, Kerrville, TX, USA

[7] Instituto Nacional de Investigaciones Forestales Agricolas y Pecuarias INIFAP, Mexico City, Mexico

Materials and methods

Animal care and use

All experimental procedures involving animals were conducted in accordance with the rules of the Brazilian National Council of Animal Experimentation (CONCEA). Animal research was approved by the Ethics Committee on Animal Use/CEUA at the Federal University of Mato Grosso do Sul, Campo Grande, MS, Brazil (protocol number 699/2015). In this study sheep, bovines and rabbits were used.

Tick rearing facility

The study was performed on the Embrapa Gado de Corte Experimental Farm (20°25′03″S, 54°42′20″W), Animal Health sector, municipality of Campo Grande, state of Mato Grosso do Sul, Brazil, between November 2014 and March 2016.

Tick source and rearing

Amblyomma mixtum engorged females were collected from naturally infested bovines at a farm in Medellin de Bravo, Veracruz, Mexico (19°01′01.6″N, 96°08′14.4″W). Engorged female ticks were weighed using an analytical scale and housed in vials with perforated covers and maintained in B.O.D. incubators at 27 °C and 80% relative humidity and a photoperiod regimen 12:12 (light:darkenss). In addition, the other tick life stages (eggs, larvae, and nymphs) were maintained under the same laboratory conditions to collect off-host data. The species was morphologically identified in accordance with the taxonomic characteristics described by Nava et al. (2014).

Hosts

Bovines (*Bos taurus*), rabbits (*Oryctolagus cuniculus*), and sheep (*Ovis aries*) were tested for their suitability as hosts for *A. mixtum* under laboratory conditions because those vertebrate species were reported to be susceptible to natural infestation or suitable laboratory hosts for North American populations formerly referred to as *A. cajennense* (Cooley and Kohls 1944; Drummond and Whetstone 1975). Ten rabbits of the New Zealand breed, with no previous tick contact and without distinction of gender were used. The chambers were fixed with atoxic glue, according to Szabó et al. (1995). The animals were kept in individual cages with free access to food and water.

Three bovines (*Bos taurus*), ranging in age from 8 to 12 months were kept in individual stalls with free access to food and water. The animals, with previous tick contact, were not treated with acaricides for at least 30 days before infestation. Plastic chambers were used as described above in an area on the back of the animal where hair had been clipped to allow tick feeding. Care and use protocols for bovines were adapted for two adult Santa Ines sheep. These animals had previous contact with ticks. A plastic chamber was also glued to a shaved portion of the sheep's back to confine ticks and allow blood feeding. Santa Ines sheep were chosen because they are sensitive to infestation by ticks (Garcia et al. 2014).

Experimental infestations

Approximately 1000 larvae, 90 nymphs and 12 adult ticks (six males and six females), were used per infestation and per animal. Ticks were placed in feeding chambers on the rabbits, cattle and sheep. All ticks used in experimental infestations were between 15 and 20 days in age. Tick fixation takes only a few minutes. Feed chambers were individually inspected daily to collect engorged ticks. All naturally detached engorged ticks from different hosts were removed from the chambers and taken to the laboratory where they were counted, weighed and identified in vials for incubation and maintained in B.O.D. incubators at 27 °C and 80% relative humidity, and a photoperiod regimen 12:12 (light:darkenss).

Parameters analyzed

Several biological parameters were assessed in larvae, nymphs, and engorged female ticks. The formulae described below were adapted and information from other studies was used to make relevant comparisons to *A. cajennense* sensu lato (s.l.) (Hooker et al. 1912; Drummond and Whetstone 1975; Szabó et al. 1995; Sanavria and Prata 1996; Gerardi et al. 2013). To calculate the biological parameters we performed a weighted average. The duration of the life cycle described by us is related only to feeding, molting, pre-oviposition and incubation, being discarded the period when unfed ticks were allowed to feed.

Larval parameters

The engorgement period was defined as the number of days from larvae infestation on the host until the collection day of detached larvae. To calculate the average duration of the engorgement period we performed a weighted average. The individual weight of engorged larvae was estimated from the total weight of larvae collected divided by the number of larvae collected. The duration of the molting process period was defined as the number of days from larvae detachment from the host until the premolt periods. The molting success was defined as the number of larvae that underwent metamorphosis divided by the number of engorged larvae that were collected multiplied by 100%.

Nymphal parameters

The feeding success was defined as the number of nymphs collected divided by the number of released nymphs multiplied by 100%.

The engorgement period was defined as the number of days from nymph infestation on the host until the collection day of released nymphs. To calculate the average duration of the engorgement period we performed a weighted average. The individual weight of engorged nymphs was defined as the total weight of nymphs collected divided by the number of nymphs collected. The duration of the molting process period was defined as the number of days from nymphs detachment from the host until the premolt periods. The molting success was defined as the number of engorged nymphs that underwent metamorphosis divided by the number of engorged nymphs collected, multiplied by 100%.

Engorged female parameters

The feeding success was defined as the number of engorged females that were collected divided by the number of released females multiplied by 100%. The engorgement period was defined as the number of days from the release of adult ticks on the host until the detachment of engorged females. To calculate the average duration of the engorgement period we performed a weighted average. Engorged female weight was determined by weighing each adult female. The pre-oviposition period was defined as the number of days from detachment until the beginning of oviposition. Egg mass weight was defined as the total egg mass deposited by the engorged female. The egg incubation period was defined as the number of days from the beginning of oviposition until larvae eclosion. Larvae eclosion percentage was determined by visual estimation and calculated from the average obtained by three examiners (Szabó et al. 1995). The efficiency index of the conversion of food reserve into eggs was defined as egg mass weight divided by engorged female weight multiplied by 100%.

Descriptive statistics were applied to summarize and describe the biological data. The life cycle parameters of *A. mixtum* obtained in the laboratory were analyzed using BioEstat 5.0 software, with Kruskal–Wallis and Mann–Whitney tests ($\alpha = 0.05$).

Results

Bovines and rabbits were more suitable than were sheep as hosts for *A. mixtum* under laboratory conditions. Sheep were found to be unsuitable because no ticks attached.

Infestation on rabbits

It took 88 days on average for *A. mixtum* to complete its life cycle when feeding on rabbits under laboratory conditions. Thus, approximately 4.1 generations/year could be produced under the laboratory conditions used in this study. Biological parameters obtained from larvae and nymphs were listed in Tables 1 and 2, respectively. The values of reproductive parameters for females are listed in Table 3.

Table 1 Mean (± SD; range in parentheses) biological parameters of *Amblyomma mixtum* larvae feeding on rabbit or bovine hosts under laboratory conditions

Host	Weight (mg)	Period (days)		Percentage
		Feeding	Molting	Molting
Rabbit	0.73 ± 0.6^{a}	6.3 ± 2.4^{a}	12.6 ± 2.3^{a}	74.6 ± 16.3^{a}
	(0.65–0.78)	(3–12)	(9–17)	(60–96.8)
Bovine	0.96 ± 0.3^{b}	5.5 ± 1.2^{b}	11.5 ± 1.2^{b}	90.5 ± 6.9^{b}
	(0.77–1.41)	(4–7)	(10–13)	(80.4–96)

Means within a column followed by different letters are significantly different ($p < 0.05$)

Table 2 Mean (± SD; range in parentheses) biological parameters of *Amblyomma mixtum* nymphs feeding on rabbit and bovine hosts under laboratory conditions

Host	Yield (%)	Weight (mg)	Period (days)		Percentage
			Feeding	Molting	Molting
Rabbit	65 ± 28.2 (27.8–92.9)	12.8 ± 2.7 (9.1–15.2)	7.45 ± 2.9 (3–13)	13.5 ± 2.3 (9–17)	92.7 ± 14.5 (71–100)
Bovine	43.3 ± 49.3 (10.3–100)	15.6 ± 1.7 (13.7–17)	6 ± 2.5 (4–10)	12.6 ± 2.4 (10–16)	92 ± 10.5 (80–100)

Infestation on cattle

The average life time of *A. mixtum* using bovine hosts under laboratory conditions was 79 days. Thus, approximately 4.6 generations/year could be produced under the laboratory conditions used in this study. For larvae and nymphs, the mean values obtained were listed in Table 1 and Table 2, respectively. The values of reproductive parameters for females are listed in Table 3.

Discussion

This is the first study to report data on the life cycle of *A. mixtum* under laboratory conditions on three different hosts. This tick species was originally described by Koch (1844) and was reinstated by Nava et al. (2014). Ticks in the genus *Amblyomma* are represented by 26 species in Mexico, and *A. mixtum* is one of the most widely distributed and prevalent species parasitizing livestock, wildlife and humans (Guzman-Cornejo et al. 2011; Rodriguez-Vivas et al. 2016).

The study of *A. mixtum* as a member of the *A. cajennense* complex is important because previous information related to *A. mixtum* in North America was reported under the *A. cajennense* specific name, including data for colonies maintained in several North American laboratories that did not highlight the potential variations between the six species that are part of the complex (Hooker et al. 1912; Cooley and Kohls 1944; Drummond and Whetstone 1975).

Cattle and sheep can be naturally infested with *A. cajennense* s.l. (Guglielmone and Nava 2006; Alvarez and Bonilla 2007; Gonzalez-Ceron et al. 2009; Ramos et al. 2016). Rabbits are small laboratory animals that are commonly used to maintain colonies of ticks (Sanavria and Prata 1996). The laboratory results presented here confirm field observations suggesting that *A. mixtum* is a generalist host. Based on our findings, rabbits may be the preferred species for long-term maintenance of *A. mixtum* colonies because of their small size, which makes them relatively easier to handle and more convenient with regard to the laboratory facilities required for housing and husbandry costs. And also because all instars of this tick specie had satisfactory results when submitted to feeding on rabbits.

According to the results of this study, the life cycle of *A. mixtum* under laboratory conditions was 88 days when infested on rabbits and 79 days when infested on cattle, unlike that presented by Almazán et al. (2016), this authors reported a life cycle for this species of tick from 133 to 193 days. For pre-oviposition, the present study reports a

Table 3 Mean (± SD; range in parentheses) reproductive parameters of *Amblyomma mixtum* females feeding to repletion on rabbit or bovine hosts under laboratory conditions

Host	Yield (%)	Weight (mg)		Period (days)			Percentage	
		Female	Eggs	Feeding	PRE-oviposition	Incubation	Larvae hatching rate	Reproductive efficiency
Rabbit	81.1 ± 20.0	550.6 ± 78.6	302.5 ± 38.1	10 ± 2.4	5.5 ± 1.2	33.1 ± 3.7	68.2 ± 30.3	55 ± 0.94
	(60–100)	(462–612.3)	(259.4–332.2)	(6–15)	(4–8)	(27–39)	(51.7–82)	(54.3–56.1)
Bovine	40 ± 0	237 ± 10.6	146.7 ± 3.1	6 ± 0	8 ± 0	30 ± 0	47.5 ± 3.5	61.9 ± 1.4
	(20)*	(229.5–244.5)	(144.5–148.9)	(6)*	(8)*	(30)*	(45–50)	(60.9–63)

* The data presented in these variables show that the ticks were collected on the same day

significantly shorter period than those obtained by Almazán et al. (2016), which showed a variation of 25–29 days for the same parameter, with the cattle as hosts.

Under the same conditions of the present study Almazán et al. (2016) reported an incubation period similar to the results presented by us, as well as a similarity in the engorgement time of all instars of this species of tick in both studies. However different values were reported for the duration of the molting process of nymphs and adults, in which these authors obtained a greater period for this parameter.

In relation to the weight of engorged ticks recovered as well as the respective masses of eggs obtained, the values presented here are significantly lower than those obtained by Almazán et al. (2016). However, the food conversion rate of *A. mixtum* in the present study has a difference of 8.6% more compared to Almazán et al. (2016).

The hypotheses for the differences between the parameters analyzed between larvae, nymphs and adults in the present work in comparison to the results obtained by Almazán et al. (2016) can be several factors, such as, management condition, different breeds of cattle used as hosts, climatic conditions of the study sites and, season of the year.

Our results were similar to those reported for *A. mixtum* (reported as *A. cajennense*) in Texas (Hooker et al. 1912; Strickland et al. 1976). Nymphs of what is now *A. mixtum* from Texas took 3–13 days to engorgement (Strickland et al. 1976), whereas, in our study, nymphs completed feeding after 4–10 days on cattle, and 3–13 days on rabbits.

The acquisition of data for the life cycle of *A. mixtum* under laboratory conditions provided an opportunity for comparative analyses with other tick species of the *Amblyomma cajennense* complex and other *Amblyomma* species under laboratory conditions.

Considering the data reported by Tarragona et al. (2015), *A. tonelliae* under laboratory conditions, when evaluated in different photoperiods before and after feeding, presented a pre-oviposition period of 7–17 days when rabbits were used as hosts. For the *A. mixtum* infesting the same host species, in the present study, the pre-oviposition period varied from 4 to 8 days.

Amblyomma tonelliae engorged female weight varied from 291 to 791 mg, while the *A. mixtum* engorged female weight when infesting rabbits varied from 462 to 612.3 mg, while when infesting bovines varied from 229.5 to 244.5 mg. The incubation period for *A. tonelliae* varied from 36 to 45 days, and for *A. mixtum* varied from 27 to 39 days when infesting rabbits.

Lisboa et al. (1998) reported that *A. sculptum* larvae feeding on rabbits required three to seven days to engorgement. Prata et al. (1996), in a study with *A. sculptum* nymphs feeding on rabbits, reported a recovery rate of 53.4% for nymphs, an engorgement period of 5 days, and a 95% molting success. Similar values to the results found in this study with *A. mixtum*.

Regarding the adult ticks, yield values and reproductive efficiencyfor *A. mixtum* were similar to the values reported for other tick species from genre *Amblyomma* under laboratory conditions (Troughton and Levin 2007; Olegario et al. 2011; Faccini et al. 2010; Labruna et al. 2004).

Comparisons of biological parameters across the life stages of *Amblyomma* reared in the laboratory show the complex adaptations associated with a three-host life cycle, which may be affected by biotic and abiotic factors (Szabó et al. 2009).

The colony of *A. mixtum* provides an opportunity to produce a continuous supply of ticks for further research on their biology, role as a vector of disease agents, and control technologies. This is especially important in parts of North America where *A. mixtum* is a vector of veterinary and public health importance, including Mexico, where laboratory colonies are not available (Gonzalez-Ceron et al. 2009; Illoldi-Rangel et al. 2012; Alonso-Diaz et al. 2013).

Significant differences have been reported in biological parameters between conspecific ticks under field conditions and those reared in the laboratory (Yu et al. 2010; Meng et al. 2014). The results showed that rabbits and bovines can be a host to maintain this tick species in laboratory, the data from this study conducted under laboratory conditions can be considered as an example for the life cycle of *A. mixtum*. However, caution must be exercised when making comparisons to the biology of *A. mixtum* in its natural habitat.

Acknowledgements To the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul (Fundect, MS) - Governo do Estado de Mato Grosso do Sul, Embrapa Gado de Corte, Instituto Nacional de Investigaciones Forestales Agricolas y Pecuarias (INIFAP-Mexico), and Consejo Nacional de Ciencia y Tecnologia (CONACyT) for financial support of the project, USDA is an equal opportunity provider and employer.

Compliance with ethical standards

Conflict of interests The authors declare to have no conflict of interest.

References

Allan SA (2014) Tick rearing and in vitro feeding. In: Sonenshine DE, Roe RM (eds) Biology of ticks, 2nd edn. Oxford University Press, New York, pp 445–473. ISBN 978-0-19-974450-3

Almazán C, Torres-Torres A, Torres-Rodriguez L, Soberanes-Cespedes N, Ortiz-Estrada M (2016) Aspectos biologicos de *Amblyomma mixtum* (Koch, 1844) en el noreste de Mexico. Queh Cient Chiapas 11:10–19

Alonso-Diaz MA, Fernandez-Salas A, Martinez-Ibanez F, Osorio-Miranda J (2013) *Amblyomma cajennense* (Acari: Ixodidae) tick populations susceptible or resistant to acaricides in the Mexican Tropics. Vet Parasitol 197:326–331. doi:10.1016/j.vetpar.2013.06.004

Alvarez CV, Bonilla MR (2007) Adultos y ninfas de la garrapata *Amblyomma cajennense* Fabricius (Acari: Ixodidae) en equinos y bovinos. Agron Costarric 31:61–69

Beck DL, Orozco JP (2015) Diurnal questing behavior of *Amblyomma mixtum* (Acari: Ixodidae). Exp Appl Acarol 66:613–621. doi:10.1007/s10493-015-9928-x

Bouchard K, Wikel S (2005) Care, maintenance and experimental infestation of ticks in the laboratory setting. Biol Dis Vectors 2:705–712

Castro DAM, Garcia SGG, Dzul-Rosado K, Aguilar A, Castillo J, Gabster A, Trejos D, Zavala-Castro J, Bermúdez SEC (2015) Questing *Amblyomma mixtum* and *Haemaphysalis juxtakochi* (Acari: Ixodidae) infected with candidatus "*Rickettsia amblyommii*" from the natural environment in Panama Canal Basin, Panama. Trop Med Health 43:217–222. doi:10.2149/tmh.2015-07

Cooley RA, Kohls GM (1944) The genus *Amblyomma* (Ixodidae) in the United States. J Parasitol 30:77–111. doi:10.2307/3272571

Drummond RO, Whetstone TM (1975) Oviposition of the cayenne tick, *Amblyomma cajennense* (F.), in the laboratory. Ann Entomol Soc Am 68:214–216. doi:10.1093/aesa/68.2.214

Estrada-Pena A, Tarragona EL, Vesco U, Meneghi D, Mastropaolo M, Mangold AJ, Guglielmone AA, Nava S (2014) Divergent environmental preferences and areas of sympatry of tick species in the *Amblyomma cajennense* complex (Ixodidae). Int J Parasitol 44:1081–1809. doi:10.1016/j.ijpara.2014.08.007

Faccini JLH, Cristina BA, Onofrio CVC, Labruna MB, Barros-Battesti DM (2010) The life cycle of *Amblyomma auricularium* (Acari: Ixodidae) using rabbits (*Oryctolagus cuniculus*) as experimental host. Exp Appl Acarol 50:71–77. doi:10.1007/s10493-009-9281-z

Garcia MV, Andreotti R, Reis FA, Aguirre AAR, Barros JC, Matias J, Koller WW (2014) Contributions of the hair sheep breed Santa Ines as a maintenance host for *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) in Brazil. Parasites Vectors 7:1–4. doi:10.1186/s13071-014-0515-5

Gerardi M, Martins MM, Nava S, Szabo MPJ (2013) Comparing feeding and reproductive parameters of *Amblyomma parvum* tick populations (Acari: Ixodidae) from Brazil and Argentina on various host species. Vet Parasitol 197:312–317. doi:10.1016/j.vetpar.2013.06.018

Gonzalez-Ceron F, Becerril-Perez CM, Torres-Hernandez G, Diaz-Rivera P (2009) Ticks infesting body regions of tropical milking criollo cattle in Veracruz, Mexico. Agrociencia 43:11–19

Guglielmone AA, Nava S (2006) Las Garrapatas Argentinas del Genero *Amblyomma* (Acari: Ixodidae): Distribucion y hospedadores. RIA 35:133–153

Guzman-Cornejo C, Robbins RG, Guglielmone AA, Montiel-Parra G, Perez TM (2011) The *Amblyomma* (Acari: Ixodida: Ixodidae) of Mexico: Identification keys, distribution and hosts. Zootaxa 2998:16–38

Hooker WA, Bishopp FC, Wood HP (1912) The life history and bionomics of some North American ticks. Bur Entomol Bull 106:72–87

Illoldi-Rangel P, Rivaldi CL, Sissel B, Fryxell RT, Gordillo-Perez G, Rodriguez-Moreno A, Montiel-Parra P, Sánchez-Cordero V, Sarkar S (2012) Species distribution models and ecological suitability analysis for potential tick vectors of lyme disease in Mexico. J Trop Med 2012:1–10. doi:10.1155/2012/959101

Koch CL (1844) Systematische Übersicht über die ordnung der zecken. Arch Naturg 10:217–239

Labruna MB, Pinter A, Teixeira RHF (2004) Life cycle of *Amblyomma cooperi* (Acari: Ixodidae) using capybaras (*Hydrochoerus hydrochaeris*) as hosts. Exp Appl Acarol 32:79–88. doi:10.1023/B:APPA.0000018228.05088.26

Labruna MB, Terassini FA, Camargo LM (2009) Notes on population dynamics of Amblyomma ticks (Acari: Ixodidae) in Brazil. J Parasitol 95:1016–1018. doi:10.1645/GE-1878.1

Lisboa CML, Leite RC, Labruna MB, Oliveira PR, Borges LMF, Rodrigues ZB, Carvalho HA, Freitas CMV, Júnior CRV (1998) Host specificity of *Amblyomma cajennense* (Fabricius, 1787) (Acari: Ixodidae) with comments on the drop-off rhythm. Mem Inst Oswaldo Cruz 93:347–351. doi:10.1590/S0074-02761998000300014

Meng H, Xu S, Yu Z, Liu Z, Liu J, Yang X, Liu J (2014) The life cycle and occurrence of *Haemaphysalis concinna* (Acari: Ixodidae) under field conditions. Ticks Tick Borne Dis 5:887–891. doi:10.1016/j.ttbdis.2014.07.007

Nava S, Guglielmone AA, Mangold AJ (2009) An overview of systematics and evolution of ticks. Front Biosci 14:2857–2877. doi:10.2735/3418

Nava S, Beati L, Labruna MB, Caceres AG, Mangold AJ, Guglielmone AA (2014) Reassessment of the taxonomic status of *Amblyomma cajennense* (Fabricius, 1787) with the description of three new species, *Amblyomma tonelliae* n. sp., *Amblyomma interandinum* n. sp. and *Amblyomma patinoi* n. sp., and reinstatement of *Amblyomma mixtum* Koch, 1844, and *Amblyomma sculptum* Berlese, 1888 (Ixodida: Ixodidae). Ticks Tick Borne Dis 5:252–276. doi:10.1016/j.ttbdis.2013.11.004

Olegario MMM, Gerardi M, Tsuruta SA, Szabo MPJ (2011) Life cycle of the tick *Amblyomma parvum* Aragao, 1908 (Acari: Ixodidae) and suitability of domestic hosts under laboratory conditions. Vet Parasitol 179:203–208. doi:10.1016/j.vetpar.2011.01.056

Parola P, Christopher D, Paddock CD, Raoult D (2005) Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. Clin Microbiol Rev 18:719–756

Prata AMC, da Silva LA, Sanavria A (1996) Parâmetros biológicos do estagio ninfal de *Amblyomma cajennense* (Fabricius, 1787) (Acari: lxodidae) em coelhos. Rev Bras Ciênc Vet 3:55–57. doi:10.4322/rbcv.2015.045

Ramos VN, Piovezan U, Franco AHA, Rodrigues VS, Nava S, Szabo MPJ (2016) Nellore cattle (*Bos indicus*) and ticks within the Brazilian Pantanal: ecological relationships. Exp Appl Acarol 68:227–240. doi:10.1007/s10493-015-9991-3

Rodriguez-Vivas RI, Perez-Cogollo LC, Rosado-Aguilar JA, Ojeda-Chi MM, Trinidad-Martinez T, Miller JR, Li AY, de León AP, Guerrero F, Klafke G (2014) *Rhipicephalus* (*Boophilus*) *microplus* resistant to acaricides and ivermectin in cattle farms of Mexico. Braz J Vet Parasitol 23:113–122. doi:10.1590/S1984-29612014044

Rodriguez-Vivas RI, Apanaskevich DA, Ojeda-Chi MM, Trinidad-Martinez I, Reyes-Novelo E, Esteve-Gassent MD, de León AAP (2016) Ticks collected from humans, domestic animals, and wildlife in Yucatan, Mexico. Vet Parasitol 215:106–113. doi:10.1016/j.vetpar.2015.11.010

Sanavria A, Prata MCA (1996) Metodologia para colonização do *Amblyomma cajenennse* (Fabricius, 1787) (Acari:Ixodidae) em laboratório. Rev Bras Parasitol Vet 5:87–90

Sonenshine DE, Roe RM (2014) Biology of ticks, 2nd edn. Oxford University Press, Oxford. ISBN 978-0-19-974405-3

Strickland RK, Gerrish RR, Hourrigan JL, Schubert GO (1976) Ticks of veterinary importance. U.S. Government Printing Office, Washington

Szabó MPJ, Mukay LS, Rosa PCS, Bechara GH (1995) Differences in the acquired resistance of dogs, hamsters and guinea pigs to repeated infestations with adults ticks *Rhipicephalus sanguineus* (Acari:Ixodidae). Braz J Res Anim Sci 32:43–50. doi:10.11606/issn.0000-0000.32143-50

Szabó MPJ, Pereira LF, Castro MB, Garcia MV, Sanches GS, Labruna MB (2009) Biology and life cycle of *Amblyomma incisum* (Acari: Ixodidae). Exp Appl Acarol 48:263–271. doi:10.1007/s10493-008-9234-y

Tarragona EL, Mangold AJ, Mastropaolo M, Guglielmone AA, Nava S (2015) Ecology and genetic variation of *Amblyomma tonelliae* in Argentina. Med Vet Entomol 29:297–304. doi:10.1111/mve.12110

Thangamani S, Bente D (2014) Establishing protocols for tick containment at biosafety level 4. Pathog Dis 71:282–285. doi:10.1111/2049-632X.12187

Troughton DR, Levin ML (2007) Life cycles of seven Ixodid tick species (Acari: Ixodidae) under standardized laboratory conditions. J Med Entomol 44:732–740. doi:10.1093/jmedent/44.5.732

Yu Z, Zheng H, Chen Z, Zheng B, Ma H, Liu J (2010) The life cycle and biological characteristics of *Dermacentor silvarum* Olenev (Acari: Ixodidae) under field conditions. Vet Parasitol 168:323–328. doi:10.1016/j.vetpar.2009.11.010

Artigo 2

Efficacy profile of Cypermethrin and Chlorpyrifos based acaricides on *Rhipicephalus microplus* control on cattle in the rearing phase, naturally infested and exposed to tick fever agents in central Brazil. Veterinary Parasitology: Regional Studies and Reports v. 12 n. 2018, p. 43-48, Feb 2018. Referente ao objetivo analisar o perfil de eficácia aos acaricidas à base da cipermetrina e clorpirifos sobre o controle de *Rhipicephalus microplus* em bovinos em fase de recria, naturalmente infestados e expostos a agentes da TPB no Brasil central, foram publicados na Revista Veterinary Parasitology: Regional Studies and Reports (Fator de impacto: 0,71)

Veterinary Parasitology: Regional Studies and Reports 12 (2018) 43–48

Contents lists available at ScienceDirect

Veterinary Parasitology: Regional Studies and Reports

journal homepage: www.elsevier.com/locate/vprsr

Original Article

Efficacy profile of Cypermethrin and Chlorpyrifos based acaricides on *Rhipicephalus microplus* control on cattle in the rearing phase, naturally infested and exposed to tick fever agents in central Brazil

Vinicius da Silva Rodrigues[a,f], Paulino Bonatte Jr[b,f], Marcos Valério Garcia[f,g], Leandro de Oliveira Souza Higa[a,f], Francisco Tobias Barradas Piña[a,c,f], Namor Pinheiro Zimmermann[d], Pâmella Oliveira Duarte[a,f], Jacqueline Cavalcante Barros[e], Renato Andreotti[e,*]

[a] *Pós-Graduação em Doenças Infecciosas e Parasitárias, Faculdade de Medicina, UFMS - Univ. Federal Mato Grosso do Sul, Campo Grande, MS, Brazil*
[b] *Pós-Graduação em Ciência Animal, Faculdade de Medicina Veterinária, UFMS - Univ. Federal Mato Grosso do Sul, Campo Grande, MS, Brazil*
[c] *Instituto Nacional de Investigaciones Forestales Agricolas y Pecuarias INIFAP, Mexico*
[d] *Centro Universitário da Grande Dourados, Dourados, MS, Brazil*
[e] *Empresa Brasileira de Pesquisa Agropecuária, Embrapa Gado de Corte, Campo Grande, MS, Brazil*
[f] *Laboratório de Biologia do Carrapato, Embrapa Gado de Corte, Campo Grande, MS, Brazil*
[g] *Bolsista DCR Fundect, Governo do estado de Mato Grosso do Sul, MS, Brazil*

ARTICLE INFO

Key-words:
Resistance
Control, cattle tick
Bovines
Acaricides

ABSTRACT

The objective of this work was to evaluate the efficacy of two cypermethrin- and chlorpyrifos-based acaricides in controlling *Rhipicephalus microplus* in a naturally infested bovine herd and in *in vitro* tests, as well as to monitor the animals for tick fever. Male bovines in the rearing phase were used, with 30 Brangus and 30 Nellore animals naturally infested. The groups were composed as follows: 15 Nellore treated, 15 Nellore control, 15 Brangus treated and 15 Brangus control. Every 18 days, the animals were monitored for tick count, acaricide treatment, weight, blood pack cell volume, and clinical signs. For *in vitro* tests, the larval packet test, adult immersion test and DNA amplification for tick fever diagnosis were performed. In the first animal treatment period, product 1 (cypermethrin, 15 g + chlorpyrifos, 25 g + citronellal, 1 g) was used; in the second period, product 2 (cypermethrin, 15 g + chlorpyrifos, 30 g + fenthion, 15 g) was used. In Brangus animals, the mean efficacy was 35.1% and 95.8% in the first and second periods, respectively, for the same animals. For Nellore animals, the efficacy in periods one and two was 51% and 97.1%, respectively. The *in vitro* results showed efficacy above 95% for the two challenged acaricides. The Brangus animals showed a high production of ticks associated with the presence of tick fever agents, which could generate risks for the disease's enzootic stability

1. Introduction

Rhipicephalus microplus is widespread, making it one of the great obstacles of the cattle industry; it is a one-host tick, parasitizing mainly bovines (Andreotti et al., 2016). In Brazil, it is found all over the country, and estimates indicate that it causes an annual loss of 3.24 billion dollars (Grisi et al., 2014). One of the major causes of such losses, the “Bovine Parasitic Sadness”, is a disease complex with high morbidity and mortality that is caused by *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale*, all of which utilize *R. microplus* as their main vector (Gonçalves, 2000; Gonçalves et al., 2011).

Western-central Brazil is an important region for beef cattle production, and the high genetic value bovine market attempts to produce breeds with better performance and high productive lineage able to generate descendants with greater weight gain and better quality (Andreotti et al., 2016, Battistelli et al., 2013, Wambura et al., 1998).

In this context, the Brangus cross showed superiority with respect to Nellore and other crosses in this region (Battistelli et al., 2013); however, cross-bred animals are generally more susceptible to ticks, and the control of these ectoparasites is achieved through the use of acaricides. This increase in infection pressure places these populations of ticks at a higher risk of developing resistance, including multi-chemical resistance to the different chemical bases used (Higa et al., 2015) in

* Corresponding author at: Empresa Brasileira de Pesquisa Agropecuária, Embrapa Gado de Corte, Avenida Rádio Maia, 830, Campo Grande, MS, CEP 79106-550, Brazil.
E-mail address: renato.andreotti@embrapa.br (R. Andreotti).

https://doi.org/10.1016/j.vprsr.2018.02.001
Received 16 April 2017; Received in revised form 2 February 2018; Accepted 5 February 2018
Available online 06 February 2018

different regions (Andreotti et al., 2011, Reck et al., 2014, Higa et al., 2016, Klafke et al., 2017) of Brazil.

Another aggravating factor in the use of acaricides is the risk of environmental contamination and residues in meat, milk and its derivatives (De Meneghi et al., 2016, Gaus and Furlong, 2002, Kunz and Kemp, 1994).

The objective of this work was to evaluate the efficacy of two cypermethrin- and chlorpyrifos-based acaricides in the control of *R. microplus* in naturally infested beef cattle and to compare the performance of these acaricides with *in vitro* tests, as well as to monitor the presence of tick fever agents.

2. Material and methods

2.1. Study area

The study was conducted between June and November 2016 on a farm owned by Agropecuária Sanyo that was located in Água Clara County, MS, Brazil at latitude 20°46′24″S longitude 52°32′24″W and an altitude of 309 m. The climate is characterized as humid tropical with a dry season of one to three months and an average temperature above 18 °C in all months of the year (IBGE, 2002; Flumignan et al., 2015).

2.2. Use of animals

All the performed procedures using animals were in accordance with the norms published by the National Council of Control of Animal Experimentation/CONCEA and were approved by the Ethics Commission of the Use of Animals/CEUA at Embrapa Gado de Corte, protocol no. 01/2016.

2.3. Experimental animals

Sixty male bovines in the rearing phase were used, with 30 Brangus and 30 Nellore animals naturally infested with *R. microplus* ticks. The animals were kept in a 48-ha picket under a continuous grazing system with *Brachiaria* spp. The rearing phase corresponded to the period of animal life post-weaning, which occurs around seven months of age, until they reached the age of 22 months. In the present study, the animals were an average of nine months of age.

2.4. Tick count, acaricide application and weighing

The experimental design was based on pre-tick counts between 4.5 and 8 mm (Wharton and Utech, 1970) throughout the body length of the two cattle sides on days −2 and −1 (pretreatment). Posteriorly, the counts on both sides of the animals were added and divided into four homogeneous groups regarding tick count and race as follows: Nellore treated, Nellore control, Brangus treated and Brangus control.

The treated Nellore and Brangus animals were treated with commercial acaricide formulations every 18 days. In the first 4 treatments (first period), product 1 (cypermethrin, 15 g + chlorpirifos, 25 g + citronellal, 1 g; Colosso® - Ouro Fino Saúde Animal Ltda, Ribeirão Preto, SP, Brazil) was applied as a *pour on* formulation; after the fourth treatment (second period), product 2 (cypermethrin, 15 g + chlorpyrifos, 30 g + fenthion, 15 g; Colosso FC-30® - Ouro Fino Saúde Animal Ltda., Ribeirão Preto, SP, Brazil) was applied by topical spray.

The products were used as recommended by the manufacturer. For the first period, the counts were performed on days +18, +36, +54, and +72 (treatment 1), and for the second period, the counts were performed on days +90, +108, +126, and +144 (treatment 2).

The acaricide efficacy percentage calculation in the field was performed according to the formula proposed by Corrêa et al. (2015) and Cruz et al. (2015):

$$\text{Efficacy percentage} = \left(1 - \frac{\mathbf{Ta} \times \mathbf{Cb}}{\mathbf{Tb} \times \mathbf{Ca}}\right) \times 100$$

In this equation, **Ta** represents the mean number of partially engorged female ticks counted on the treated animals after medication; **Tb** is the mean number of partially engorged female ticks counted on the treated animals during the two days that preceded the treatment; **Ca** is the mean number of partially engorged female ticks counted on the control group after the experiment was initiated; and **Cb** is the mean number of partially engorged females counted on the untreated animals (control) during the two days that preceded the treatment.

Simultaneously, with the counts (every 18 d), the animals were individually weighed using a Coimma® digital scale.

2.5. Blood collection

Blood was collected from the animals every 36 days for the detection of tick fever agents. All material was subjected to DNA extraction and subsequent polymerase chain reaction (PCR). A hematocrit evaluation was also performed, and hematocrit concentration was obtained using a Daiki® CM-12000 microhematocrit centrifuge (Alves et al., 1986).

2.6. Detection of pathogens

2.6.1. DNA extraction

For the genomic DNA extraction, 300 µl of bovine blood plus 2 µl of proteinase K (20 mg/ml) and 500 µl of sodium dodecyl sulfate (20%) were used; the samples were incubated for 1 h in a water bath at 65 °C. After the incubation period, 800 µl of chloroform was added.

The samples were vigorously vortexed for homogenization, 350 µl of protein precipitation solution (6 ml of potassium acetate, 1.1 ml of glacial acetic acid, and 2.9 ml of water) was added, and the mixture was centrifuged at 18,000 x g (10 min). The aqueous phase was transferred to a new tube, 1 ml of 100% ice-cold ethanol was added, and the samples were kept in a freezer at −20 °C overnight for DNA precipitation.

Afterwards, the samples were centrifuged at 13,000 rpm (5 min), the supernatant was discarded for the addition of 1 ml of 70% ethanol, and the mixture was centrifuged at 13,000 rpm (2 min). The pellet was oven-dried at 37 °C, and the DNA was resuspended in 50 µl of ultrapure water and eluted in a water bath for 30 min at 65 °C. The samples were quantified by spectrophotometry (NanoDrop ND-1000, Uniscience) and diluted to 100 ng for PCR performance.

2.6.2. Polymerase chain reaction (PCR)

For pathogen detection, specific pairs of *primers* for *Anaplasma marginale* (Echaide et al., 1998), *Babesia bige*mina and *Babesia bovis* (Guerrero et al., 2007) were used to amplify fragments of 458, 262 and 217 base pairs (bp), respectively.

To perform PCR, the following reagent concentrations were used: 2.5 µl of 10× buffer (1×); 0.75 µl of MgCl2 (50 mM); 0.5 µl of dNTPs (2.5 mM, Invitrogen by Life Technologies™); 0.5 µl of forward and reverse primers (10 picomoles); 0.3 µl of Taq (Ludwig Biotec); 1 µl of DNA (100 ng) and ultrapure water. The final volume was 25 µl.

The reactions were performed in a Bio Rad T100™ thermal cycler. For *A. marginale,* the following program was used: 95 °C/3 min; followed by 40 cycles of 95 °C/30 s; 65 °C/1 min; 72 °C/45 s; with a final extension of 72 °C/10 min. For *B. bigemina*, the performed program was as follows: 95 °C/2 min; followed by 40 cycles of 95/1 min; 60 °C/30 s; 72 °C/1.5 min; with a final extension of 72 °C/7 min. For *B. bovis,* the program that was used was as follows: 95 °C/2 min; followed by 40 cycles of 94/1 min; 60 °C/30 s; 72 °C/1 min; with a final extension of 72 °C/7 min. After amplification, the products were visualized on a 1.5% agarose gel stained with ethidium bromide (EtBr).

2.7. Field bioassays

To verify acaricide efficiency in the different field situations, the adult immersion test (AIT) was performed. For each AIT, three groups of ten engorged female ticks (triplicates), totaling 30 engorged female ticks per treatment, were used in an adapted test from Drummond et al. (1973).

The product tested in the field bioassay was product 2, and the engorged female ticks used in the test were manually collected just before the animals were topically sprayed. Three treatments were performed: dirty solution, clean solution and control.

For controls, water was used for engorged female tick immersion. For the clean solution treatment, the solution was collected directly from the spray pump at the time of spraying. For the dirty solution, the solution that had been drained from the body of the animal treated at the time of the spraying was collected.

After being submitted to the AIT, the engorged female ticks were conditioned in Petri dishes, sent to the laboratory and maintained in ideal conditions of temperature and humidity (28 °C and 80% relative humidity) to evaluate the reproductive parameters and posterior acaricide efficiency.

The evaluation of the *in vitro* efficacy of product 1 on engorged females was carried out according to Garcia et al. (2011). To evaluate the hatchability rate of larvae, a technique described by Szabó et al. (1995) was used.

2.8. Laboratory bioassays

To verify acaricide efficacy throughout the experiment (product 1 and product 2), engorged female ticks were collected and taken to the laboratory for *in vitro* bioassays. To obtain efficacy, the adult immersion test (AIT) (Drummond et al., 1973) was performed, and a sample of engorged female ticks were kept in BOD to obtain larvae and later for use in the larval packet test (LPT) (FAO, 2004).

2.9. Statistical analysis

For the statistical analysis, the program Bioestat 5.0 was used. The Kruskal-Wallis and Mann-Whitney tests were performed.

3. Results

3.1. Tick count

The general tick production in average numbers is shown in Table 1, and the number of ticks during the whole experiment is shown in Fig. 1. In Table 2, the acaricide efficacy throughout the experiment is shown.

When evaluating the tick counts with their averages, a daily average in the first period of 121.5 ticks/animal in the Brangus control was observed, while for the treated Brangus animals, in the same period, an average production of 80.7 ticks/animal was observed, demonstrating an average efficacy of 35.1% for product 1 when used in Brangus. For Nellore animals, in the same period, the counting averages were 14.7 and 7 ticks/animal for control and treated animals, respectively. Thus, an average efficacy of 51% was obtained.

In the second period, the counting averages were 98.7 and 4 ticks/animal for the control and treated Brangus animals, respectively, and the average efficacy was 95.8%. For the Nellore animals of the control group during the same period, a mean of 20.7 ticks/animal was obtained, while the treated ones showed an average of 0.7 tick/animal, demonstrating an average efficacy of 97.1% for product 2.

The Nellore group of control animals produced an average of 17.7 ticks/animal throughout the experimental period, while in that same period, the average of the control Brangus was 110.1 ticks/animal. The results showed that Nellore animals presented 16.1% of the total production of Brangus ticks, meaning that Brangus produced 6.2 times

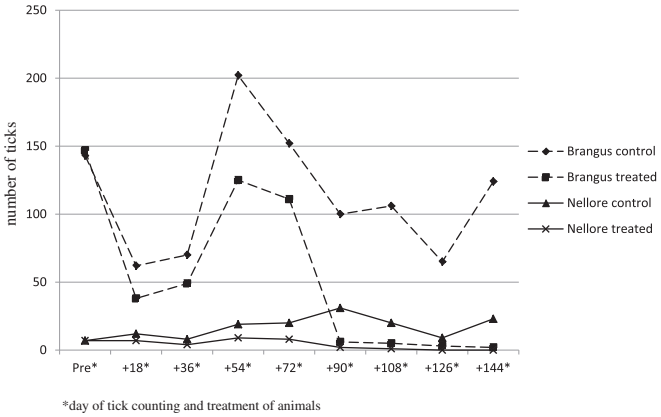


Fig. 1. Profile of the average number of *Rhipicephalus microplus* ticks on cattle in the rearing phase after treatment with product 1 (cypermethrin, 15 g + chlorpyrifos, 25 g + citronellal, 1 g) and product 2 (cypermethrin, 15 g + chlorpyrifos, 30 g + fenthion, 15 g) in the city of Água Clara, MS, Brazil.

Table 2
Treatment efficacy with product 1 (cypermethrin, 15 g + chlorpyrifos, 25 g + citronellal, 1 g) and product 2 (cypermethrin, 15 g + chlorpyrifos, 30 g + fenthion, 15 g) on *Rhipicephalus microplus* in Brangus and Nellore animals in the rearing phase, Água Clara county, MS, Brazil.

	Efficacy in percentage (%)							
	Product 1				Product 2			
	+18[a]	+36[a]	+54[a]	+72[a]	+90[a]	+108[a]	+126[a]	+144[a]
Brangus	40,0	31,9	39,8	28,9	94,1	95,4	95,5	98,4
Nellore	41,6	50,0	52,6	60,0	93,5	95,0	100	100

[a] Day of tick counting and treatment of animals.

Table 1
Mean of ticks, *Rhipicephalus microplus*, in cattle in the rearing phase after treatment with product 1 (cypermethrin, 15 g + chlorpyrifos, 25 g + citronellal, 1 g) and product 2 (cypermethrin, 15 g + chlorpyrifos, 30 g + fenthion, 15 g) in the municipality of Água Clara, MS, Brazil.

	Average tick count								
	Pre[b]	Product 1				Product 2			
		+18[a]	+36[a]	+54[a]	+72[a]	+90[a]	+108[a]	+126[a]	+144[a]
Brangus control	143	62	70	202	152	100	106	65	124
Brangus treated	147	38	49	125	111	6	5	3	2
Nellore control	7	12	8	19	20	31	20	9	23
Nellore treated	7	7	4	9	8	2	1	0	0

[a] Day of tick counting and treatment of animals.
[b] Pretreatment (days −1 e-2).

more ticks than Nellore when both were untreated and exposed to the same conditions.

With regard to the two acaricide treatments, a significant difference ($p < .05$) was observed between the treated animals of the first and second periods, and in the second period, treatment with product 2 showed satisfactory efficacy (averaging over 95%).

The presence of myiasis was observed during the experiment in 5 of 15 animals of the Brangus breed belonging to the control group, which had a high tick infestation.

3.2. Results of the field bioassays

The results of the field bioassays with product 2 were 100% effective in AIT, both for the clean and dirty solutions. The engorged female ticks from the control groups performed posture, and the larval hatching rate was 98%. Both of the groups from the clean and dirty solutions showed 100% mortality.

3.3. Results of the laboratory bioassays

All AIT bioassays for both products, performed in the laboratory, presented 100% efficacy. The engorged female ticks used for the control performed posture, and the hatching rate was of 98%. With respect to the LPT bioassays, there was an efficacy above 95% for both products.

3.4. Results for tick fever

Regarding the detection of pathogenic agents by PCR, the circulating agents in the experimental animals were, mainly, *A. marginale* and *B. bigemina*, and both were detected in all animals. *B. bovis* was detected only once in a Brangus animal of the treated group. Despite the molecular detection of tick fever agents, no animal showed clinical signs of disease during the experiment.

3.5. Hematocrit

In the present study, no correlation was observed between the parasitic load and the presence of below-normal values in the hematocrit. The animals, regardless of the parasitic load, had normal hematocrit values.

3.6. Weight gain of the animals in the period

When comparing the weight gain of the animals in the rearing phase in which the experiment was performed, there was no significant difference between the different groups (treated and controls) and breeds ($p > 0.05$).

4. Discussion

During the whole experimental period, an average of 110 ticks/day were found for Brangus animals, which is well above the amount reported by Gomes et al. (1989), who, using the same animal and breed categories, observed 59.7 ticks/day in infested animals in the field in the same region of the present study.

Higher values were also obtained for Nellore animals, with this study yielding 17.7 ticks/day, while Gomes et al. (1989) reported 3.3 ticks/day. The differences in the number of ticks found between the studies may be due to changes in the biological and ecological behavior of the ectoparasite (Rodrigues et al., 2017), as well as changes in the host, given the constant genetic improvements only with respect to the animals' weight gain.

The treatment of the Brangus and Nellore animals with product 2 provided an average efficiency above 95%, which was similar to the *in vitro* tests and in compliance with the requirements and recommendations of the competent authorities (Ministry of Agriculture, Livestock and Food Supply, Brazil, 1997 and FAO, 2004). On the other hand, the use of product 1 showed an efficacy above 95% *in vitro*, but when used on animals in the field, it presented much lower results, with only 35.1% and 51% effectiveness for Brangus and Nellore, respectively.

The significant difference of the *in vitro* and *in vivo* (in the field) results suggests that this difference may be associated with the application method, since in the field, product 1 was used *via* the *pour on* system, and product 2 was applied by topical spray. These data corroborate results presented by Corrêa et al. (2015), who evaluated the performance of acaricides *in vivo* using animals in the field and using the stall test method, as well as *in vitro* examination, and concluded that depending on the formulations used, the *in vitro* tests can overestimate the effectiveness of a certain acaricide or formulation, thus generating unreliable results.

According to the methodology used and the degree of susceptibility of a *R. microplus* tick population to the acaricide, differences in the effectiveness of the products in the different tests can classify a tick population as sensitive or resistant (Corrêa et al., 2015). According to Corrêa et al. (2015), AIT provides a greater penetration of acaricides in ticks, and the five-minute immersion time may actually be more than sufficient for the tests using acaricides in association.

The acaricides efficacy evaluation studies in Brazil are not systematic, and the presence of resistance to different chemical bases is a reality reported in several regions of the country (Andreotti et al., 2011, Reck et al., 2014, Higa et al., 2015, 2016, Klafke et al., 2017). In the region in which this study was conducted, there are populations of this tick that are highly resistant to cypermethrin-based acaricides, a fact that was previously identified by Andreotti et al. (2011) and Mendes et al. (2013).

If we were to consider only the efficacy results of product 1 in the field, excluding the *in vitro* test, product 1 could be considered ineffective in tick control because of the low efficacy values presented. According to studies by Gomes et al. (2011) and Higa et al. (2016) in the same region of the study were found populations resistant to this acaricide formulation.

For the same formulation in question, Brito et al. (2011) observed a minimum and maximum efficacy of 72.4% and 86.3%, respectively, in a study carried out in the Northern region of the country, confirming the need for resistance monitoring using bioassays for each locality and indistinct tick population.

The results obtained in this study regarding acaricide efficacy in the field and *in vitro* demonstrate that this tick population is sensitive to the formulation of cypermethrin associated with chlorpyrifos and fenthion, corroborating the data of Heidmann et al. (2016).

It is also worth emphasizing that the enzootic stability for tick fever was demonstrated in the present study, since the presence of the causative agents was verified through the DNA amplification of samples collected from the animals; however, no bovine from the present study presented clinical signs of the disease or the presence of hematocrit values below normal.

Animals with high infestation (> 100 adult ticks in the total count) received preventive treatment for tick fever to reduce their risk of acquiring the disease during the experiment; however, this preventive treatment consisted of the subcutaneous administration of imidocarb dipropionate at a dosage of 1.5 mg/kg of the animal's live body weight, and animals of the Brangus breed that presented a high rate of tick infestation associated with the presence of tick fever agents were potentially at risk in terms of the enzootic stability of the disease. Madruga et al. (1987) noted that Nellore is a breed with a high resistance to ticks and a mean infestation rate that is sufficient to maintain tick fever at a stable enzootic level.

Also in relation to the high infestations, the presence of myiasis can be observed in the control group animals that presented intense infestations. This result corroborates the data presented by Reck et al. (2014) who report a positive relationship between the high rate of tick infestation and the presence of myiasis. These authors also found that

animals with high parasitic loads are about four times more likely to have myiasis than animals with low parasitism.

Regarding cattle weight, treated and control animals in the short experimental period did not have significant differences in weight gain following either treatment (product 1 and product 2); in contrast, Jonsson (2006) estimated that tick parasitism led to a loss of 1.18 g/tick/day.

Although the *in vitro* tests are low cost and have a certain practicality in the development of the techniques, the results obtained need to be carefully analyzed because they do not always reliably reflect the results obtained in field trials, thus making it necessary to make careful assessments and observations, both in bioassays and in the acaricide application methods (*e.g.*, *pour on* or topical spray).

The formulation of a commercial product containing an acaricide, or mixtures thereof, can influence its performance. How the applicator handles the product to treat the animal can also influence product performance. However, performance variability and other factors can influence the acaricidal formulation and treatment method of choice for cattle (De Meneghi et al., 2016).

We demonstrate here the importance of tick monitoring in a cattle production system in Central Brazil, especially for animals of more sensitive breeds such as Brangus. In these animals, high infestations and, in some cases, association with myiasis can be observed. Therefore, the correct use of acaricides at treatment intervals not exceeding 21 days (as demonstrated in the study) proved to be efficient in significantly reducing infestation of the animals.

Preventive treatment against cattle tick fever in animals with high infestations of ticks proved to be effective during the experimental period and may be an alternative for tick fever prevention, however additional studies should be performed to demonstrate the efficacy of this treatment in the bovine production system.

Acknowledgment

To the Agropecuária Sanyo, Ernesto Lyo Nakasshima, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul (Fundect, MS) - Governo do Estado de Mato Grosso do Sul.

Conflict of interests

The authors declare to have no conflict of interest.

Ethical statement

All the performed procedures using animals were in accordance with the norms published by the National Council of Control of Animal Experimentation/CONCEA and were approved by the Ethics Commission of the Use of Animals/CEUA at Embrapa Gado de Corte, protocol no. 01/2016.

References

Alves, J.A., Souza-Filho, S.D., Oliveira, H.M., Carvalho-Neto, J.L., 1986. Determinação de microhematócritos em centrifuga convencional. Rev. Bras. Anal. Clin. 18, 47–48.

Andreotti, R., Garcia, M.V., Reis, F.A., Rodrigues, V.S., Barros, J.C., 2016. Proposta de controle de carrapatos para o Brasil Central em sistemas de produção de bovinos associados ao manejo nutricional no campo. In: Embrapa Gado de Corte, 34 p. (Documentos/Embrapa Gado de Corte, ISSN 1983-974;214). http://www.cnpgc.embrapa.br/publicacoes/doc/DOC214.pdf>.

Andreotti, R., Guerrero, F.D., Soares, M.A., Barros, J.C., Miller, R.J., Léon, A.P., 2011. Acaricide resistance of *Rhipicephalus* (*Boophilus*) *microplus* in State of Mato Grosso do Sul, Brazil. Rev. Bras. Parasitol. Vet. 20, 127–133.

Battistelli, J.V.F., Torres-Junior, R.A.A., Menezes, G.R.O., Reggiori, M.R., Souza-Junior, M.D., Silva, L.O.C., 2013. Alternativas de cruzamento utilizando raças taurinas adaptadas ou não sobre matrizes Nelores para a produção de novilhos precoce – Fase de cria e recria, p. 10–23. In: Simpósio Brasileiro de Melhoramento Animal, 10., Uberaba, 2013. Anais... Uberaba, MG, agosto de 2013.

Brazil, 1997. Ministério da Agricultura e Abastecimento. Secretaria de Defesa Agropecuária, Portaria (n.°48, 12/05/1997).

Brito, L.G., Barbieri, F.S., Rocha, R.B., Oliveira, M.C.S., Ribeiro, E.S., 2011. Evaluation of the efficacy of acaricides used to control the cattle tick, *Rhipicephalus microplus*, in dairy herds raised in the Brazilian Southwestern Amazon. Vet. Med. Int. 2011, 1–6.

Corrêa, R.R., Lopes, W.D.Z., Teixeira, W.F.P., Cruz, B.C., Gomes, L.V.C., Felipelli, G., Maciel, W.G., Favero, F.C., Buzzulini, C., Bichuetti, M.A., Soares, V.E., Costa, A.J., 2015. A comparison of three different methodologies for evaluating *Rhipicephalus* (*Boophilus*) *microplus* susceptibility to topical spray compounds. Vet. Parasitol. 207, 115–124.

Cruz, B.C., Lopes, W.D.Z., Maciel, W.G., Felipelli, G., Favero, F.C., Teixeira, W.F.P., Carvalho, R.S., Ruivo, M.A., Colli, M.H.A., Sakamoto, C.A.M., Costa, A.J., Oiveira, G.P., 2015. Susceptibility of *Rhipicephalus* (*Boophilus*) *microplus* to ivermectin (200, 500 and 630 μg/kg) in field studies in Brazil. Vet. Parasitol. 207, 309–317.

De Meneghi, D., Stachurski, F., Adakal, H., 2016. Experiences in tick control by Acaricide in the traditional cattle sector in Zambia and Burkina Faso: possible environmental and public health implications. Front. Public Health 4, 1–11. http://dx.doi.org/10.3389/fpubh.2016.00239.

Drummond, R.O., Ernst, S.E., Trevino, J.L., Gladney, W.J., Graham, O.H., 1973. *Boophilus annulatus* and *Boophilus microplus*: laboratory tests of insecticides. J. Econ. Entomol. 66, 130–133. http://dx.doi.org/10.1093/jee/66.1.130.

Echaide, S.T., Knowles, D.P., McGuirre, T.C., Palmer, G.H., Suarez, C.E., McElwain, T.F., 1998. Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5. J. Clin. Microbiol. 36, 777–782.

Flumignan, D.L., Fietz, C.R., Comunello, E., 2015. O clima na região do Bolsão de Mato Grosso do Sul. Embrapa Agropecuária do Oeste. 42p. (Documentos/Embrapa Agropecuária do Oeste, ISSN 1679-043X; 127).

Food and Agriculture Organization of the United Nations – FAO, 2004. Resistance management and integrated parasite control in ruminants: guidelines. Module 1. Ticks: acaricide resistance: diagnosis, management and prevention [online]. pp. 25–77. [cited 2016 Jan 4]. Available from: ftp://ftp.fao.org/docrep/fao/010/ag014e/ag014e05.pdf.

Garcia, M.V., Monteiro, A.C., Szabó, M.P.J., Mochi, D.A., Simi, L.D., Carvalho, W.M., Tsuruta, S.A., Barbosa, J.C., 2011. Effect of *Metarhizium anisopliae* fungus on off-host *Rhipicephalus* (*Boophilus*) *microplus* from tick-infested pasture under cattle grazing in Brazil. Vet. Parasitol. 181, 10–1016. http://dx.doi.org/10.1016/j.vetpar.2011.04.031.

Gomes, A., Honer, M.R., Schenk, M.A., Curvo, J.B., 1989. Populations of the cattle tick (*Boophilus microplus*) on purebred Nellore, Ibage and Nellore X European crossbreds in the Brazilian savanna. Trop. Anim. Health Prod. 21, 20–24. http://dx.doi.org/10.1007/BF02297336.

Gomes, A., Koller, W.W., Barros, A.T.M., 2011. Susceptibilidade de *Rhipicephalus* (*Boophilus*) *microplus* a carrapaticidas em Mato Grosso do Sul, Brasil. Cienc. Rural. 41, 1447–1452.

Gonçalves, P.M., 2000. Epidemiologia e controle da Tristeza Parasitária Bovina na região Sudeste do Brasil. Cienc. Rural. 30, 187–194.

Gonçalves, R.C., Silva, A.A., Ferreira, D.O.L., Chiacchio, S.B., Lopes, R.S., Borges, A.S., Amorim, R.M., 2011. Tick fever in cattle in the region of Botucatu – SP: a retrospective study of 1986–2007. Semin: Cienc. Agrar. 32, 307–312.

Grisi, L., Leite, R.C., Martins, J.R.S., Barros, A.T.M., Andreotti, R., Cançado, P.H.D., Leon, A.A.P., Pereira, J.B., Villela, H.S., 2014. Reassessment of the potential economic impact of cattle parasites in Brazil. Rev. Bras. Parasitol. Vet. 23, 150–156.

Guerrero, F.D., Bendele, K.G., Davey, R.B., George, J.E., 2007. Detection of *Babesia bigemina* infection in strains of *Rhipicephalus* (*Boophilus*) *microplus* collected from outbreaks in south Texas. Vet. Parasitol. 145, 156–163.

Heidmann, M.J., Souza, A.A.D., Nascimento, C.G., Bragaglia, G.N., Lima, L.M.S., Toma, S.B., Rosa, S.C., Castro, B.G., 2016. Eficácia acaricida da associação de Cipermetrina, Fenthion e Clorpirifós em bovinos naturalmente infestados por *Rhipicephalus microplus* na Amazônia Matogrossense. 23. Vet. e Zootec., pp. 713–721.

Higa, L.O.S., Garcia, M.V., Barros, J.C., Koller, W.W., Andreotti, R., 2015. Acaricide resistance status of the *Rhipicephalus microplus* in Brazil: a literature overview. Med. Chem. 5, 326–333.

Higa, L.O.S., Garcia, M.V., Barros, J.C., Koller, W.W., Andreotti, R., 2016. Evaluation of *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) resistance to different acaricide formulations using samples from Brazilian properties. Rev. Bras. Parasitol. Vet. 25, 163–171. http://dx.doi.org/10.1590/S1984.29612016026.

IBGE, 2002. Instituto Brasileiro de Geografia e Estatística. In: Mapa de Clima do Brasil, Acesso em 20/03/2017. ftp://geoftp.ibge.gov.br/informacoes_ambientais/climatologia/mapas/brasil/clima.pdf.

Jonsson, N.N., 2006. The productivity effects of cattle tick (*Boophilus microplus*) infestation on cattle, with particular reference to *Bos indicus* cattle and their crosses. Vet. Parasitol. 137, 1–10.

Klafke, G., Webster, A., Agnol, B.D., Pradel, E., Silva, J., La Canal, L.H., Becker, M., Osório, M.F., Mansson, M., Barreto, R., Scheffer, R., Souza, U.A., Corassini, V.B., Santos, J., Reck, J., Martins, J.R., 2017. Multiple resistance to acaricides in field populations of Rhipicephalus microplus from Rio Grande do Sul state, Southern Brazil. Ticks Tick Borne Dis. 8, 73–80.

Kunz, S.E., Kemp, D.H., 1994. Insecticides and acaricides: resistance and environmental impact. Rev. Sci. Tech. Off. Int. Epiz. 13, 1249–1286.

Madruga, C.R., Honer, M.R., Schenk, M.A.M., Curvo, J.B.E., 1987. Avaliação preliminar de parâmetros epidemiológicos da tristeza parasitária bovina no Mato Grosso do Sul. Embrapa Gado de Corte, 7p. (Pesquisa em Andamento n. 38/Embrapa Gado de corte).

Mendes, M.C., Duarte, F.C., Martins, J.R., Klafke, G.M., Fiorini, F.C., Barros, A.T.M., 2013. Characterization of the pyrethroid resistance profile of *Rhipicephalus* (*Boophilus*)

microplus populations from the states of Rio Grande do Sul and Mato Grosso do Sul, Brazil. Rev. Bras. Parasitol. Vet. 22, 379–384.

Reck, J., Klafke, G.M., Webster, A., Dall'Agnol, B., Scheffer, R., Souza, U.A., Corassini, V.B., Vargas, R., Santos, J.S., Martins, J.R.S., 2014. First report of fluazuron resistance in *Rhipicephalus microplus*: a field tick population resistant to six classes of acaricides. Vet. Parasitol. 201, 128–136. http://dx.doi.org/10.1016/j.vetpar.2014.01.012.

Reck, J., Marks, F.S., Rodrigues, R.O., Souza, U.A., Webster, A., Leite, R.C., Gonzales, J.C., Klafke, G.M., Martins, J.R., 2014. Does *Rhipicephalus microplus* tick infestation increase the risk for myiasis caused by *Cochliomyia hominivorax* in cattle? Prev. Vet. Med. (1), 59–62. http://dx.doi.org/10.1016/j.prevetmed.2013.10.006.

Rodrigues, V.S., Garcia, M.V., Cruz, B.C., Maciel, W.G., Zimmermann, N.P., Koller, W.W., Barros, J.C., Andreotti, R., 2017. Life cycle and parasitic competence of *Dermacentor nitens* Neumann, 1897 (Acari: Ixodidae) on different animal species. Ticks Tick Borne Dis. 8, 379–384. http://dx.doi.org/10.1016/j.ttbdis.2016.12.014.

Szabó, M.P.J., Mukai, L.S., Rosa, P.C.S., Bechara, G.H., 1995. Differences in the acquired resistance of dogs, hamsters and guinea pigs to repeated infestations with adult ticks *Rhipicephalus sanguineus* (Acari: Ixodidae). Braz. J. Vet. Res. Anim. Sci. 32, 43–50.

Wambura, P.N., Gwakisa, O.S., Silayo, R.S., Rugaimukamu, E.A., 1998. Breed-associated resistance to tick infestation in *Bos indicus* and their crosses with *Bos taurus*. Vet. Parasitol. 77, 63–70.

Wharton, R.H., Utech, K.B.W., 1970. The relation between engorgement and dropping of *Boophilus microplus* (Canestrini) (Ixodidae) to the assessment of the tick numbers on cattle. J. Aust. Ent. Soc. 9, 171–182.

Artigo 3

Probable acaricide resistance in different life stages of *Amblyomma mixtum* (Acari: Ixodidae) collected from the State of Veracruz, Mexico. Referente ao Os resultados gerados derivados da determinação da resistência as diferentes famílias de acaricidas comerciais em diferentes estágios da vida alimentados e não alimentados de *Amblyomma mixtum* foram submetido na revista Tick and Ticks Borne Disease (Qualis B1)

Manuscript Details

Manuscript number	TTBDIS_2018_232
Title	Probable acaricide resistance in different life stages of Amblyomma mixtum (Acari: Ixodidae) collected from the State of Veracruz, Mexico
Article type	Research Paper

Abstract

The objective of this study was to adapt the larval packet test (LPT) as a methodology for diagnosis of acaricide resistance in Amblyomma mixtum. Tests were carried out with different life stages (unfed and fed larvae; nymphs and unfed adult females) of a tick colony originated in the state of Veracruz, Mexico. Ticks were challenged in filter papers impregnated with aqueous solutions of the following commercial acaricides diluted at their concentration of use in the field: 1. an acaricide mixture (cypermethrin + chlorpyrifos + citronella + piperonyl butoxide); 2. amitraz; 3. cypermethrin; and 4) an organophosphate mixture (dichlorvos + chlorpyrifos). Mortality of ticks was recorded after 24 hours of exposure to the acaricides. In general, the higher mortality rates were obtained for all life stages exposed to the acaricide mixture (ranging between 87.6% and 100%) and organophosphates (98.8 to 100%). Lower mortality rates were obtained across all life stages for amitraz (0 to 24.4%) and cypermethrin (2.2% to 40%), indicating the potential presence of resistant individuals to these two compounds in the population. The mortality of ticks was equivalent regardless of the life stage used, with no significant differences within the same acaricide group. It was possible to conclude that the use of larvae, nymphs and adults as biological source for resistance detection with a modified packet test is possible. The proposed methodology is an alternative to optimize the diagnosis of resistance in three-host ticks as A. mixtum.

Keywords	Ticks, Amblyomma spp., efficacy, bovines, Mexico

Corresponding Author	francisco tobias Barradas-Piña
Corresponding Author's Institution	inifap
Order of Authors	francisco tobias Barradas-Piña, Marcos Valerio-Garcia, Renato Andreotti, Dora Romero-Salas, Leandro Higa, Jacqueline Barros, Guilherme Klafke, Vinicius Rodrigues, Robert MIller, Adalberto Perz de Leon

Submission Files Included in this PDF

File Name [File Type]

Cover letter.doc [Cover Letter]

Paper Dx Rest.docx [Manuscript File]

Table 2.docx [Table]

To view all the submission files, including those not included in the PDF, click on the manuscript title on your EVISE Homepage, then click 'Download zip file'.

COVER LETTER

Campo Grande - MS, May 28th, 2018.

From the authors:

Francisco Tobias Barradas Piña, Leandro de Oliveira Souza Higa, Vinicius da Silva Rodrigues, Marcos Valério Garcia, Renato Andreotti, Dora Romero Salas, Jacqueline Cavalcante Barros Guilherme M. Klafke, Robert John Miller, Adalberto Perez de Leon

Contacts: Embrapa Beef Cattle, Avenida Rádio Maia, 830 Vila Popular, CEP 79106-550 - Campo Grande, MS, Brazil, fone/fax: 55 (67) 33682169.

Corresponding Author: Francisco Tobias Barradas Piña

E-mail Adress: fcobarradast@gmail.com

Dear Editor.

We are forwarding the manuscript entitled "Probable acaricide resistance in different life stages of Amblyomma mixtum (Acari: Ixodidae) collected from the State of Veracruz, Mexico". This manuscript was written according to the journal guidelines and edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified native person. All authors permit use of figures and tables.

If accepted for publication, we pledge to consider all questions of the reviewers and respond as soon as possible.

Without more and available for necessary adjustments to the manuscript.

Sincerely,

The authors.

Probable acaricide resistance in different life stages of Amblyomma mixtum (Acari: Ixodidae) collected from the State of Veracruz, Mexico

Francisco Tobias Barradas Piña[1,6], Marcos Valério Garcia[2,3], Renato Andreotti[1,3], Dora Romero Salas[5], Leandro de Oliveira Souza Higa[1], Jacqueline Cavalcante Barros[3] Guilherme M. Klafke[7], Vinicius da Silva Rodrigues[3], Robert John Miller[4*], Adalberto Perez de Leon[4]

1Pós-Graduação em Doenças Infecciosas e Parasitárias-Faculdade de Medicina, UFMS -Universidade Federal Mato Grosso do Sul, Campo Grande, MS, Brazil.
2 Bolsista DCR, FUNDECT - Governo do Estado de Mato Grosso do Sul, Brazil.
3Laboratorio de biologia do carrapato, Embrapa Gado de Corte, Campo Grande, MS, Brazil.
4 USDA-ARS, Knipling-Bushland U.S. Livestock Insects Research Laboratory, and Veterinary Pest Genomics Center, Kerville, TX, USA.
5 Facultad de Medicina Veterinaria y Zootecnia, Universidad Veracruzana, Veracruz, México.
6 Instituto Nacional de Investigaciones Forestales Agricolas y Pecuarias INIFAP-Campo Experimental La Posta, México.
7 Instituto de Pesquisas Veterinarias Desiderio Finamor; Secretaria da Agricultura do Estado do Rio Grande do Sul. Estrada do Conde, 6000, Eldorado do Sul, RS, Brazil. 92990-000.

Abstract

The objective of this study was to adapt the larval packet test (LPT) as a methodology for diagnosis of acaricide resistance in *Amblyomma mixtum*. Tests were carried out with different life stages (unfed and fed larvae; nymphs and unfed adult females) of a tick colony originated in the state of Veracruz, Mexico. Ticks were challenged in filter papers impregnated with aqueous solutions of the following commercial acaricides diluted at their concentration of use in the field: 1. an acaricide mixture (cypermethrin + chlorpyrifos + citronella + piperonyl butoxide); 2. amitraz; 3. cypermethrin; and 4) an organophosphate mixture (dichlorvos + chlorpyrifos). Mortality of ticks was recorded after 24 hours of exposure to the acaricides. In general, the higher mortality rates were obtained for all life stages exposed to the acaricide mixture (ranging between 87.6% and 100%) and organophosphates (98.8 to 100%). Lower mortality rates were obtained across all life stages for amitraz (0 to 24.4%) and cypermethrin (2.2% to 40%), indicating the potential presence of resistant individuals to these two compounds in the population. The mortality of ticks was equivalent regardless of the life stage used, with no significant differences within the same acaricide group. It was possible to conclude that the use of larvae, nymphs and adults as biological source for resistance detection with a modified packet test is possible. The proposed methodology is an alternative to optimize the diagnosis of resistance in three-host ticks as *A. mixtum*.

Keywords: Ticks, Amblyomma spp., efficacy, bovines, Mexico

Introduction

Currently in Mexico there are 68 tick species belonging to the order Ixodidae (Pérez et al., 2014). Among these, only two species from different genus, *Rhipicephalus microplus* and *Amblyomma mixtum* are known to be responsible for economic losses in the cattle business due to their parasitism (Guglielmone & Nava, 2006; Álvarez & Bonilla, 2007; Hernandez et al., 2013; Tapias & Vaca, 2013; Rodrígues-Vivas et al., 2010; Guzmán-Cornejo et al., 2011; Rodriguez-Vivas et al., 2014).

A. mixtum is distributed through the south of Texas, Mexico, Panama, Ecuador and some Caribbean Islands (Nava et al., 2014). It is three-host tick, being a species of generalist habit, infesting birds and mammals, including human beings. This species also deserves attention regarding public health since it is vector of pathogens such as *Rickettsia ricktesii*, which causes spotted fever in humans (Parola et al., 2005; Rodríguez-Vivaz et al., 2016).

Tick control is made mainly with the use of acaricides (FAO, 1987), and development of resistance in *R. microplus* in Mexico has been extensively reported (Guerrero et al., 2002; Rodríguez-Vivaz et al., 2006; 2013; 2014; Fernández-Salas et al., 2012; Perez-Cogollo et al., 2010; Miller et al., 2013); on the other hand, just a few number of studies were aimed on the resistance status of *Amblyomma mixtum* in Mexico (Aguirre et. al., 1996; Martinez-Ibañez et al., 2010; Alonso-Diaz et al., 2013).

Diagnostic methods to detect resistance in one-host ticks can be adapted and carried out with multi-host ticks (Natala et al., 2005; Miller et al., 2002; Bittencourt et al., 1989). These tests are the adult immersion test (AIT), larval immersion test (LIT) and the larval packet test (LPT) (FAO, 2004; Klafke et al., 2012). Most of the studies were performed with larvae and adults, with none using the nymph stage.

Considering: I) the lack of information about acaricide resistance in *Amblyomma mixtum* II) the fact that most of the control strategies has only been conceived, proposed and established for *R. microplus*, and III) that the existence of co-parasitism of these

two species is very common in cattle producing areas of Mexico; there is an urgent need for a broader vision regarding the control of these two important ectoparasites of cattle. The detection of resistance is one of the most important components of acaricide management; therefore, the development and/ or improvement of techniques to test different life stages of a three-host tick species is much needed. In the present study, we evaluated the toxicity of different commercial formulations in fed and unfed immature stages as well as unfed adults of *A. mixtum* using a modified version of the larvae packet test.

Materials and Methods

Study Location

The study was conducted in the Tick Biology Laboratory, Animal Health Department, at Embrapa Beef Cattle, Campo Grande, Mato Grosso do Sul, Brazil.

Sample collection and maintenance

A. mixtum engorged females were collected in January 2016 from naturally infested bovines from a ranch in Medellín of Bravo, Veracruz, Mexico (19°01′01.6″N; 96°08′14.4″W). The ranch produces zebu and taurine crosses of cattle (Brown-Swiss x Zebu and Holstein x Zebu). The control of ticks is mainly performed with the use of acaricides (mostly amitraz and pyrethroids), with treatments every 14 days.

The *A. mixtum* engorged females were maintained in incubators (27 °C and 80% UR) for oviposition, incubation and larvae hatching. To maintain the *A. mixtum* colony, New Zealand rabbits were used. To obtain three instars of this tick, rabbits were infested using artificial feeding chambers, as described by (Szabó et al., 1995; Barradas et al., 2017). All procedures using animals are in accordance with the norms of the Animal Experimentation National Brazilian Council (Conselho Nacional Brasileiro de Experimentação Animal - CONCEA). The study was approved by the Animal Use Ethics Commission (Comissão de Ética no Uso de Animais / CEUA) at the Universidade Federal de Mato Grosso do Sul, protocol nº 699/2015.

Acaricides

Commercial acaricides in its emulsifiable concentrate formulations were used diluted in distilled water: 1) cypermethrin 15g + chlorpyriphos 25g + piperonil butoxyde 15g + citronella 1g (1:1000 dilution) (Cyperclor® Plus Pulverização; Vetbrands Saude Animal); 2) amitraz 12.5g (1:500) (Clipatic®, Fagra); 3) cypermethrin 15g (1:1000) (Maximo® Pulverização, Biovet) and; 4) dichlorvos 60g + chlorpyriphos 20g (1:400) (Ectobat® Champion Saúde Animal).

Modified larval packet test (LPT)

The larval packet test established by FAO (2004) was modified to analyze the toxicity of commercial formulations of acaricides in *A. mixtum* ticks (fed and non-fed larvae, fed and non-fed nymphs and non-fed adults). The following numbers of ticks from each live stage were used: i) 100 larvae (three-days fed and unfed), ii) 30 nymphs (three days-fed and unfed) and iii) five *A. mixtum* adult couples (20 days after molting) were used for each treatment with three repetitions each.

In the adapted methodology, it was considered to use the commercial acaricide diluted in water in order to simulate the field conditions of treatment to ticks. The formulations were diluted in distilled water according table 1 and for each treatment three repetitions were made. Filter papers (85 mm x 75 mm) were impregnated with 0.67 mL of each acaricide solution. Control group was impregnated with distilled water. Filter papers were placed at room temperature for 24 hours to allow them to dry. After 24 hours from impregnation, larvae were put inside the packets and sealed using clips, and posteriorly incubated at 28oC; 80% relative humidity. After 24 hours, the mortality of ticks was recorded.

Statistical analysis

The mean mortality for each treatment and life stage was calculated and the statistical significance of difference among treatments was analyzed using the Kruskall-Wallis variance test was made using the Bioestat 5.0 program. The diagnose of susceptibility/ resistance of the *Amblyomma mixtum* life stages was performed according the criteria used by Alonso-Diaz et al.,. 2013 for the same species of ticks: i) acaricides causing

<80% mortality were classified as ineffective under "potential status of resistance"; (ii) acaricides causing a mean mortality between 80% and 97% were classified using "status of probable resistance" and; (iii) acaricides with a mean mortality >97% were classified using "status of susceptibility".

Results

The results obtained with the bioassays are presented in the table 2. For the mixture of acaricides (cypermethrin + chlorpyriphos + citronella + piperonyl butoxide), it was observed a mortality rate of 87.4% for the unfed larvae. At this mortality rate, this living stage was considered at "status of probable resistance". Nevertheless, engorged nymphs had a mortality rate of 97.8% and 100% of the engorged larvae, unfed nymphs and adults were dead after exposure to this chemical. Considering all instars, the present isolate was considered susceptible to this mixed formulation of acaricides.

For amitraz, the mortality of ticks of all living stages tested was low. No mortality was recorded for the adults. The unfed nymphs presented a mortality of 7.5%. Unfed larvae died at a rate of 8.6%, and there was 12% mortality of engorged larvae and 24.4% mortality of unfed larvae. The tick isolate was considered resistant to amitraz. For cypermethrin, the response to treatment was similar to the observed for amitraz. The mortality was 2.2% for the engorged larvae, 9.8% for unfed larvae, 26.7% for engorged nymphs, 31% of unfed nymphs and 40% of the adults and the strain can be categorized as resistant to cypermethrin.

In the case of the mixture of organophosphates (dichlorvos + chlorpyriphos), the strain was completely susceptible. The mortality of unfed larvae was 98.8% and the rest of the instars showed 100% mortality. In spite of some variation, there were no significant differences on the mortality of the different life stages (larvae, nymphs, adults) within the tests with the same acaricide (table 2).

Discussion

One of the biggest challenges in the cattle producing areas in the world is tick parasitism. In Mexico, both *R. microplus* and *A. mixtum* cause great economic losses and control problems, especially in locations where the co-parasitism occurs (González-Ceron et al., 2009; Almazán et al., 2016). It is important to note that these two species

of ticks have different parasitic life cycles and a marked difference in seasonality (Alonso-Diaz et al., 2007; Almazan et al., 2016; Barradas et al., 2017). These differences impact on the establishment of control strategies that generally focus on *R. microplus* species only. This can be an important factor for *Amblyomma ssp* to generate resistance to acaricides.

Different studies have been carried out with different life stages of *Amblyomma* species to diagnose acaricide resistance. Natala et al., (2005) used the larval packet technique with immature stages of *A. variegatum* (larvae and nymphs) and detected resistance to amitraz in both life stages. Ntodini et al., (2008) determined the level of resistance to Amitraz, cypermethrin, and Chlorfenvinphos, in Eastern Cape Province, South Africa, using larvae from ticks collected from different hosts of ticks: *Rhipicephalus microplus*, *R. evertsi evertsi* and *R. appendiculatus*, using The Shaw Larval Immersion Test for larvae. Showing that *R. microplus* was resistant to amitraz and cypermethrin, *R. evertsi* was resistant to chlorfenvinphos and *R. appendiculatus* for the cypermethrin family.

In this study, we observed low mortality of *A. mixtum* exposed cypermethrin and amitraz. It is noteworthy that the ranch from where the colony was isolated had problems with the control *A. mixtum* and the usage of amitraz and cypermethrin was frequent. It is possible this species has developed resistance in a production system of the Mexican tropic where the control protocols are designed for the one-host tick, *R. microplus*.

Interestingly, of the *A. mixtum* that showed low susceptibility to amitraz and cypermethrin, all life stages presented low susceptibility and did not show difference on mortality levels among them. These results contrast to those of Bittencourt et al. (1989) where the author showed a lower susceptibility of *A. mixtum* (*A. cajennense*) blood-fed individuals comparing to the unfed. Nevertheless, the technique used was the immersion test what can determine difference in the exposure to the acaricides and return in differences of mortality. Burridge et al., (2004) reported resistance to amitraz and pyrethroids in males and females of *A. americanum*, *A. cajennense*, and *A. maculatum*, using the immersion test.

It was demonstrated that for the isolate of *A. mixtum* tested in the present study, the organophosphates showed a satisfactory effect. This chemical class is used globally for

the control of *R. microplus*; however, there have been resistance reports for this species since the 1980 in Mexico and other countries (Aguirre & Santamaría 1986; Rodrígues-Vivaz et al., 2006; Rodríguez-Vivaz et al., 2007; Klafke et al., 2016).

A study carried out by Alonso-Díaz et al., (2013) aimed to detect acaricide resistance in *A. mixum* using the larvae packet test in the state of Veracruz, Mexico. In that survey, the frequency of resistant populations was 72.6% for pyrethroids; 100% for diazinon, 91.7% for chlorpyriphos and 12.5% for coumaphos. This, in addition to the results found here, demonstrates that ticks of the *A. mixtum* species can develop resistance to these acaricides.

With some refinement, this study supports the use of other life stages beyond larvae of three-host ticks (nymphs and adults) in toxicological bioassays as being a reliable technique to detect resistance in economically important or public health relevant tick species.

Conclusions

We can conclude that with refinement and verification using a known susceptible strain the bioassays adopted in the present study can be used to evaluate the susceptibility to acaricides of *A. mixtum* using different life-stages, and may contribute to early resistance diagnosis and control management of this economically important tick species.

Conflict of interests

The authors declare to have no conflict of interest.

Acknowlegdments

To the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul (Fundect, MS) - Governo do Estado de Mato Grosso do Sul and Embrapa Gado de Corte, Instituto Nacional de Investigaciones Forestales, Agricolas y Pecuarias

(INIFAP-Mexico), USDA-ARS, Knipling-Bushland U.S. Livestock Insects Research Laboratory, and Veterinary Pest Genomics Center, Kerville, TX, USA.

References

Aguirre, E.J., Santamaría, V.M., 1996. Purificación y caracterización toxicológica de garrapatas *B. microplus* resistentes a ixodicidas organofosforados y organoclorados. In: Memorias de IV Reunión Anual de la Asociación Mexicana de Parasitología Veterinaria.

Almazán, C., Torres-Torres, A., Torres-Rodríguez, L., Soberanes-Céspedes, N., Ortiz-Estrada, M., 2016. Aspectos biológicos de *Amblyomma mixtum* (Koch, 1844) en el noreste de México. Quehacer Cient. en Chiapas. 11, 2.

Alonso-Diaz, M.A., Fernández-Salas, A., Martínez-Ibáñez, J.F., Osorio-Miranda., 2013. *Amblyomma cajennense* (Acari: Ixodidae) tick populations susceptible or resistant to acaricides in the Mexican Tropics. Vet. Parasitol. 197, 326-331.

Alonso-Díaz, M.A., López-Silva, B.J., de Magalhães-Labarthe, L.A.C.L., Rodriguez-Vivas, R.I., 2007. Infestación Natural de hembras de *Boophilus microplus* Canestrini, 1887 (Acari:Ixodidae) en dos genotipos de bovinos en el trópico de Veracruz, México. Vet. Mex. 38, 4.

Álvarez, C.V., Bonilla, M.R., 2007. Adultos y Ninfas de la Garrapata *Amblyomma Cajennense* Fabricius (Acari: Ixodidae) en Equinos y Bovinos. Agro. Costarricense. 31, 61-69.

Barradas P.F.T., Rodrigues, da S.V., Higa, de O.S.L., Garcia, V.M., Barros, C.J., Pérez de León, A.A., Andreotti, R., 2017. Life cycle of *Amblyomma mixtum* (Acari: Ixodidae) parasitizing different hosts under laboratory conditions. Exp Appl Acarol.

Bittencourt, V.R.E.P., Massard, L.C., And Grisi, L., 1989. Atividade *in Vitro* de alguns piretrõides sintéticos no carrapato *Ambl yomma cajennense* (Fabricius, 1787). Pesq.Agropec.Bras., Brasília, 24, 1193-1199.

Burridge, J.M., Leigh-Anne, S., and Allan, A.S., 2004. Efficacy of Acaricides for Control of Four Tick Species of Agricultural and Public Health Significance in the United States. J. Agric. Urban. Entomol. 20, 4, 207-219.

Davey, B.R., George, E.J., Miller, R.J., 2006. Comparison of the reproductive biology between acaricide-resistant and acaricide-susceptible *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae). Vet Parasitol. 139 211–220.

Fernández-Salas, A., Rodríguez-Vivas, R.I., Alonso-Diaz, M.A., 2012. Resistance of *Rhipicephalus microplus* to amitraz and cypermethrin in tropical cattle farms in Veracruz, Mexico. J. Parasitol. 98, 1010-1014.

GenBank accesion nos. (KF200160,), *Amblyomma cajennense* voucher CAS-T071 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial, (KF200097) Amblyomma cajennense voucher CAS-T070 cytochrome oxidase subunit 1 (COI) gene,

partial cds; mitochondrial, (KF200124.1,), *Amblyomma cajennense* voucher CAS-T073 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial, (KF20097).

González-Cerón, F., Becerril-Pérez, C.M., Torres-Hernández, G., Díaz-Rivera, P., 2009. Garrapatas que Infestan Regiones Corporales del Bovino Criollo Lechero Tropical en Veracruz, México. Agrociencia. 43, 11-19.

Guerrero, F., Li, Y.A., Hernandez, R., 2002. Molecular Diagnosis of Pyrethroid Resistance in Mexican Strains of *Boophilus microplus* (Acari: Ixodidae). J. Med. Entomol. 39, 770-776.

Guglielmone, A.A., Nava, S., 2006. Las Garrapatas Argentinas del Género *Amblyomma* (Acari: Ixodidae): Distribución y Hospedadores. Ria. 35, 133-153.

Guzmán-Cornejo, C., Robbins, R.G., Guglielmone, A.A., Montiel-Parra, G., Pérez, T.M., 2011. The *Amblyomma* (Acari: Ixodida: Ixodidae) of Mexico: Identification Keys, Distribution and Hosts. *Zootaxa.* 2998, 16-38.

Hernández, K., Arece, J., Simón, L., Hernández, L., Valdés, O., 2013. Comportamiento de ixódidos en diferentes genotipos de ganado mayor en silvopastoreo (Nota Técnica). Pastos y Forrajes. 36, 72-76.

Klafke, M.G., Castro-Janer, E., Mendes, C.M. and Namindome, A., 2012. Applicability of *in vitro* bioassays for the diagnosis of ivermectin resistance in *Rhipicephalus microplus* (Acari: Ixodidae).Vol 184, pp 212-220.

Klafke, G., Webster, A., Agnol, D.B., Pradel, E., Silva, J., de la Canal, L.H., Becker, M., Osório, M.F., Mansson, M., Barreto, R., Scheffer, R., Souza, A.U., Corassini, B.V., dos Santos, J., Reck, J., Martins, R.J., 2016. Multiple resistance to acaricides in field populations of *Rhipicephalus microplus* from Rio Grande do Sul state, Southern Brazil. pp 1-8.

Martínez-Ibañez, F., Osorio, M.J., Peláez, F.A., Delabra, V.G., Chiu, A.D., 2010. Respuesta toxicológica de garrapatas *Boophilus microplus* y *Amblyomma cajennense* en ranchos con infestaciones mixtas en Tamaulipas, Puebla y Veracruz. Congreso Nacional de Buiatría, CINTERMEX, Monterrey, Nuevo León, México pp. 191.

Miller, J.R., Davey, B.R., and George, E.J., 2002. Modification of the Food and Agriculture Organization Larval Packet Test to Measure Amitraz-Susceptibility Against Ixodidae; Journ. of Medic. Entomol. 39(4), 645-651.

Miller, J.R., Almazán, C., Ortíz-Estrada, M., Davey, B.R., George, E.J., Peréz de León, A., 2013. First report of fipronil resistance in *Rhipicephalus (Boophilus) microplus* of Mexico. Vet. Parasitol. 191, 97-101.

Natala, A.J., Agyei, A.D., Awumbila, B., 2005. Susceptibility of *Amblyomma variegatum* ticks to acaricides in Ghana. Exp. and App. Acarol. 3, 259-268.

Nava, S., Beati, L., Labruna, M.B., Cáceres, A.G., Mangold, A.J., Guglielmone, A.A., 2014. Reassessment of the taxonomic status of *Amblyomma cajennense* (Fabricius,

1787) with the description of three new species, *Amblyomma tonelliae* n. sp., *Amblyomma interandinum* n. sp. and *Amblyomma patinoi* n. sp., and reinstatement of *Amblyomma mixtum* Koch, 1844, and *Amblyomma sculptum* Berlese, 1888 (Ixodida: Ixodidae). Ticks and Tick Borne Dis., 5, 252-276.

Ntondini, N., van Dalen, E.M.S.P., Horak, I.G., 2008. The extent of acaricide resistance in 1-, 2- and 3-host ticks on communally grazed cattle in the eastern region of the Eastern Cape Province, South Africa. Tydskr.S.Afr.vet.Ver. 79(3), 130-135.

Parola, P., Christophe,r D., Paddock, C.D., Raoult, D., 2005. Tick-Borne Rickettsioses around the World: Emerging Diseases Challenging Old Concepts. Clinic. Microbiol. Rev. 719-756.

Perez-Cogollo, L.C., Rodriguez-Vivas, R.I., Ramirez-Cruz, G.T., Rosado-Aguilar, J.A., 2012. Survey of *Rhipicephalus microplus* resistance to ivermectin at cattle farms with history of macrocyclic lactones use in Yucatan, Mexico. Vet. Parasitol. 172, 109-113.

Pérez, M.T., Guzmán-Cornejo, C., Montiel-Parra, G., Paredes-León, R., and Rivas, G., 2014. Biodiversidad de ácaros en México. Revista Mexicana de Biodiversidad, Supl. 85, S399-S407.

Rodriguez-Vivas, R.I., Alonso-Diaz, M.A., Rodriguez-Arevalo, F., Fragoso-Sanchez, H., Santamaria, V.M., Rosario-Cruz, R., 2006. Prevalence and potential risk factors for organophosphate and pyrethroid resistance in *Boophilus microplus* ticks on cattle ranches from the State of Yucatan, Mexico. Vet. Parasitol. 136, 335-342.

Rodríguez-Vivas, R.I., Apanaskevich, D.A., Ojeda-Chi, M.M., Trinidad-Martínez, I., Reyes-Novelo, E., Esteve-Gassent, M.D., Pérez de León, A.A., 2016. Ticks collected from humans, domestic animals, and wildlife in Yucatan, Mexico. Vet. Parasitol. 215, 106-113.

Rodriguez-Vivas, R.I., Arieta-Roman, J.R., Perez-Cogollo, L.C., Rosado-Aguilar, J.A., Ramirez-Cruz, G.T., Basto-Estrella, G., 2010. Uso de lactonas macrociclicas para el control de la garrapata *Rhipicephalus (Boophilus) microplus* en el ganado bovino. Arch. Med. Vet. 42, 115-123.

Rodriguez-Vivas, R.I., Li, Y.A., M.M, Ojeda-Chi, M.M., Trinidad-Martinez, I., Rosado-Aguilar, J.A., Miller, R.J., Pérez de León, A.A., 2013. In vitro and in vivo evaluation of cypermethrin, amitraz, andpiperonyl butoxide mixtures for the control of resistant *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) in the Mexican tropics. Vet. Parasitol. 197, 288-296.

Rodríguez-Vivas, R.I., Pérez-Cogollo, L.C., Rosado-Aguilar, J.A., Ojeda-Chi, M.M., Trinidad-Martinez, I., Miller, J.R., Li, Y.A., Perez de León, A., Guerrero, F., Klafke, G., 2014. *Rhipicephalus (Boophilus) microplus* resistant to acaricides and ivermectin in cattle farms of Mexico. Braz. J. Vet. Parasitol. 23, 113-122.

Rodríguez-Vivas, R.I., Rivas, A.L., Chowell, G., Fragoso, S.H., Rosario, C.R., García, Z., Smith, S.D., Williams, J.J., Schwager, S.J., 2007. Spatial distribution of acaricide

profiles (*Boophilus microplus* strains susceptible or resistant to acaricides) in southeastern Mexico. Vet. Parasitol. 146, 158-169.

Szabó, M.P.J., Mukay, L.S., Rosa, P.C.S., Bechara, G.H., 1995. Diferences in the acquired resistence of dogs, Hamsters and guinea pigs to repeated infestations with adults ticks *Rhipicephalus sanguineus* (Acari:Ixodidae). *Braz. J. Res. Anim. Sci*. 32(1): 43-50.

Tapias VMC, Vaca RJL. Carga de Ixódidos en Bovinos de Predios Lecheros, Provincia Cercado, Beni, Bolivia, 2009. Agrociencias Amazonia, 2013, 1(2), 25-34.

Table 2: Different acaricide families' efficacy evaluation on different *Amblyomma mixtum* instars from the region of Veracruz, Mexico.

Ínstar	Acaricides (Packet Test)				
	cypermethrin 15g + chlorpyrifos 25g + PBO* 1 mL	formamidine 12,5g	cypermethrin 15g	dichlorvos 60g + chlorpyrifos 20g	Control
Larvae	87.4 %[a]	8.6 %[b,c]	9.8 %[b]	98.8 %[a]	1.9 %[b,c]
Engorged larvae	100.0 %[a]	12.0 %[b,c]	2.2 %[b]	100.0 %[a]	2.3 %[b,c]
Nymphs	100.0 %[a]	7.5%[b,c]	31.0 %[b]	100.0 %[a]	2.5 %[b,c]
Engorged nymphs	97.8 %[a]	24.4 %[b,c]	26.7 %[b]	100.0 %[a]	4.4 %[b,c]
Non-fed adults	100.0 %[a]	0.0[b,c]	40.0 %[b]	100.0 %[a]	0.0[b,c]

*PBO: piperonyl butoxide; equal letters among values do not present a significant difference ($p>0.05$).

Artigo 4

Immune response and efficacy of a synthetic peptide based on the aquaporin protein of *Amblyomma sculptum*.Os resultados obtidos em relação ao objetivo avaliação da resposta imune de um peptídeo sintético baseado na proteína aquaporina em *Amblyomma sculptum*, foram submetidos à revista Vaccine (Qualis A2)

Elsevier Editorial System(tm) for Vaccine
Manuscript Draft

Manuscript Number:

Title: Immune response and efficacy of a synthetic peptide based on the aquaporin protein of Amblyomma sculptum.

Article Type: Original article

Keywords: peptide vaccine, Amblyomma cajennense complex, immunoinformatics, tick control.

Corresponding Author: Dr. Renato Andreotti, PhD

Corresponding Author's Institution: Emprapa Beef Cattle

First Author: Francisco B Piña, Master and Science

Order of Authors: Francisco B Piña, Master and Science; Leandro O Higa, Master and Science; Vinicius S Rodrigues, Master and Science; Andre A Aguirre, Doutor; Barbara G Csordas, Master and Science; Marcos V Garcia, Doutor; Jason Tidwell, Master and Science; Adalberto A PerezdeLeon, PhD; Renato Andreotti, PhD

Abstract: Amblyomma sculptum is a tick with a heteroxene life cycle that parasites wild animals, domestic animals and humans. It is considered the main vector of Rickettsia rickettsii in different states of Brazil. The established control methods are based on avoiding the human contact with ticks and the use of acaricides, which generate the problem of development of resistance and environmental degradation as a disadvantage. The use of vaccines is an alternative tool for the control of ticks, and the present work aims to design a synthetic peptide based on aquaporin B and T cell epitopes and evaluate its immune response and efficacy. Additional analyzes of the aquaporin protein were performed using algorithms provided within Geneious Pro 4.8.5 software (Biomatters). The software tools used predicted antigenic properties of the primary protein structure using features such as hydrophobicity, linear regions and predicted secondary structures (alpha helix, beta and spiral chains). Rabbits were used as laboratory model to evaluate the effect of the immune response against A. sculptum (vaccinated and non-vaccinated group). three doses were applied at 21 day intervals. Efficacy was evaluated with an established formula for heteroxene ticks. The immune response in the present study was 100% in the vaccinated group, showing an immune response time of 83 days, and the peptide efficacy was 68.1%. In the present work it can be observed the first result of efficacy of a vaccine formulation against the tick A. sculptum, the main vector of Brazilian spotted fever, using immunoinformatics tools to" design of synthetic peptides.

Suggested Reviewers: Rodrigo C Cunha PhD
Prfessor, Parasitology, Universidade Federal de Pelotas
rodrigocunha_vet@hotmail.com
Parasitologisty

Marcelo B Labruna PhD
Researcher, Parasitology, USP

labruna@usp.br
Parasitologisty

Dora R Salas PhD
Professor, Parasitology, UV
dromero@uv.gov.mx
Parasitologisty

Wilson W Koler PhD
Researcher, Parasitology, Embrapa
willson.koller@embrapa.br
Parasitologisty

Opposed Reviewers:

COVER LETTER

Campo Grande - MS, May 28th, 2018.

From the authors:

Francisco Tobias Barradas Piña, Leandro de Oliveira Souza Higa, Vinicius da Silva Rodrigues, André de Abreu Rangel Aguirre, Bárbara Guimarães Csordas, Marcos Valério Garcia; Jason Tidwell; Adalberto Angel Pérez de León; Renato Andreotti.

Contacts: Embrapa Beef Cattle, Avenida Rádio Maia, 830
Vila Popular, CEP 79106-550 - Campo Grande, MS,
Brazil, fone/fax: 55 (67) 33682169, e-mails:

Dear Editor.

We are forwarding the manuscript entitled "Immune response and efficacy of a synthetic peptide based on the aquaporin protein of *Amblyomma sculptum*". This manuscript was written according to the journal guidelines and edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified native person. All authors permit use of figures and tables.

If accepted for publication, we pledge to consider all questions of the reviewers and respond as soon as possible.

Without more and available for necessary adjustments to the manuscript.

Sincerely,

The authors.

***Suggested Reviewers**

Suggested Reviewers:

Rodrigo Casqueiro Cunha:
Universidade Federal de Pelotas
E-mail adress: rodrigocunha_vet@hotmail.com

Marcelo Bahia Labruna
Unicersidade federal de São Paulo
E-mail adress:labruna@usp.br

Matias Pablo Szabó
Universidade]federal de Uberlandia
E-mail adress:szabo@famev.ufu.br

Fabio Leivas Leite
Universidade Federal de Pelotas
E-mail adress:fabio.leite@ufpel.br

Abstract

Amblyomma sculptum is a tick with a heteroxene life cycle that parasites wild animals, domestic animals and humans. It is considered the main vector of *Rickettsia rickettsii* in different states of Brazil. The established control methods are based on avoiding the human contact with ticks and the use of acaricides, which generate the problem of development of resistance and environmental degradation as a disadvantage. The use of vaccines is an alternative tool for the control of ticks, and the present work aims to design a synthetic peptide based on aquaporin B and T cell epitopes and evaluate its immune response and efficacy. Additional analyzes of the aquaporin protein were performed using algorithms provided within Geneious Pro 4.8.5 software (Biomatters). The software tools used predicted antigenic properties of the primary protein structure using features such as hydrophobicity, linear regions and predicted secondary structures (alpha helix, beta and spiral chains). Rabbits were used as laboratory model to evaluate the effect of the immune response against *A. sculptum* (vaccinated and non-vaccinated group). three doses were applied at 21 day intervals. Efficacy was evaluated with an established formula for heteroxene ticks. The immune response in the present study was 100% in the vaccinated group, showing an immune response time of 83 days, and the peptide efficacy was 68.1%. In the present work it can be observed the first result of efficacy of a vaccine formulation against the tick *A. sculptum*, the main vector of Brazilian spotted fever, using immunoinformatics tools to" design of synthetic peptides.

Key words: peptide vaccine, *Amblyomma cajennense* complex, immunoinformatics, tick control.

*Title Page

Immune response and efficacy of a synthetic peptide based on the aquaporin protein of *Amblyomma sculptum*.

Immune response and efficacy of a synthetic peptide based on the aquaporin protein of *Amblyomma sculptum*.

Francisco Tobias Barradas Piña[1,2,8], Leandro de Oliveira Souza Higa[1,2], Vinicius da Silva Rodrigues[1,2], André de Abreu Rangel Aguirre[1,5], Bárbara Guimarães Csordas[1,2], Marcos Valério Garcia[3,4]; Jason Tidwell[7]; Adalberto Angel Pérez de León[7]; Renato Andreotti[6].

1 Pós-Graduação em Doenças Infecciosas e Parasitarias - Faculdade de Medicina, UFMS - Universidade Federal Mato Grosso do Sul, Campo Grande, MS, Brazil

2 Laboratório de Biologia do Carrapato, Embrapa Gado de Corte, Campo Grande, MS, Brazil

3 Laboratório de Biologia do Carrapato, Empresa Brasileira de Pesquisa Agropecuária, Embrapa Gado de Corte, Campo Grande, MS, Brazil

4 Bolsista DCR Fundect, MS – Governo do estado de Mato Grosso do Sul, Campo Grande, Brazil

5 Fundação Oswaldo Cruz, Fiocruz Rondônia, Rua da Beira, No. 7671, Bairro Lagoa, CEP 76812-245 Porto Velho, RO, Brazil

6 Empresa Brasileira de Pesquisa Agropecuária, Embrapa Gado de Corte, Avenida Radio Maia, 830, Campo Grande, MS CEP 79106-550, Brazil

7 USDA-ARS, Knipling-Bushland U.S. Livestock Insects Research Laboratory, and Veterinary Pest Genomics Center, Kerville, TX, USA.

8 Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias INIFAP-México.

Abstract

Amblyomma sculptum is a tick with a heteroxene life cycle that parasites wild animals, domestic animals and humans. It is considered the main vector of *Rickettsia rickettsii* in different states of Brazil. The established control methods are based on avoiding the human contact with ticks and the use of acaricides, which generate the problem of development of resistance and environmental degradation as a disadvantage. The use of vaccines is an alternative tool for the control of ticks, and the present work aims to design a synthetic peptide based on aquaporin B and T cell epitopes and evaluate its

immune response and efficacy. Additional analyzes of the aquaporin protein were performed using algorithms provided within Geneious Pro 4.8.5 software (Biomatters). The software tools used predicted antigenic properties of the primary protein structure using features such as hydrophobicity, linear regions and predicted secondary structures (alpha helix, beta and spiral chains). Rabbits were used as laboratory model to evaluate the effect of the immune response against *A. sculptum* (vaccinated and non-vaccinated group). three doses were applied at 21 day intervals. Efficacy was evaluated with an established formula for heteroxene ticks. The immune response in the present study was 100% in the vaccinated group, showing an immune response time of 83 days, and the peptide efficacy was 68.1%. In the present work it can be observed the first result of efficacy of a vaccine formulation against the tick *A. sculptum*, the main vector of Brazilian spotted fever, using immunoinformatics tools to" design of synthetic peptides.

Key words: peptide vaccine, *Amblyomma cajennense* complex, immunoinformatics, tick control.

1. Introduction

Amblyomma sculptum is characterized by a biological cycle of three hosts, which can parasitize wildlife (reptiles, tapirs, capybaras, etc.) as well as domestic animals, mainly horses (1). This tick species affects humans being the vector for *Riclettsia rickettsii*, the causative agent of Brazilian spotted fever which are endemic in some states of Brazil (2-3-4).

In agriculture production and domestic animals, the main method of tick control is the use of acaricides, which have several disadvantages namely: development of tick resistance to different chemical classes, negative environmental impacts, economic

prices, and they can accumulate in the meat and milk (5). Pasture management is also a strategic method for tick control (6). An alternative method is the use of anti-tick vaccines, introduced to the market since 1990 and commercially known as Gavac©. These commercial vaccines are used only for the cattle tick *Rhipicephalus microplus*. Their development was based on the tick gut glycoprotein BM86 and BM95 (7-8-9).

In the case of anti-tick vaccines, one of the main objectives is to have an impact on tick development by reducing productive parameters such as: recovery rate, period of engorgement, engorged female weight, weight in egg mass, and reduction in larvae hatching rate (10-11-12). Other objectives include to improve strategic tick control, reduce the negative environmental impact of acaricide use, and diminish economic losses in cattle breeding (13).

An adult tick of the *Amblyomma* spp. can ingest blood volumes up to 600 times that of larvae. Ticks need large amounts of blood to maintain efficient water transport mechanisms in order to concentrate blood components for efficient digestion. Within this context, the aquaporin protein, recently used as antigen for the development of a vaccine for tick control, with the purpose of altering the transport of water through cell membranes (14).

Bioinformatics and immunoinformatics generate tools that uses the sequence established in the database, using *in silico* algorithms to identify potentially immunogenic epitopes, antibody binding sites, determination of B and T cell epitopes, origin, binding of MHC classes I and II, gene expression, etc. The present work aims to use immunoinformatic tools to develop the first time a synthetic peptide from the aquaporin protein of the *A. cajennense sensu lato* (s.l.) tick and evaluate its efficacy against *A. scupltum* using rabbits as experimental host under laboratory conditions.

2. Materials and methods

Animals: Nine New Zealand rabbits of different sexes were used. The animals were randomly divided into two groups (vaccinated and control) and kept in individual cages under laboratory conditions. Sterilized feed and water were offered to the rabbits *ad libitum*. The management processes of the experiment were approved by the Ethics Committee (CEUA nº 699/2015).

Tick source and rearing: Engorged female ticks of *A. sculptum* were weighed using an analytical scale, housed in vials with perforated covers, and incubated at 27 °C and 80% relative humidity. In addition, the other tick life stages (eggs, larvae, and nymphs) were maintained under the same laboratory conditions to collect off-host data. The species was morphologically identified in accordance with the taxonomic characteristics described by (15).

Immunoinformatics analysis of the aquaporin protein and peptide synthesis: The *A. cajennense* s.l. aquaporin amino acid sequence was obtained from GenBank (access number: JAC21377.1) analyzed by several bioinformatics softwares (figure 1), including: Predictions for binding to MHC class II were used to predict regions in the aquaporin protein that are recognized by MHC-II host molecules by using net MHCIIpan algorithm. The mouse H-2 alleles IAb, IAd, IAs, IAu and IEk were used for this purpose. The values obtained were based on binging affinity IC50 values in nanomolar (nM) values. (16-17-18,).

Further analyzes of the aquaporin protein were performed using algorithms provided within the Geneious Pro 4.8.5 software (Biomatters, Auckland, New Zealand). The software tools used predicted antigenic properties from the primary protein structure, utilizing characteristics such as hydrophobicity, linear regions, and predicted secondary structures (alpha helix, beta chains, and coils).

SignalP 4.1 (www.cbs.dtu.DK/services/SignalP) and TMHMM v2.0 (www.cbs.dtu.dk/services/TMHMM), for the prediction of signal peptide and intracellular, extracellular or transmembrane, regions with a 0.5 cut-off for SignalP. The Bepipred Linear Epitope Prediction and Emini Surface Accessibility Prediction Results softwares (IEDB Analysis Resource–Antibody Epitope Prediction, http://tools.iedb.org/bcell) were used to detect linear B-cell epitopes that are predicted to be presented at the surface of the aquaporin tertiary molecule structure, which could increase the likelihood of immunogenicity using a cutoff of 0.0 and 0.250 for 15 consecutive amino acids.

The peptide was synthesized by GenOne (GenOne, BRA). The chemically synthesized peptide in this study contains 17 amino acids with a cysteine at the amino-terminal.The carrier-conjugated peptide consisted of the peptide with cysteine attached to the amino terminal together with m-maleimidobenzoyl-N-hydoxysuccinimide linker to the Keyhole Limpet Hemocyanin (KLH) carrier.

To verify if there are properties that would generate inespecific immunogenicity, the BLAST program was used to detect possible regions of the aquaporin protein that were highly similar to rabbit proteins.

Aquaporin alignment with orthologs was performed with Clustal W2 to verify that the peptide sequence had sufficient identity to elicit an immune response against other *Amblyomma* spp. The high identity is to seek an antigen that works against distinct

populations of ticks, including *A. scultpum*, and to control other distinct species of the *A. cajennense* complex Figure 1.

Inoculation and infestation of animals: Rabbits were randomized into two groups (vaccinated group 5 rabbits, unvaccinated group 4 rabbits), the two groups were infested with larvae, nymphs and adults ticks of *A. sculptum*. The inoculation schedule had three applications with a 21-day interval for each inoculation, considering the first dose (day 0); second dose (day 21); third dose (day 42). For rabbit inoculation, the KLH-conjugated peptide was diluted in PBS and emulsified with Montanide adjuvant (Seppic, Paris) at an adjuvant/peptide ratio of 60/40 v/v. Each animal received a dose of 500 μL containing 100 μg of peptide conjugated to KLH to ensure the immune response. The inoculation in the animals was intramuscular.

For evaluation of the vaccine, each animal was infested with 100 larvae, 100 nymphs and six adult couples *A. sculptum*, placed in feed chambers for each instar at the same time. Feeding chambers were inspected daily for the collection of naturally detached engorged ticks. The ticks were taken to the laboratory where they were counted, weighed and identified in flasks for incubation and evaluation of the biological cycle (parameters described below).

Immunoassays: Blood samples from the animals were collected on day 0, 21, 42, 63, and 84. The serum was separated by centrifugation and stored in the freezer for the analysis of the immune response .

Microtiter plates (Costar®) were coated with a solution of 1 μg of the peptide in 20 mM carbonate buffer (pH 9.6 - 100 μL per well) overnight at 4 ° C. To verify whether the antibodies induced by cattle immunization were able to recognize epitopes

from Aquaporin, we conducted western blotting and indirect ELISA using the method previously described (19) with some modifications.

Efficacy calculation of vaccine efficacy against *A. sculptum*: In order to calculate the peptide efficacy, several parameters of *A. sculptum* fitness were used, which were the effect of each immunogen on larval vitality (VL), yield of nymphs (RN), yield of engorged females (RA), viability of adults (VA), oviposition of females (OA), and fertility of eggs (FE). With these parameters, we used the equation E (%) = 100 x [1 - (RL*VL*RN*VN*RA*OA*FE)] for peptide efficacy (20).

Parameters evaluated: Each Rabbit was challenged with *A. sculptum* 21-days after the third inoculation with 100 larvae, 100 nymphs, and six adult couples in chambers of artificial feeding placed on the backs of rabbits (21). The chambers were checked daily until the last engorged tick detached naturally. The life cycle parameters evaluated were: (1) Recovery Rate in Larvae, Nymphs and Engorged females. (2) Molting in Larvae and Nymphs. (3) Weight of eggs mass. (4) Larval hatchability rate and egg maturation period. Using the methodology for *A. cajennense* sensu lato (sl) (22-23-24-25-26).

Statistical analysis: Data on the reproductive parameters of ticks recovered from vaccinated and control groups were analyzed using a t-test for unequal variances for Kruskal-Wallis for *A. scupltum*. Differences were considered significant when $p < 0.05$. Mean antibody levels were determined for each group and compared using analysis of variance with 2 factors (ANOVA), and the F-test were used to determine the significance of any differences observed between the groups. Differences were

considered significant at a P value < 0.01. The analyses were conducted using GraphPad Prism 1 Version 7 for Windows (La Jolla, USA).

3. Results

The immunoinormatic approaches were analyzed together to predict an antigenic MHC II epitope (figure 1). We chose a peptide containing 17 amino acids located between residues 120-136. This region shared the most antigenic characteristics: prediction to bind on murine MHC class II, prediction to bind on B-cell receptor, linear epitope on the surface protein and located totally on extracellular region. The predicted peptide sequence obtained is: N-RQVLGEKGTAGIFATYP-C.

The figure 2 shows that immune responses of animals 3, 4, and 5 were greater within the first 18 days after the first application and the response remained constant until the last blood collection (day 83). For animals 1 and 2, peaks occurred in the first 18 days, but the response have diminished during the following days and presented lower antibody titers. The control group did not present an immune response during the study.

Effectiveness formula:

Efficacy: 100 x [1- (72,5*48,6) x (73*86,9) x (70,8*58,8) x (56,4*58,9) x (70*58,3) x (56,4*239,3) x (62,7*80,5)] = 68,1

Table 1 shows the mean recovery rates for engorged larvae, nymphs and engorged females. The vaccinated group had a larvae recovery rate of 72.5% with a range of 55.5% to 82.5%, nymphs recovery rate of 70.8% with a range of 51% to 87%, and the teleogines recovery rate of 70% with a range of 16.7% to 100%. The recovery rates for the control group for larvae, nymphs, and teleogines were 48.6%, 58.7% and

58.4% respectively. The larval and nymphal hatching rate of the vaccinated group was 73.1% and 56.8%, respectively. The hatching rate for the control group of larvae and nymphs was 86.9% and 58.8% respectively. The overall mean weight of the egg mass was 117.4 mg for the vaccinated group and 244 mg of the control group. The hatchability in the vaccinated group was 62.7% and 80.5% in the control group.

4. Discussion

The present study evaluated for the first time the response immune and efficacy of a synthetic peptide designed using the immunoinformatic tools based on aquaporin sequence from *A. sculptum*; using rabbits as a laboratory model. The target species of the experiment was *A. sculptum*, evaluating productive parameters in larvae, nymphs and adults. The immune response was assessed by means of the Elisa test (identification of IgG antibodies).

In the present experiment it is observed that 100% of the inoculated rabbits responded positively to the inoculation of the peptide, showing IgG antibody titers from the first inoculation until 23 days after the third inoculation, this result shows that the development of synthetic peptides using bioinformatics as a tool contributes satisfactorily to the design of antigens that in future studies can be potentially immunogenic in order to obtain an alternative method for the control of ticks with heteroxen life cycle.

In the present work total number of vaccinated rabbits presented an immune response, being higher in three rabbits (figure 1). Studies done by (19-27) specified that the immune response may manifest differently for each animal, while the efficacy results observed in a study conducted in Brasil, using a RmAQP1 antigen-based peptide against *R. microplus* were evaluated in cattle, where the The authors identified a marked

reduction in tick recovery rate in animals of the vaccinated group, with a difference of 29% of the recovery rate of the control group (14).

The use of tools such as B and T cell epitope prediction for the development of synthetic peptides may contribute as alternative methods for the control of ticks from different life cycles (monoxenes, heteroxenes), generating greater identification opportunities using *in silico* approaches to select new antigens potentially immunogenic against ticks (28). The development and use of tick vaccines can be an effective complement to the planning of strategic schedules for tick control in domestic animals, generating low costs, better management of infested animals, reduction in parasite rate, reduction of pathogen transmission and production with mixed infestations. generating better cost-benefit efficiency contributes to environmental pollution (29).

Biological parameters showed that, larvae of vaccinated group presented 13.8% lower hatch rate than the control group. The nymphs of the vaccinated group expressed the ecdise rate of 2.5% less than control group. The weight mean of egg mass was 126.5 mg lower in the vaccinated group and reproductive efficiency presented 49.6% in the vaccinated group, considering these biological parameters in the formula proposed (19), it was determined that the efficiency of the synthetic peptide based on aquaporin was 68.1%. Results presented by (30) show relatively greater efficacy using commercial vaccines for monoxene ticks. the present work showed decreasing in the tick population due to the vaccine effect. However its considerable to develop new studies the efficiency of the peptide can be improved in order to reduce the high parasitoses in the hosts and indirectly could contribute in the transmission of pathogens of impact in the public health.

The formula to evaluate the effectiveness of three-host life cycle ticks was established by (20), where they establish the standard comparison of the different

parameters to evaluate in ticks exposed to the vaccinated group and in the control group. The behavior of parameters such as molting rate in larvae and nymphs were higher in the control group than in the vaccinated group, obtaining a smaller amount of ticks in the vaccinated group after the metamorphosis process. Average egg mass weight was similar for both groups. In a similar work performed by (19), a synthetic peptide based on ATAQ protein for the control of *R. sanguineus*, obtaining an efficiency of 47%, technique used in the present work for the development of the synthetic peptide based on the aquaporin was such higher, obtaining an efficiency of 68.1%.

In the present work the established formula for determining the efficacy of a vaccine in the control of heterexene ticks was used for the first time. The use of reverse vaccination is a tool that contributed to the development of a synthetic peptide generated a high immune response, however it is important to develop new experiments to increment the efficacy.

5. References

1. Cunha AP, Ana CP, de Paiva B, Romário CL, Bastinetto E, Cândido A, Leite RC, de Freitas CM, Oliveira PRV. Controle Estratégico de *Amblyomma cajennense* (Fabricius, 1787) (Acari: Ixodidae) em Eqüinos, Minas Gerais, Brasil-Parte I; Rev Bras Parasitol Vet 2007;16:221-228.

2. Guedes E, Leite RC, Prata MCA, Pacheco RC, Walker DH, Labruna MB, Detection of *Rickettsia rickettsii* in the tick *Amblyomma cajennense* in a new Brazilian spotted fever-endemic area in the state of Minas Gerais. Mem do Inst Oswaldo Cruz 2005;100:841-845.

3. Oliveira MC, Soares AFM, Rodrigues F, Guedes E. Aspectos Gerais da Febre Maculosa Brasileira. CES Revista 2006;133-146.

4. Freitas, EdePS, Zapata MTAG, Fernandes FdeF. Monitoring of resistance or susceptibility of adults and larvae of *Amblyomma cajennense* (Acari: Ixodidae) to synthetic acaricides in Goiás, Brazil. Exp Appl Acarol 2011;53:189-202.

5. Grisi L, Leite RC, Martins JRdeS, de Barros ATM, Andreotti R, Cançado PHD, de León AAP, Pereira JB, Villela HS. Reassessment of the potential economic impact of cattle parasites in Brazil. Braz. J. Vet. Parasitol 2014;23:150-156.

6. Gauss BLC, Furlong J. Comportamento de larvas infestantes de *Boophilus microplus* em pastagem de *Brachiaria decumbens*. Ciên Rur Santa Maria 2002;32:467-472.

7. Rodriguez M, Penichet ML, Mouris AE, Labarta V, Luaces L, Rubiera R, Cordová C, Sánchez PA, Ramos E, Soto A, Canales M, Palenzuela D, Triguero A, Lleonart R, Herrera L, de la Fuente J. Control of *Boophilus microplus* populations ingrazing cattle vaccinated with a recombinant Bm86 antigen preparation. Vet Parasitol 1995;57:339-349.

8. García-García ACJ, Carlos M, Redondo M, Vargas M, Canales M, Boue O, Rodríguez M, Joglar M, Machado HI, González L, Valdés M, Méndez L, de la Fuente JA. Control of ticks resistant to mmunization with Bm86 in cattle

vaccinated with the recombinant antigen Bm95 isolated from the cattle tick, *Boophilus microplus*. Vacc 2000;18:2275-2287.

9. de la Fuente J, Almazán C, Canales M, Pérez LJM, Kocan MK, Willadsen P. A ten-year review of commercial vaccine performance for control of tick infestations on cattle. Anim Health Research Rev 2007;8:23-28.

10. Redondo M., Fragoso H, Ortíz M, Montero C, Lona J, Medellín JA, Fría R, Hernández V, Franco R, Machado H, Rodríguez M, de la Fuente J. Integrated control of acaricide-resistant *Boophilus microplus* populations on grazing cattle in Mexico using vaccination with Gavac™ and amidine treatments. Exp and App Acarol 1999;23:841-849.

11. Rodríguez MV, Méndez L, Váldez M, Redondo R, Esponosa CM, Vargas M, Cruz RLL, Barrios HP, Seoane G, Ramírez ES, Boue O, Vigil JL, Machado H, Nordelo CB, Piñeiro MJ. Integrated control of *Boophilus microplus* ticks in Cuba based on vaccination with the anti-tick vaccine Gavac^{TM} . Exp and App Acarol 2004;34:375-382.

12. Andreotti R, Pedroso M, Caetano AR, Martins NF. Comparison of predicted binders in *Rhipicephalus (Boophilus) microplus* intestine protein variants BM86 Campo Grande Strain, BM86 and BM95. Rev Bras Parasitol Vet 2008;17:93-98.

13. Rodriguez-Valle Moolhuijzen MP, Piper EK, Weiss O, Vance M, Bellgard M, Lew-Tabor A. *Rhipicephalus microplus* lipocalins (LRMs): Genomic

identification and analysis of the bovine immune response using *in silico* predicted B and T cell epitopes. Int J for Parasitol 2013;43:739-752.

14. Guerrero FD, Andreotti R, Bendele GK, Cunha CR, Miller RJ, Yeater K, de León PA, *Rhipicephalus* (*Boophilus*) *microplus* aquaporin as an effective vaccine antigen to protect against cattle tick infestations. Parasit and vect 2014;7:2-12.

15. Nava S, Beati L, Labruna MB, Cáceres AG, Mangold AJ, Guglielmone AA. Reassessment of the taxonomic status of *Amblyomma cajennense* (Fabricius, 1787) with the description of three new species, *Amblyomma tonelliae* n. sp., *Amblyomma interandinum* n. sp. and *Amblyomma patinoi* n. sp., and reinstatement of *Amblyomma mixtum* Koch, 1844, and *Amblyomma sculptum* Berlese, 1888 (Ixodida: Ixodidae). Ticks and Tick-borne Dis 2014;5:252-276.

16. Vordermeier M, Whelan AO, Hewinson RG. Recognition of mycobacterial epitopes by T Cells across mammalian species and use of a program that predicts human HLA-DR binding peptides to predict bovine epitopes. Inf and Immu 2003:1980-1987.

17. Nene V, Svitek N, Toye P, Golde WT, Barlow J, Harndahl M, Buus S, Nielsen M. Designing bovine T cell vaccines via reverse immunology. Ticks and Tick-borne Diseases 2012:3;188-192.

18. Rodriguez-Valle M, Moolhuijzen P, Piper EK, Weiss O, Vance M, Bellgard M, Lew-Tabor A. *Rhipicephalus microplus* lipocalins (LRMs): Genomic identification and analysis of the bovine immune response using *in silico* predicted B and T cell epitopes. Int J for Parasitol 2013:43;739-752.

19. Aguirre AdeAR, Lobo PF, Cunha CR, Garcia VM, Andreotti R. Design of the ATAQ peptide and its evaluation as an immunogen to develop a *Rhipicephalus* vaccine. Vet Parasitol 2016;221:30-38.

20. Aguirre AdeAR, Garcia VM, Szabó MPJ, Barros CJ, Andreotti R. Formula to evaluate efficacy of vaccines and systemic substances against three-host ticks. Int J for Parasitol 2015;45:357-359.

21. Bechara GH, Zsabó MPJ, Ferreire BR, Garcia MV. *Rhipicephalus sanguineus* tick in Brazil: Feeding and reproductive aspects under laboratorial conditions. Rev Bras Parasitol Vet. 1995;4:61-66.

22. Hooker WA, Bishopp FC, Wood HP. The life history and bionomics of some North American ticks. Bur Entomol Bull 1912;106:72-87.

23. Drummond RO, Whetstone TM. Oviposition of the cayenne tick, *Amblyomma cajennense* (F.), in the laboratory. Ann Entomol Soc Am 1975;68:214-216.

24. Szabó MPJ, Mukay LS, Rosa PCS, Bechara GH. Differences in the acquired resistance of dogs, hamsters and guinea pigs to repeated infestations with adults

ticks *Rhipicephalus sanguineus* (Acari:Ixodidae). Braz J Res Anim Sci 1995;32:43-50.

25. Sanavria A, Prata MCA. Metodologia para colonização do *Amblyomma cajenennse* (Fabricius, 1787) (Acari:Ixodidae) em laboratório. Rev Bras Parasitol Vet 1996;5:87-90.

26. Gerardi M, Martins MM, Nava S, Szabo MPJ. Comparing feeding and reproductive parameters of *Amblyomma parvum* tick populations (Acari: Ixodidae) from Brazil and Argentina on various host species. Vet Parasitol 2013;197:312-317.

27. Andreotti R, Cunha RC, Soares MA, Guerrero FD, Leite FPL, Pérez de León AA. Protective immunity against tick infestation in cattle vaccined with recombinant trypsin inhibitor of *Rhipicephalus microplus*. Vacc 2012;30:6678-6685.

28. Maritz-Olivier C, Zyl WV, Stutzer Ch. A systematic, functional genomics, and reverse vaccinology approach to the identification of vaccine candidates in the cattle tick, *Rhipicephalus microplus*. Ticks and Tick-borne Diseases 2012;3:179-187.

29. de la Fuente J, Kocan KM, Contreras M. Prevention and control strategies for ticks and pathogen transmission. Rev Sci Tech Off Int Epiz 2015;34:249-264.

30. Andreotti R. Performance of two Bm86 antigen vaccin formulation against tick using crossbreed bovines in stall test. Rev Bras Parasitol Vet 2006;15:97-100.

Tables

Table 1: Biological parameters of engorged larvae, nymphs and adult females of *A. sculptum* evaluated on rabbits vaccinated with aquaporin and control group.

Group	Recovering			Hatching		Weight eggs	% EcoE
	Larvae	Nymphs	Female	Larvae	Nymphs		
Vaccinated	72.5 (± 10.7) 55.5 – 82.5	70.8 (± 14) 51 - 87	70 (± 32.0) 16.7 - 100	73.1 (± 14.8) 51.8 – 87.3	56.4 (± 35.8) 17.2 – 96.1	269.3 (± 158.4) 20.1 – 450.4	47 (± 14) 21 – 61.2
Control	48.6 (± 36.7) 4.5 - 84	58.7 (± 32.8) 21 - 99	58.4 (± 48) 16 - 100	86.9 (± 10.2) 72.3 - 95	58.8 (± 46.3) 15.2 - 100	269.8 (± 141.2) 156.6 – 476.2	46.2 (± 12.7) 19.3 – 58.2

Figures

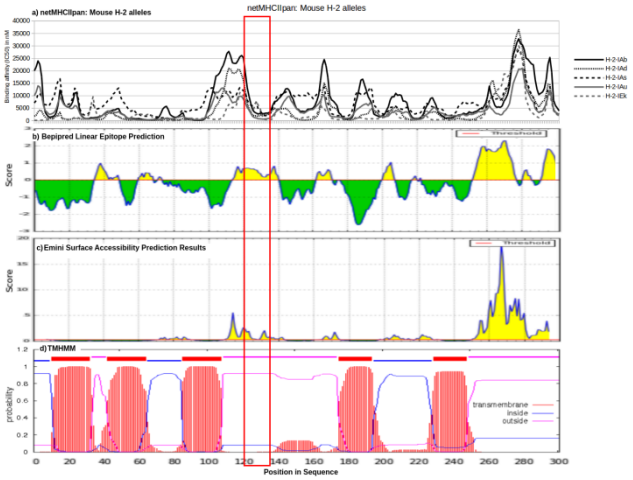


Figure 1. Algorithms used for aquaporin-based peptide production insertion of the peptide between nucleotide 120 and 140.

Figures

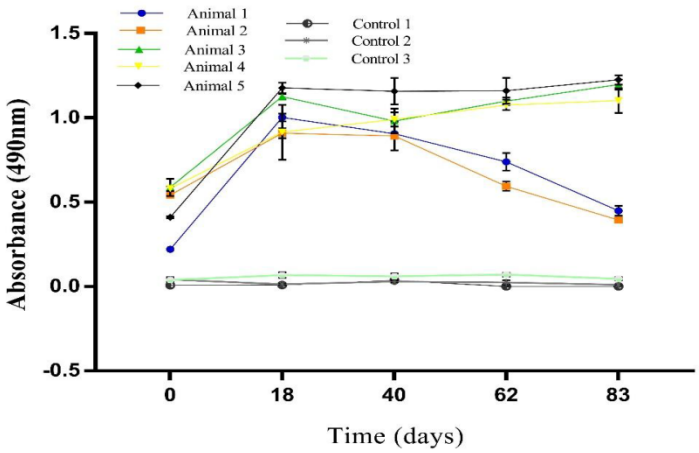


Figure 2: Immunoenzymatic test (ELISA) of animals vaccinated with peptide Rc18-KLH. The ELISA was performed using only the peptide (1ug), without the KLH carrier. The sera of the animals were diluted (1: 200).

7 DISCUSSÃO

Os carrapatos são ectoparasitas que apresentam uma diversa gama de hospedeiros e portanto uma ampla distribuição geográfica, indicando que estes podem ser considerados organismos bem-sucedidos (WANG, NUTTAL, 1999; ALI *et al*., 2016). Durante a hematofagia, algumas implicações em consequência deste processo podem ocorrer, como: paralisia do hospedeiro devido a liberação de neurotoxinas ("tick paralysis"), transmissão de patógenos, danos físicos (ex: couro, reação alérgica) etc (WALL, SHEARER, 1997).

Dentro da temática da saúde pública, espécies como *A*. *mixtum* e *A. sculptum* parasitam animais domésticos como equinos e caninos podem ser associados como um importante vetor entre o contato de humanos e carrapatos, (GONZALEZ-CERÓN *et al*., 2009; GUZMÁN-CORNEJO *et al*., 2011). Sendo assim, o controle dessas espécies é de grande relevância para saúde pública e médico veterinária (MEDINA-SANCHEZ *et al*., 2005; SANCHEZ-MONTES *et al*., 2016).

Atualmente, em termos de controle do carrapato, existem diversas formas de combate que vão desde a utilização de nematóides, fungos entomopatogênicos, extratos de plantas, vacinas e acaricidas (PATARROYO *et al*., 2002; RODRIGUEZ *et al*., 2004; BROGLIO-MICHELETTI *et al*., 2009; PARIZI *et al*., 2012). O uso de acaricidas (químicos), como já mencionado, constitui a principal forma de controle dos carrapatos em todo mundo. Para o controle de carrapatos de importância na saúde em geral, *R*. *microplus* é utilizado como modelo de estudos nessa área devido a sua importância econômica e larga utilização de acaricidas desde o século XX (DRUMMNOND *et al*., 1973; KUNZ, KEMP, 1994; MILLER *et al*., 2002; RODRÍGUEZ-VIVAS *et al*., 2010).

Devido ao problema constante do desenvolvimento de resistência por parte das populações de carrapatos tratadas com acaricidas a diversas bases químicas é cada vez mais comum (WARTON, ROULSTON, 1970; SOBERANES *et al*., 2002; RECK *et al*., 2014; HIGA *et al*., 2015; HIGA *et al*., 2016; KLAFKE *et al*., 2017), algumas indicações são importantes para evitar o avanço da resistência nas populações de carrapatos. O uso do conhecimento do ciclo do carrapato a favor do controle estratégico se torna imprescindível para um controle eficaz do parasito. O carrapato

bovino apresenta um ciclo biológico de 21 dias, e estudos que utilizaram tratamentos sistemáticos dentro deste período se mostraram eficazes (KUNS, KEMP, 1994; RODRÍGUEZ-VIVAS *et al*., 2010).

O presente estudo realizado manifesta a diferença significativa entre dois tratamentos de base química semelhantes em bovinos naturalmente infestados por *R*. *microplus*, demonstrando a importância da escolha da base química adequada e diagnóstico da resistência da população de carrapatos (RODRIGUES *et al*., 2018). Neste estudo, ambos produtos químicos apresentavam formulação baseada na associação entre piretróide e organofosforado (cipermetrina + clorpirifós), porém com a presença de fenthion no acaricida apresentado como de maior eficácia. A população de carrapatos em questão foi tratada a cada 18 dias, impedindo assim o desenvolvimento completo dos carrapatos durante o período, uma vez que o ciclo parasitário compreende em 21 dias.

O presente trabalho manifesta que o uso de ferramentas como ciclo biologico, uso de raças cruzadas (*Bos taurus taurus* X *Bos taurus indicus*), raças naturalmente resistentes (*Bos taurus indicus*), diagnóstico de resistência, monitoramento dos carrapatos de forma direta no campo e métodos de aplicação dos acaricidas contribuem no controle dos mesmos e podem aumentar a eficiência até um 60% aproximadamente em campo (RODRIGUES et al., 2017). Os métodos de diagnóstico e as outras ferramentas estabelecidas para carrapatos com ciclo de vida monoxenos (*R. microplus*), podem-se adaptar essas ferramentas para carrapatos com ciclo de vida heteroxeno (RODRIGUES *et al* 2017; NATALA et al 2005).

Outro carrapato que afeta bovinos, é o *A*. *mixtum*. Este carrapato parasita animais concomitantemente ao *R*. *microplus*, causando prejuízos econômicos. *A*. *mixtum* apresenta um ciclo de vida de 88 dias em condições de laboratório (BARRADAS *et al*., 2017). Além disso, a dinâmica populacional durante as estações do ano diferem do carrapato bovino no México, apresentando picos nos meses de Março-Maio e Junho-Agosto duas gerações por ano (ALMAZÁN *et al*., 2016). Nesse contexto, a importância do conhecimento da biologia da espécie de forma geral se faz muito importante, pois além de causar perdas a pecuária, este carrapato está envolvido com a transmissão de patógenos aos seres humanos (GONZALES-CERON *et al*., 2009; SANCHES-MONTES *et al*., 2016; RODRIGUEZ-VIVAS *et al*., 2016).

O controle químico no México é realizado de acordo com a infestação por *R*. *microplus*, não considerando a possibilidade de co-infestação por *A*. *mixtum*. Esse controle gera uma pressão de seleção artificial, podendo ocorrer o fenômeno da resistência. A resistência pode ser definida resumidamente como a capacidade de uma população de carrapatos em sobreviver e gerar descendentes mesmo perante a presença de doses comerciais ou superiores de acaricidas (SOBERANES *et al*., 2002; FERNÁNDEZ-SALAS *et al*., 2012b; ABBAS *et al*., 2016).

Métodos de diagnóstico como TIA e TPL são amplamente empregados e conhecidos para diferentes carrapatos (monóxeno ou heteróxeno) (DRUMMOND, 1981; BITTENCOURT *et al*., 1989; ALONSO-DÍAZ *et al*., 2013). Para a realização do bioensaio TIA com carrapato heteróxeno (*Amblyomma* spp.), uma limitação pode ser considerada para o bioensaio: a quantidade de teleóginas. Para tal, coelhos (*Oryctolagus cuniculus*) podem servir como hospedeiros e assim adquirir as teleóginas necessárias. A inclusão deste processo, além de aumentar o tempo total do teste imersão (que vai desde o banho in vitro, oviposição até a eclosão das larvas), fica dependente de outros fatores como taxa de recuperação, período de ingurgitamento, taxa de postura de ovos, anteriormente descritas no ciclo biológico deste carrapato (BARRADAS *et al*., 2017). Por meio dos testes supracitados, foi possível identificar a presença de resistência em *R*. *microplus* e *A*. *mixtum* que co-parasitavam bovinos para as mesmas bases químicas: amitraz e cipermetrina. Tal fato comprova a capacidade de responder a seleção artificial para ambos os carrapatos, porém apresentam dinâmica e importância na saúde diferentes.

O ciclo de vida e a diversidade de hospedeiros entre *R*. *microplus* e *A*. *mixtum*, é um fator que inflinge diretamente no controle. Pensando nessa problemática e na questão da dificuldade para obtenção de teleóginas anteriormente citada, uma adaptação do teste de pacote de larvas foi realizada: o teste de pacote de ninfas (TPN). Em amostras de carrapatos *A*. *mixtum* testadas, foi demonstrado uma concordância entre a presença ou não de resistência as bases químicas testadas nos diferentes instares. Foi encontrada uma eficácia de 100% para organosforados para larvas alimentadas, ninfas e adultos, as larvas apresentaram 86% de susceptibilidade. O princípio ativo amitraz também apresentou a presença de resistência nos três instares, sendo que larvas, larvas ingurgitadas, ninfas, ninfas ingurgitadas e adultos

sem alimentar apresentaram mortalidade de 8.6%, 12%, 7.5%, 24.4%, 0.0%, para larvas, ninfas e adultos respectivamente (dados não publicados). Os baseados no bioensaio TPN sugerem aplicabilidade do teste com ninfas, o qual ainda necessita de mais estudos futuramente, pois esta foi à primeira vez em que esta adaptação de metodologia foi testada no mundo.

Com o advento do TPN mencionado, outros carrapatos heteróxenos também podem ser testados e a realização de um controle estratégico para *Amblyomma* spp. em diferentes partes do mundo pode começar a ser traçada, uma vez que este teste, aliado ao TIA e TPL e dinâmica populacional, podem fornecer informações sobre qual base química e qual instar é mais susceptível aos acaricidas.

Apesar do avanço nas técnicas de diagnóstico, a resistência em espécies pertencentes ao complexo *Amblyomma cajennense* já pode ser considerada uma realidade (BITTENCOURT *et al*., 1989; da CUNHA *et al*., 2007; FREITAS *et al*., 2011; ALONSO-DÍAZ *et al*., 2013). Assim como no carrapato bovino, formas de controle alternativo são necessárias para auxiliar e promover o controle dos carrapatos, complementando as ferramentas já existentes e acometendo o ectoparasita de diferentes formas. A eficácia de antígenos para o controle de carrapatos monoxenos podem diminuir o uso dos produtos acaricidas, diminuindo o impacto ambiental negativo, custos e de forma indireta a transmissão de doenças (RODRÍGUEZ-VALLE *et al*., 2004).

Pesquisas desde 1990 com uso de vacinas contra carrapatos monoxenos têm sido realizadas mostrando diferentes resultados de eficácia; Andreotti *et al*. (2002) reportaram uma eficácia de 72% contra larvas de *R. microplus*. Canales *et al*. (2009) reportaram uma eficácia de 85.2% com a vacina Gavac®. No presente trabalho avaliamos um peptideo sintetico a base de aquaporin desenhado com a ferramenta de vacinologia reversa utilizando reportadas em GenBank. Para avaliação da eficácia considerando o ciclo biológico de *A. sculptum* foi utilizada a fórmula sugerida por Aguirre *et al*. 2016, obtendo uma eficacia de 68.1%.

Atualmente um trabalho por Aguirre *et al*. (2016) reportaram que o um peptideo sintético avaliado em espécies de *R. microplus* e *R. sanguineus*, mostraram uma eficácia no ciclo biológico de 37% e 45% respectivamente, considerando que o peptideo foi testado em carrapatos com ciclo de vida diferente, nosso trabalho com

metodologia similar apresentou uma eficacia de 68.1% utilizando a formula proposta por Aguirre *et al.* (2016), considerando que o peptideo foi desenvolvido utilizando outro tipo de antigeno. Finalmente, o presente trabalho apresenta resultados de baixo impacto para estabelecer estratégias que contribuiram no controle de parasitoses mistas em sistemas de produção e em animais hospedeiros das espécies *A. mixtum* e *A. sculptum* respectivamente. É importante considerar que pode-se realizar novos estudos com o intuito de melhorar a eficacia do peptídeo sintetico.

8 CONCLUSÕES

Os resultados obtidos da caracterização biológica de *A. mixtum* mostraram que coelhos e bovinos podem ser um hospedeiro para manutenção da colonia de carrapatos em condições de laboratório.

O uso de acaricidas com base no controle estratégico, utilizando intervalos de tratamento não superiores a 21 dias, como demostrado no estudo, mostrou-se eficiente em reduzir significativamente a infestação dos animais.

O Teste de Pacote de Ninfas foi utilizado pela primeira vez com sucesso. O uso de TPN pode ser utilizado durante o ano todo, para avaliar a suceptibilidade aos acaricidas de *A. mixtum* usando diferentes estádios de vida para carrapatos com ciclo de vida trioxeno. As raças sensíveis como Brangus precisam ser monitoreadas na carga parasitaria para reduzir danos na cadeia produtiva. As asociações dos acaricidas com base na cipermetrina, respondem com relaão a eficacia de forma diferente dependendo do sinergismo das bases químicas empregadas podendo comprometer a eficacia do produto.

A vacinologia reversa é uma ferramenta que contribui para desenhar peptídeos sintéticos contra carrapatos de ciclo de vida heteróxeno. O peptídeo de antígeno aquaporin utilizado produziu resposta imune de 100% nos animais vacinados e mostrou uma redução na produção de carrapatos de 68.1%.

REFERÊNCIAS

ABBAS, R. Z.; ZAMAN, M. A.; COLWELL, D. C.; GILLEARD, J.; IQBAL, Z. Acaricide resistance in cattle ticks and approaches to its management: The state of play. **Veterinary Parasitology**, v. 203, n. 1-2, p. 6–20, June 2014.

ABDU, F. A.; BEARD, C. B. Rickettsial pathogens and their arthropod vectors. **Emerging Infectious Diseases**, v. 4, n. 2, p. 179-186, Apr-June. 1998.

AGUIRRE, A. d. A. R.; GARCIA, M. V.; SZABÓ, M. P. J.; BARROS, J. C.; ANDREOTTI, R. Formula to evaluate efficacy of vaccines and systemic substances against three-host ticks. **International Journal for Parasitology**, v. 45, p. 357-359, 2015.

AGUIRRE, A. d. A. R.; LOBO, F. P.; CUNHA, R. C.; GARCIA, M. V.; ANDREOTTI, R. Design of the ATAQ peptide and its evaluation as an immunogen to develop a *Rhipicephalus* vaccine. **Veterinary Parasitology**, v. 221, p. 30, 38, May, 2016.

AGUIRRE, A. d. A. R.; LOBO, F. P.; CUNHA, R. C.; GARCIA, M. V.; ANDREOTTI, R. Biological parameters of *Amblyomma coelebs* Neumann, 1906 (Acari: Ixodidae) under experimental conditions. **Brazilian Journal Veterinary Parasitology**, v. 27. n. 1, p. 81-86, Jan-Mar, 2018.

ALI, A., PARIZI, L. F.; FERREIRA, B. R.; JUNIOR, I. DA S. V. A revision of two distinct species of Rhipicephalus: *R. microplus* and *R. australis*. **Ciência Rural**, v. 46, n. 7, p.1240-1248, 2016.

ALLAN, S. A. Tick rearing and *in vitro* feeding. In: SONENSHINE D. E., ROE, R. M. (Ed) **Biology of ticks**, 2nd edn. New York: Oxford University Press, New York, p. 445-473, 2014.

ALMAZÁN, C.; TORRES-TORRES, A.; TORRES-RODRÍGUEZ, L.; SOBERANES-CÉSPEDES, N.; ORTIZ-ESTRADA, M. Aspectos biológicos de *Amblyomma mixtum* (Koch, 1844) en el noreste de México. **Quehacer Cientifico en Chiapas**, v. 11, n. 2, p. 10-19, 2016.

ALONSO-DIAZ, M. A. A.; FERNÁNDEZ-SALAS, A.; MARTÍNEZ-IBÁÑEZ, F.; OSORIO-MIRANDA, J. *Amblyomma cajennense* (Acari: Ixodidae) tick populations susceptible or resistant to acaricides in the Mexican Tropics. **Veterinary Parasitology**, v. 197, n. 1-2, p. 326-331, 2013.

ALONSO-DÍAZ, M. A.; LÓPEZ-SILVA, B. J.; MAGALHÃES-LABARTHE, A. C. L.; RODRÍGUEZ-VIVAS, R. I. Infestación Natural de hembras de *Boophilus microplus* Canestrini, 1887 (Acari:Ixodidae) en dos genotipos de bovinos en el trópico de Veracruz, México. **Veterinaria México**, v. 38, n. 4, p. 503-509, 2007.

ALVAREZ, C. V.; BONILLA, M. R. Adultos y ninfas de la garrapata *Amblyomma cajennense* Fabricius (Acari: Ixodidae) en equinos y bovinos. **Agronomia Costarricense**, v. 31, n. 1, p. 61-69, 2007.

ALVES, J. A.; SOUZA, S. D. F.; de OLIVEIRA, H. M.; CARVALHO, J. L. N. Determinação de microhematócritos em centrifuga convencional. **Revista Brasileira de Analises Clinico**, v. 18, n. 2, p. 47-48, 1986.

ANDREOTTI, R. Performance of two Bm86 antigen vaccine formulation against tick using crossbreed bovines in stall test. **Revista Brasileira Parasitologia Veterinaria**, v. 15, n. 3, p. 97-100, 2006.

ANDREOTTI, R.; PEDROSO, M.; CAETANO, A. R.; MARTINS, N. F. Comparison of predicted binders in *Rhipicephalus (Boophilus) microplus* intestine protein variants BM86 Campo Grande Strain, BM86 and BM95. **Revista Brasileira de Parasitologia Veterinaria**, v.17, n.1, p. 93-98, 2008.

ANDREOTTI, R.; GARCÍA, M. V.; REIS, F. A.; RODRIGUES, V. S.; CAVALCANTE, J. B. Proposta de controle de carrapatos para o Brasil Central em sistemas de produção de bovinos associados ao manejo nutricional no campo.: **Embrapa Gado de Corte**, v. 34, p. 8-34, set.2016.

ANDREOTTI, R.; GOMES, A.; MALAVAZI-PIZA, K. C.; SASAKI, S. D.; CLAUDIO, A. M. SAMPAIO, C. A. M.; TANAKA A. S. BmTI antigens induce a bovine protective immune response against *Boophilus microplus* tick. **International Immunopharmacology**, v. 2, p. 557- 563, 2002.

ANDREOTTI, R.; GUERRERO, F. D.; SOARES, M. A.; BARROS, J. C.; MILLER, R. J.; PÉREZ DE LÉON, A. Acaricide resistance of *Rhipicephalus* (*Boophilus*) *microplus* in State of Mato Grosso do Sul, Brazil. **Revista Brasileira Parasitologia Veterinaria**, v. 20, p. 127-133, 2011.

BAFFI, M. A.; SOUZA, G. R. L.; SOUZA, C. S.; CERON, C. R.; BONETTI, A. M. Esterase enzymes involved in pyrethroid and organophosphate resistance in a Brazilian population of *Riphicephallus* (*Boophilus*) *microplus* (Acari, Ixodidae). **Molecular & Biochemical Parasitology**, v. 160, p. 70-73, 2008.

BARRADAS, P. F. T.; RODRIGUES, V. da S.; HIGA, L. de O. S.; GARCIA, M. V.; BARROS, J. C.; PÉREZ de LEÓN, A. A.; ANDREOTTI, R. Life cycle of *Amblyomma mixtum* (Acari:Ixodidae) parasitizing different hosts under laboratory conditions. **Experimental Applied Acarology**, v. 73. n. 2, p. 257-267, 2017.

BARROS-BATTESTI, M. D.; ARZUA, M.; BECHARA, G. H. **Carrapatos de Importância Medico-Veterinaria da Região Neotropical**: um guia ilustrado para identificação de espécies, 2006.

BASSO, L. M. de S.; MONTEIRO, A. C.; BELO, M. A. de A.; SOARES, V. E.; GARCIA, M. V.; MOCHI, D. A. Controle de larvas de *Boophilus microplus* por *Metarhizium anisopliae* em pastagens infestadas artificialmente. **Pesquisa Agropecuaria Brasileira,** v. 40, n. 6, p. 595-600, 2005.

BATTISTELLI, J. V. F.; JUNIOR, R. A. de A. T.; MENEZES, G, R. de O.; REGGIORI, M. R.; JUNIOR, M. D. S.; da SILVA L. O. C. Alternativas de cruzamento utilizando raças taurinas adaptadas ou não sobre matrizes Nelores para a produção de novilhos

precoce-Fase de cria e recria In: Simpósio Brasileiro de Melhoramento Animal, 10., Uberaba. **Anais,** Uberaba, MG, p. 10-23, 2013.

BEATI, L., NAVA, S.; BURKMAN, E. J.; BARROS-BATTESTI, D. M.; LABRUNA, M. B.; GUGLIELMONE, A. A.; CÁCERES, A. G.; GUZMÁN-CORNEJO, C. G.; LEÓN, R.; DURDEN, L. A.; FACCINI, J. L. H. *Amblyomma cajennense* (Fabricius, 1787) (Acari: Ixodidae), the Cayenne tick: phylogeography and evidence for allopatric speciation. **BMC Evolutionary Biology**, v. 13, n. 267, p. 1-20, 2013.

BECK, D. L.; OROZCO, J. P. Diurnal questing behavior of *Amblyomma mixtum* (Acari: Ixodidae). **Experimental Applied Acarology**, v. 66, n. 4, p.613-621, Aug. 2015.

BITTENCOURT, V. R. E. P.; MASSARD, C. L.; GRISI, L. Atividade *in vitro* de alguns piretróides sintéticos no carrapato *Amblyomma cajennense* (Fabricius, 1787). **Pesquisa Agropecuaria Brasil**, v. 24, p. 1193-1199, 1989.

BOUCHARD, K.; WIKEL, S. Maintenance and experimental infestation of ticks in the laboratory setting. **Biology Disease Vectors**, v. 2, p. 705-712, 2005.

BRITO, L. G.; BARBIERI, F. S.; ROCHA, R. B.; OLIVEIRA, ; RIBEIRO, E. S. Evaluation of the efficacy of acaricides used to control the cattle tick, *Rhipicephalus microplus*, in dairy herds raised in the Brazilian South western Amazon. **Veterinary Medicine International**, v. 2011, n. 2011, p. 1-6, 2011.

BROGLIO-MICHELETTI, S. M. F.; VALENTE, E. C. N.; de SOUZA, L. A.; DIAS, N. da S.; de ARAUJO, A. M. N. Extratos de plantas no controle de *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini, 1887) (Acari: Ixodidae) em laboratório. **Revista Brasileira de Parasitologia Veterinaria**, v. 18, n. 4, p. 44-48, 2009.

CANALES, M.; ALMAZÁN, C.; NARANJO, V.; JONGEJAN, F.; de la FUENTE, J. Vaccination with recombinant *Boophilus annulatus* Bm86 ortholog protein, Ba86, protects cattle against *B. annulatus* and *B. microplus* infestations. **BMC Biotechnology**, v. 9, n. 29, p. 1-8, 2009.

CASTAGNOLLI, C. K.; de FIGUEIREDO, L. B.; DANILO, A.; SANTANA, D. A.; MÁRCIO, B., de CASTRO, M. B.; ROMANO, M. A.; SZABÓ, M. P. J. Acquired resistance of horses to *Amblyomma cajennense* (Fabricius, 1787) ticks. **Veterinary Parasitology**, v. 117, n, 4, p. 271-283, 2003.

CASTRO, D. A. M.; GARCÍA, S. G. G.; DZUL-ROSADO, K.; AGUILAR, A.; CASTILLO, J.; GABSTER, A.; TREJOS, D.; ZAVALA-CASTRO, J.; BERMÚDEZ, C. S. E. Questing *Amblyomma mixtum* and *Haemaphysalis juxtakochi* (Acari: Ixodidae) infected with candidatus ‘‘*Rickettsia amblyommii*’’ from the natural environment in Panama Canal Basin, Panama. **Tropical Medicine Health**, v. 43, n. 4, p. 217-222, 2015.

COOLEY, R. A.; KOHLS, G. M. The genus *Amblyomma* (Ixodidae) in the United States. **Journal Parasitology**, v. 30, n. 2, p. 77-111, Apr.1944.

CORRÊA, R. R.; LOPES, W. D. Z.; TEIXEIRA, W. F. P.; CRUZ, B. C.; GOMES, L. V. C.; FELIPPELLI, G.; MACIEL. W. G.; FÁVERO, F. C.; BUZZULINI, C.; BICHUETTE,

M. A.; SOARES, V. E.; COSTA, A, J. A comparison of three different methodologies for evaluating *Rhipicephalus* (*Boophilus*) *microplus* susceptibility to topical spray compounds. **Veterinary Parasitology**, v. 207, n. 1-2, p. 115-124, Jan. 2015.

CRUZ, B. C.; LOPES, W. D.; MACIEL, W. G.; FELIPPELLI, G.; FÁVERO, F. C.; TEIXEIRA, W. F.; CARVALHO, R. S.; RUIVO, M. A.; COLLI, M. H.; SAKAMOTO, C. A.; da COSTA, A. J.; de OLIVEIRA, G. P. Susceptibility of *Rhipicephalus* (*Boophilus*) *microplus* to ivermectin (200, 500 and 630 µg/kg) in field studies in Brazil. **Veterinary Parasitology**, v. 207, n. 3-4, p. 309-317, Jan. 2015.

Da CUNHA, A. P.; BELLO, A. C. P. de P.; LEITE, R. C.; BASTIANETTO, E.; RIBEIRO, A. C. C. L.; de FREITAS, C. M. V.; OLIVEIRA, P. R. Controle estratégico de *Amblyomma cajennense* (Fabricius, 1787) (Acari: Ixodidae) em eqüinos, Minas Gerais, Brasil - Parte I; **Revista Brasileira de Parasitologia Veterinária**, v. 16, n. 4, p. 221-228, 2007.

De La FUENTE, J.; ALMAZÁN, C.; CANALES, M.; PÉREZ De La LASTRA, J. M.; KOCAN, K. M.; WILLADSEN, P. A ten-year review of commercial vaccine performance for control of tick infestations on cattle. **Animal Health Research Reviews**, v. 8, n.1, 23-28, 2007.

De La FUENTE, J.; RODRÍGUEZ, M.; REDONDO, M.; MONTERO, C.; GARCÍA-GARCÍA, J. C.; MÉNDEZ, L.; SERRANO, E.; VALDÉS, M.; ENRIQUEZ, A.; CANALES, M., RAMOS, E.; BOUÉ, O.; MACHADO, H.; LLEONART, R.; de ARMAS, C. A.; REY, S., RODRÍGUEZ, J. L.; ARTILES, M.; GARCÍA, L. Field studies and cost-effectiveness analysis of vaccination with Gavac™ against the cattle tick *Boophilus microplus*. **Vaccine**, v. 16, n. 4, p. 366-373. Feb. 1998.

De La FUENTE, J.; KOCAN, K. M. Strategies for development of vaccines for control of ixodid tick species. **Parasite Immunology**, v. 28, n. 7, p. 275-283, 2006.

De La FUENTE, J.; KOCAN, K. M.; CONTRERAS, M. Prevention and control strategies for ticks and pathogen transmission. **Review Science Technical Of International Epizotic**, v.34, n.1, p. 249-264, 2015.

De MENEGHI, D.; STACHURSKI, F.; ADAKA, H. Experiences in tick control by acaricide in the traditional cattle sector in Zambia and Burkina Faso: possible environmental and public health implications. **Frontiers in Public Health**, v. 4, n. 239, p. 1-11, Nov. 2016.

De VOS, S.; ZEINSTRA, L.; TAOUFIK, O.; WILLADSEN, P.; JONGEJAN, F.; Evidence for the utility of the Bm86 antigen from *Boophilus microplus* in vaccination against other tick species. **Experimental and Applied Acarology**, v. 25, n. 3, p. 245-261, 2001.

DOMÍNGUEZ-GARCÍA, D. I.; ROSARIO-CRUZ, R.; ALMAZÁN-GARCÍA, C.; OAXACA, J. A. S.; De La FUENTE, J. *Boophilus microplus:* Aspectos biológicos y moleculares de la resistencia a los acaricidas y su impacto en la salud animal. **Tropical and Subtropical Agroecosystems**, v.12, n. 2, p. 181-192, Mayo-Ago. 2010.

DRUMMOND, R. O.; ERNST, S. E.; TREVlNO, J. L.; GLADNEY, W. J.; GRAHA, O. H. *Boophilus annulatus* and *Boophilus microplus*: laboratory tests of insecticides. **Journal Economy Entomology**, v. 66, n. 1, p. 130-133, Feb. 1973.

DRUMMOND, R. O.; WHETSTONE, T. M. Oviposition of the cayenne tick, *Amblyomma cajennense* (F.), in the laboratory. **Annual of the Entomology Society America**, v. 68, n. 2, p. 214-216, 1975.

DRUMMOND, R. O. Susceptibility of the Cayenne Tick[1] to Acaricides, **Journal of Entomology Economic**, v. 74, n. 4, p. 469-472, 1981.

ECHAIDE, S. T.; KNOWLES, D. P.; MCGUIRE, T. C.; PALMER, G. H.; SUAREZ, C. E., MCELWAIN, T. F. Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a competitive enzyme-linked immuno asorbent assay using recombinant major surface protein 5. **Journal Clinical Microbiology**, v. 36, n. 3, p. 777-782, 1998.

ESTRADA-PEÑA, A.; TARRAGONA, E. L.; VESCO, U.; de MENEGHI, D.; MASTROPAOLO, M.; MANGOLD, A, J.; GUGLIELMONE, A. A.; NAVA, S. Divergent environmental preferences and areas of sympatry of tick species in the *Amblyomma cajennense* complex (Ixodidae). **International Journal Parasitology**, v. 44, n. 14, p.1081-1809, Sep. 2014.

FACCINI, J. L. H.; CARDOSO, A. C. B.; ONOFRIO, V. V. C.; LABRUNA, M. B.; BARROS-BATTESTI, D. M. The life cycle of *Amblyomma auricularium* (Acari: Ixodidae) using rabbits (*Oryctolagus cuniculus*) as experimental host. **Experimental Applied Acarology**, v. 50, v. 1, p. 71-77, Jun. 2010.

FERNÁNDEZ-SALAS, A. R. I.; RODRÍGUEZ-VIVAS, R. I.; ALONSO-DÍAZ, M. A.; BASURTO-CAMBEROS, H. Resistance of *Rhipicephalus microplus* to amitraz and cypermethrin in tropical cattle farms in Veracruz, Mexico. **Journal Parasitology**, v. 98, .n 5, p. 1010-1014, Apr. 2012a.

FERNANDEZ-SALAS, A.; RODRIGUEZ-VIVAS, R. I.; ALONSO-DIAZ, M. A. First report of a *Rhipicephalus microplus* tick population multi-resistant to acaricides and ivermectin in the Mexican tropics. **Veterinary Parasitology**, v. 183, p. 338-342. 2012b.

FLUMIGNAN, D. L.; FIETZ, C. R.; COMUNELLO, E. **O clima na região do Bolsão de Mato Grosso do Sul**. 1 Ed. Embrapa Agropecuária do Oeste, 2015.

FOOD and AGRICULTURE ORGANIZATION of the UNITED NATIONS – FAO. **Resistance management and integrated parasite control in ruminants**: guidelines. Module 1. Ticks: acaricide resistance: diagnosis, management and prevention [online]. p. 25-77, 2004.

FRAGOSO, H.; HOSHMAN RAD, P.; ORTIZ, M.; RODRIGUEZ, M.; REDONDO, M.; HERRERA, L.; De La FUENTE, J. Protection against *Boophilus annulatus* infestations in cattle vaccinated with the *B. microplus* Bm86-containing vaccine Gavac. **Vaccine**, v. 16, v. 20, p.1990-1992, 1998.

FREITAS, S. E. P.; ZAPATA, M. T.; FERNANDES, F. de F. Monitoring of resistance or susceptibility of adults and larvae of *Amblyomma cajennense* (*Acari: Ixodidae*) o synthetic acaricides in Goiás, Brazil. **Experimental Applied Acarology**, v. 53, n. 2, 11, p. 189-202, Feb. 2011.

GAUSS, B. L. C.; FURLONG, J. Comportamento de larvas infestantes de *Boophilus microplus* em pastagem de *Brachiaria decumbens*. **Ciência Rural Santa Maria**, v. 32, n.3, p. 467-472, 2002.

GARCIA, M. V.; MONTEIRO, A. C.; SZABÓ, M. P.; MOCHI, D. A.; SIMI, L. D.; CARVALHO, W. M.; TSURUTA, S. A.; BARBOSA, J. C. Effect of *Metarhizium anisopliae* fungus on off host *Rhipicephalus* (*Boophilus*) *microplus* from tick-infested pasture under cattle grazing in Brazil. **Veterinary Parasitology**, v. 181, n. 2-4, p. 10-1016, Sep. 2011.

GARCIA, M. V.; ANDREOTTI, R.; REIS, F. A.; AGUIRRE, A. de A. R.; BARROS, J. C.; MATIAS, J.; KOLLER, W. W. Contributions of the hair sheep breed Santa Ines as a maintenance host for *Rhipicephalus* (*Boophilus*) *microplus* (Acari:Ixodidae) in Brazil. **Parasites and Vectors**, v. 7, n. 515, p.1-4, 2014.

GASTEIGER, E.; GASTEIGER, E.; HOOGLAND, C.; GATTIKER, A.; DUVAUD, S.; WILKINS, M. R.; APPEL, R. D.; BAIROCH, A. Protein identification and analysis tools on the ExPASy Server: John M. Walker (ed): **The Proteomics Protocols Handbook,** Totowa, NJ: Humana Pres, p. 571-607, 2005.

GARCÍA-GARCÍA, A. C. J.; CARLOS, M.; REDONDO, M.; VARGAS, M.; CANALES, M.; BOUÉ, O.; RODRÍGUEZ, M.; JOGLAR, M.; MACHADO, H. I.; GONZÁLEZ, L.; VALDÉS, M.; MÉNDEZ, L.; De La FUENTE, J. A. Control of ticks resistant to mmunization with Bm86 in cattle vaccinated with the recombinant antigen Bm95 isolated from the cattle tick, *Boophilus microplus*. **Vaccine**, v.18, p. 2275-2287, 2000.

GenBank accesion. (KF200160), *Amblyomma cajennense* voucher CAS-T071 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial, (KF200097) *Amblyomma cajennense* voucher CAS-T070 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial, (KF200124.1,), *Amblyomma cajennense* voucher CAS-T073 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial, (KF20097).

GEORGE, J. E.; POUND, J. M.; DAVE, R. B. Chemical control of ticks on cattle and the resistance of these parasites to acaricides. **Parasitology**, v. 129, p. 353-366, 2004.

GERARDI, M.; MARTINS, M. M.; NAVA, S.; SZABÓ, M. P. J. Comparing feeding and reproductive parameters of *Amblyomma parvum* tick populations (Acari: Ixodidae) from Brazil and Argentina on various host species. **Veterinary Parasitology**, v. 197, n. 1-2, p. 312-317, Oct. 2013.

GOMES, A.; KOLLER, W. W.; BARROS, A. T. M. Susceptibilidade de *Rhipicephalus* (*Boophilus*) *microplus* a carrapaticidas em Mato Grosso do Sul, Brasil. **Ciencia Rural**, v. 41, p. 1447-1452, 2011.

GOMES, A.; HONER, M. R.; SCHENK, M. A.; CURVO, J. B. Populations of the cattle tick (*Boophilus microplus*) on purebred Nellore, Ibage and Nellore X European crossbreds in the Brazilian savanna. **Tropical Animal Health Production**, v. 21, n. 1, p. 20-24, Feb. 1989.

GONÇALVES, P. M. Epidemiologia e controle da Tristeza Parasitária Bovina na região Sudeste do Brasil. **Ciencia Rural**, v. 30, p. 187-194, 2000.

GONÇALVES, R. C.; da SILVA, A. A.; FERREIRA, D. O. L.; CHIACCHIO, S. B.; LOPES, R. S.; BORGES, A. S.; AMORIM, R. M. Tick fever in cattle in the region of Botucatu-SP: A retrospective study of 1986-2007. **Seminario: Ciencias Agrarias**, v. 32, n. 1, p. 307-312, 2011.

GONZÁLEZ-CERÓN, F.; BECERRIL-PÉREZ, C. M.; TORRES-HERNÁNDEZ, G.; DÍAZ-RIVERA, P. Garrapatas que infestan regiones corporales del bovino criollo lechero tropical en Veracruz, México. **Agrociencia**, v. 43, n. 1, p. 11-19, 2009.

GRAF, J. F.; GOGOLEWSKI, R.; LEACH-BING, N.; SABATINI, G. A.; M. B. MOLENTO, M. B.; BORDIN, E. L.; ARANTES, G. J. Tick control: an industry point of view. **Parasitology**, v. 129, n. 1, p. 427-442, 2004.

GRISI, L.; MARTINS, J. R. de S.; de BARROS, A. T. M.; ANDREOTTI, R.; CANÇADO, P. H. D.; PÉREZ De LEÓN, A. A.; PEREIRA, J. B.; VILLELA, H. S. Reassessment of the potential economic impact of cattle parasites in Brazil. **Revista Brasileira Parasitolgia Veterinaria**, v. 23, n. 2, p. 150-156, 2014.

GUEDES, E.; LEITE, R. C.; PRATA, M. C. A.; PACHECO, R. C.; WALKER, D. H.; LABRUNA, M. B. Detection of *Rickettsia rickettsii* in the tick *Amblyomma cajennens;* in a new Brazilian spotted fever-endemic area in the state of Minas Gerais. **Memórias do Instituto Oswaldo Cruz**, v. 100. n. 8, p. 841-845, 2005.

GUERRERO, F. D.; ANDREOTTI, R.; BENDELE, K. G.; CUNHA, R. C.; MILLER, R. J.; YEATER, K.; PÉREZ De LEÓN, A. A. *Rhipicephalus* (*Boophilus*) *microplus* aquaporin as an effective vaccine antigen to protect against cattle tick infestations. **Parasites and vectors**, v. 7, n. 475, p. 1-12, Oct. 2014.

GUERRERO, F. D.; BENDELE, K. G.; DAVEY, R. B.; GEORGE, J. E. Detection of *Babesia bigemina* infection in strains of *Rhipicephalus* (*Boophilus*) *microplus* collected from outbreaks in south Texas. **Veterinary Parasitology**, v. 145, p. 156-163, 2007.

GUERRERO, F.; LI, A. Y.; HERNÁNDEZ, R. H. Molecular Diagnosis of Pyrethroid Resistance in Mexican Strains of *Boophilus microplus* (Acari: Ixodidae). **Journal of Medicine Entomology**, v. 39, n. 5, p. 770-776, 2002.

GUGLIELMONE, A. A.; MANGOLD, A. J.; GARCIA, M. D. The life cycle of *Amblyomma parvum* Aragão, 1908 (Acari: Ixodidae) under laboratory conditions. **Experimental & Applied Acarology**, v. 13, n. 2, p. 129-136, 1991.

GUGLIELMONE, A. A.; NAVA, S. Las garrapatas argentinas del genero *Amblyomma* (Acari: Ixodidae): Distribucion y hospedadores. **RIA**, v. 35, n. 3, p. 133-153, 2006.

GUZMAN-CORNEJO, C.; ROBBINS, R. G.; GUGLIELMONE, A. A.; MONTIEL-PARRA, G.; PÉREZ, T. M. The *Amblyomma* (Acari: Ixodida: Ixodidae) of Mexico: Identification keys, distribution and hosts. **Zootaxa**, v. 2998, p. 16-38, 2011.

HALL, T. A. BioEdit: A user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series. 41, 95-98, 1999.

HERNÁNDEZ, K.; ARECE, J.; SIMÓN, L.; HERNÁNDEZ, L.; VALDÉS, O. Comportamiento de ixódidos en diferentes genotipos de ganado mayor en silvopastoreo (Nota Técnica). **Pastos y Forrajes**, v. 36, n. 1, 72-76, Marzo. 2013.

HIGA, L. De O. S.; GARCIA, M. V.; BARROS, J. C.; KOLLER, W. W.; ANDREOTTI, R. Acaricide resistance status of the *Rhipicephalus microplus* in Brazil: a literature overview. **Medicine Chemistry**, v. 5, p. 326-333, 2015.

HIGA, L.O.S.; GARCIA, M. V.; BARROS, C. J.; KOLLER, W. W.; ANDREOTTI, R. Evaluation of *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) resistance to different acaricide formulations using samples from Brazilian properties. **Brazilian Journal Veterinary Parasitology**, v. 25, p. 163-171, 2016.

HOOKER, W. A.; BISHOPP, F. C.; WOOD, H. P. The life history and bionomics of some North American ticks. **Bureau of Entomology Bulletin**, v. 7, n. 106, p. 72-87, 1912.

IBGE. Instituto Brasileiro de Geografia e Estatística. **Mapa de Clima do Brasil**, 2002.

JONSSON, N. N. The productivity effects of cattle tick (*Boophilus microplus*) infestation on cattle, with particular reference to *Bos indicus* cattle and their crosses. **Veterinary Parasitology**, v. 137, n. 1-2, p. 1-10, 2006.

JONSSON, N. N.; MATSCHOSS, A. L.; PEPPER, P.; GREEN, P. E.; ALBRECHT, M. S., HUNGERFORD, J.; ANSELL, J. Evaluation of TickGARD plus, a novel vaccine against *Boophilus microplus*, in lactating Holstein-Friesian cows. **Veterinary Parasitology**, v. 88, n. 3-4, p. 275-285, 2000.

KOCH, C. L. Systematische Ubersicht uber die ordnung der zecken. **Arch Naturgeschichte,** v. 10, p. 217-239, 1844.

KLAFKE, G.; WEBSTER, A.; AGNOL, B. D.; PRADEL, E.; SILVA, J.; De La CANAL, L. H.; BECKER, M.; OSÓRIO, M. F.; MANSSON, M.; BARRETO, R.; SCHEFFER, R.; SOUZA, U. A.; BAMBERG, V.; Dos SANTOS, C. J.; RECK, J., MARTINS, J. R. Multiple resistance to acaricides in field populations of *Rhipicephalus microplus* from Rio Grande do Sul State, Southern Brazil. **Ticks Tick Borne Disease**, v. 8, n. 1, p. 73-80, 2017.

KLAFKE, M. G.; CASTRO-JANER, E.; MENDES, M. C.; NAMINDOME, A.; SCHUMAKER, T. T. S. Applicability of *in vitro* bioassays for the diagnosis of ivermectin resistance in *Rhipicephalus microplus* (Acari: Ixodidae). **Veterinary Parasitology**, v. 184, p. 212-220, 2012.

KLAFKE, M. G.; GUSTAVO, A.; SABATINI, G. A.; THAIS, A.; De ALBUQUERQUE, T. A.; MARTINS, J. R.; DAVID, H.; KEMP, D. H.; MILLER, R. J.; SCHUMAKER, T. T. S. Larval immersion tests with ivermectin in populations of the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) from State of Sao Paulo, Brazil. **Veterinary Parasitology**, v. 142, n. 3-4, p. 386-390, 2006.

KRAWCZAK, F. S.; MARTINS, T. F.; OLIVEIRA, C. S.; BINDER, L. C.; COSTA, F. B.; NUNES, P. H.; GREGORI, F.; LABRUNA B. M. *Amblyomma yucumense* n. sp. (Acari: Ixodidae), a parasite of wilds mammals in southern Brazil. **Journal of Medical Entomology**, v. 1 n. 3, p. 28-37, 2015.

KUNZ, S. E.; KEMP, D. H., Insecticides and acaricides: resistance and environmental impact. **Science Technical Office International Epizotology**, v. 1, n. 3, p. 1249-1286, 1994.

LABRUNA, M. B.; AMAKU, M.; METZNER, J. A.; PINTER, A.; FERREIRA, F. Larval behavioral diapause regulates life cycle of *Amblyomma cajennense* (Acari: Ixodidae) in Southeast Brazil. **Journal of Medical Entomology**, v. 40, n. 2, p.170-178, 2003.

LABRUNA, M. B.; TERASSINI, F. A.; CAMARGO, L. M. A. Notes on population dynamics of Amblyomma ticks (Acari: Ixodidae) in Brazil. **Journal Parasitology,** v. 95, p.1016-1018, 2009.

LABRUNA, M. B.; NAVA, S.; MARCILI, A.; BARBIERI, A. R. M.; NUNES, P. H.; HORTA, M. C.; VENZAL, J. M. A new argasid tick species (Acari: Argasidae) associated with the rock cavy, *Kerodon rupestris Wied-Neuwied* (Rodentia: Caviidae), in a semiarid region of Brazil. **Parasites & Vectors**, v. 9, n. 511, p. 1-15, 2016.

LABRUNA, M. B.; PINTER, A.; TEIXEIRA, R. H. F. Life cycle of *Amblyomma cooperi* (Acari: Ixodidae) using capybaras (*Hydrochoerus hydrochaeris*) as hosts. **Experimental Applied Acarology**, v. 32, n. 1-2, p. 79-88, 2004.

LAMBERTZ, Ch.; CHONGKASIKIT, N.; JITTAPALAPONG, S.; GAULY, M. Immune response of *Bos indicus* cattle against the Anti-tick antigen Bm91 derived fromLocal *Rhipicephalus* (*Boophilus*) *microplus* ticks and its effect on tick reproduction under natural infestation. **Journal of Parasitology Research**, v. 2012, p. 1-9, 2012.

LI, X.; MARY, A.; SCHULER, A.M.; BERENBAUM, R.M. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. **Annuals Review Entomology**, v. 52, p. 231-53, 2007.

LISBOA, C. M. L.; LEITE, R. C.; LABRUNA, M. B.; De OLIVEIRA, P. R.; BORGES, L. M. F., RODRIGUES, Z. B.; de CARVALHO, H. A.; De FREITAS, C. M. V.; JÚNIOR, C. R. V. Host specificity of *Amblyomma cajennense* (Fabricius, 1787) (Acari: Ixodidae) with comments on the drop-off rhythm. **Memorias Instituto Oswaldo Cruz**, v. 93, n. 3, p. 347-351, 1998.

LÓPEZ-DOMÍNGUEZ, A.; PASANTES H. Acuaporinas y edema cerebral. **Revista Eclésiastica Brasileira**, v. 28. n. 4 p. 132-140, 2009.

MADRUGA, C. R.; HONNER, M. R.; SCHENK, M. A. M.; CURVO, J. B. E. **Avaliação preliminar de parâmetros epidemiológicos da tristeza parasitária bovina no Mato Grosso do Sul**. Embrapa Gado de Corte, n. 38. 1987.

MARTINS, T. F.; OLIVEIRA, C. S.; BINDER, L. C.; COSTA, F. B.; NUNES, P. H.; GREGORI, F.; LABRUNA M. B. *Amblyomma yucumense* n. sp. (Acari: Ixodidae), a parasite of wild Mammals in Southern Brazil. **Journal of Medical Entomology**, v 52, n 1, p. 28-37, 2015.

MARITZ-OLIVIER, C.; ZYL, W. V.; STUTZER, Ch. A systematic, functional genomics, and reverse vaccinology approach to the identification of vaccine candidates in the cattle tick, *Rhipicephalus microplus*. **Ticks and Tick-borne Diseases**, v, 3, n. 2012, p. 179-187, 2012.

MEDINA-SÁNCHEZ, A.; DONALD, H.; BOUYER, D. H.; ALCANTARA-RODRIGUEZ, V. A.; MAFRA, C.; ZAVALA-CASTRO, J.; WHITWORTH, T.; POPOV, V. L.; FERNANDEZ-SALAS, I.; WALKER, D. H. Detection of a Typhus Group *Rickettsia* in *Amblyomma* Ticks in the State of Nuevo Leon, Mexico. **Annais of the New York Academic of Science**, v. 1063, p. 327-332, 2005.

MEKONNEN, S.; BRYSON, N. R.; FOURIE, L. J.; PETER, R. J.; SPICKETP, A. M.; TAYLOR, R. J.; STRYDOM, T.; HORAK, I. G. Acaricide resistance profiles of single- and multi-host ticks from communal and commercial farming areas in the Eastern Cape and North-West Provinces of South Africa. **Onderstepoort Journal of Veteterinary Research**. v. 69, n. 2, p. 99-105, June. 2002.

MENDES, M. C.; DUARTE, F. C.; MARTINS, J. R.; KLAFKE, G. M.; FIORINI, L. C.; De BARROS, A. T. M. Characterization of the pyrethroid resistance profile of *Rhipicephalus* (*Boophilus*) *microplus* populations from the states of Rio Grande do Sul and Mato Grosso do Sul, Brazil. **Revista Brasileira Parasitologia Veterinaria**, v. 22, n. 3, p. 379-384, 2013.

MENG, H.; XUA, S.; YUA, Z.; LIU, Z.; YANG, X.; LIU J. The life cycle and occurrence of *Haemaphysalis concinna* (Acari: Ixodidae) under field conditions. **Ticks and Ticks Borne Disease**, v. 5, n. 6, p. 887-891, 2014.

MILLER, J. R.; ALMAZÁN, C.; ORTÍZ-ESTRADA, M.; DAVEY, R. B.; GEORGE, J. E.; PÉREZ De LEÓN A. First report of fipronil resistance in *Rhipicephalus (Boophilus) microplus* of Mexico. **Veterinary Parasitology**, v. 191, n. 2, p. 97-101, 2013.

MILLER, J. R.; DAVEY, R. B.; GEORGE, J. E. Modification of the Food and Agriculture Organization Larval Packet Test to measure amitraz-susceptibility against Ixodidae. **Journal of Medicine Entomology**, v. 39, n. 4, p. 645-651, 2002.

MILLER, R. J.; DAVEY, R. B.; GEORGE, J. E. Characterization of pyrethroid resistance and susceptibility to coumaphos in Mexican *Boophilus microplus* (Acari: Ixodidae). **Journal of Medical Entomology**, v. 36, p. 533-538, 1999.

MONTEIRO, C. M. de O.; RODRIGUES, A. F. S. F.; GUEDES, E. Aspectos gerais da febre maculosa brasileira. **Centro de Ensino Superior Revista**, p. 133-147, 2006.

MUÑOZ-LEAL, S.; TOLEDO, L. F.; VENZAL, J. M.; MARCILI, A.; MARTINS, T. F.; ACOSTA, I. C. L.; PINTER, A.; LABRUNA, M, L. Description of a new soft tick species (Acari: Argasidae: Ornithodoros) associated with stream-breeding frogs (Anura: *Cycloramphidae*: *Cycloramphus*) in Brazil. **Ticks and Tick-borne Diseases**, v. 8, n. 5, p. 682–692, 2017.

NATALA, A. J.; AGYEI, A. D.; AWUMBILA, B. Susceptibility of *Amblyomma variegatum* ticks to acaricides in Ghana. **Experimental and Applied Acarology**, v. 3, p.n. 3, 259-268, 2005.

NAVA, S, BEATI, L.; LABRUNA, M. B.; CÁCERES, A. G.; MANGOLD, A. J.; GUGLIELMONE, A. A. Reassessment of the taxonomic status of *Amblyomma cajennense* (Fabricius, 1787) with the description of three new species, *Amblyomma tonelliae* n. sp., *Amblyomma interandinum* n. sp. and *Amblyomma patinoi* n. sp., and reinstatement of *Amblyomma mixtum* Koch, 1844, and *Amblyomma sculptum* Berlese, 1888 (Ixodida: Ixodidae). **Ticks and Tick-borne Disease**, v. 5, n. 3, p. 252-276, Apr. 2014.

NAVA, S.; GUGLIELMONE, A. A.; MANGOLD, A. J. An overview of systematics and evolution of ticks. **Frontiers Bioscience**, v. 1, n.14, p. 2857-2877, 2009.

NUTTAL, P. A.; TRIMNELL, A. R.; KAZIMIROVA, M.; LABUDA, M. Exposed and concealed antigens as vaccine targets for controlling ticks and tick-borne diseases. **Parasite Immunology**, v. 28, n. 4, p. 155-163, 2006.

OLEGARIO, M. M. M.; GERARDI, M.; TSURUTA, S. A.; SZABÓ, M. P. J. Life cycle of the tick *Amblyomma parvum* Aragão, 1908 (Acari: Ixodidae) and suitability of domestic hosts under laboratory conditions. **Veterinary Parasitology**, v. 179, p. 203-208, 2011.

OLIVEIRA, P. R.; BORGES, L. M.; LEITE, R. C.; FREITAS, C. M. Seasonal dynamics of the Cayenne tick, *Amblyomma cajennense* on horses in Brazil. **Medical and Veterinary Entomology**, v. 17, n. 4, 412-416, 2003.

OLIVEIRA, P. R.; BORGES, L. M.; LOPES, C. M.; LEITE, R. C. Population dynamics of the free-living stages of *Amblyomma cajennense* (Fabricius, 1787) (Acari: Ixodidae) on pastures of Pedro Leopoldo, Minas Gerais State, Brazil. **Veterinary Parasitology**, v. 92, n. 4, p. 295-301, 2000.

OLIVO, C. J.; CARVALHO, N. M.; da SILVA, J. H. S.; VOGE, F. F.; MASSARIO, P.; MEINERZ, G.; AGNOLI, C.; MOREL, F. C.; VIAU, L. V. Óleo de citronela no controle do carrapato de bovinos. **Ciência Rural**, v. 38, n. 2, p. 406-410, 2008.

ORTIZ, E. M.; SANTAMARÍA, V. M.; ORTIZ, N. A.; SOBERANES, C. N.; OSORIO, M. J., FRANCO, B. R.; MARTÍNEZ, I. F.; QUEZADA, D. R.; FRAGOSO, S. H. Caracterización de la resistencia de *Boophilus microplus* a ixodicidas en México. In: Memorias de IV seminario internacional de parasitología animal, resistencia y control en garrapatas y moscas de importancia veterinaria. Acapulco, Guerrero, México. p. 58-66, 1995.

PARIZI, L. F.; RECK, J. J. R.; OLDIGES, D. P.; GUIZZO, M. G.; SEIXAS, A.; LOGULLO, C.; De OLIVEIRA, P. L.; TERMIGNONI, C.; MARTINS, J. R.; VAZ, I. S. J. R. Multi-antigenic vaccine against the cattle tick *Rhipicephalus* (*Boophilus*) *microplus*: a field evaluation. **Vaccine**, v. 30, n. 48, p, 6912– 6917, 2012.

PAROLA, P.; PADDOCK, C. D.; RAOULT, D. Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. **Clinical Microbiolgy Reviews**, v. 18, p. n. 4, 719-756, Oct. 2005.

PATARROYO, J. H.; PORTELA, R. W.; De CASTRO, R. O.; PIMENTEL, J. C.; GUZMAN, F.; PATARROYO, M. E.; VARGAS, M. I.; PRATES, A. A.; MENDES, M. A. D. Immunization of cattle with synthetic peptides derived from the *Boophilus microplus* gut protein (Bm86). **Veterinary Immunology and Immunopathology**, V. 88, P. 163-172, 2002.

PEREZ-COGOLLO, L. C.; RODRIGUEZ-VIVAS, R. I.; RAMIREZ-CRUZ, G. T.; ROSADO-AGUILAR, J. A. Survey of *Rhipicephalus microplus* resistance to ivermectin at cattle farms with history of macrocyclic lactones use in Yucatan, Mexico. **Veterinary Parasitology**, v. 172, n. 1-2, p. 109-113, Aug. 2012.

PINNA, H. M. Incidência e distribuição de *Amblyomma cajennense* em regiões corporais de eqüinos das raças Mangalarga Marchador e Bretão Postier, naturalmente infestados. **Parasitology Latinoamericana**, v. 59, n. 1-2, p. 21-25, Ene. 2004.

PINTER, A.; LABRUNA, B. M. Isolation of *Rickettsia rickettsii* and *Rickettsia bellii* in Cell Culture from the Tick *Amblyomma aureolatum* in Brazil. **Annais of the New York Academic of Science**, v. 1078, p. 523-530, 2006.

PIRES, M. S.; dos SANTOS, T. M.; SANTOS, H. A.; VILELA, J. A. R.; PEIXOTO, P. M.; ROIER, E. C. R.; Da SILVA, C. B.; BARREIRA, J. D.; De LEMOS, E. R. S.; MASSARD, C. L. *Amblyomma cajennense* infestation on horses in two microregions of the state of Rio de Janeiro, Brazil. **Revista Brasileira de Parasitologia Veterinaria**, v. 22, n. 2, p. 235-242, 2013.

PRATA, A. M. C.; ALONSO, L. Da S.; SANAVRIA, A. Parâmetros biológicos do estagio ninfal de *Amblyomma cajennense* (Fabricius, 1787) (Acari: Ixodidae) em coelhos. **Revista Brasileira Ciência Veterinaria**, v. 3, n. 2, p. 55-57, Maio-Ago. 1996.

RAMOS do N. V.; PIOVEZAN, U.; FRANCO, A. H. A.; OSAVA, C. F.; HERRERA, H. M.; SZABÓ, M. P. J. Feral pigs as hosts for *Amblyomma sculptum* (Acari:Ixodidae) populations in the Pantanal, Mato Grosso do Sul, Brazil. **Experimental Applied Acarology**, v. 64, n. 3, p. 393-406, 2014.

RAMOS, V. N.; PIOVEZAN, U.; FRANCO, A. H.; RODRIGUES, V. S.; NAVA, S.; SZABÓ, M. P. Nellore cattle (*Bos indicus*) and ticks within the Brazilian Pantanal: ecological relationships. **Experimental Applied Acarology**, v. 68, n. 2, p. 227-240, Feb. 2016.

RECK, J.; KLAFKE, G. M.; WEBSTER, A.; DALL'AGNOL, B.; SCHEFFER, R.; SOUZA, U. A.; CORASSINI, V. B.; VARGAS, R.; dos SANTOS, J. S.; MARTINS, J. R. First

report of fluazuron resistance in *Rhipicephalus microplus*: a field tick population resistant to six classes of acaricides. **Veterinary Parasitology**, v. 201, n. 1-2, p. 128-136, Mar. 2014a.

RECK, J.; MARKS, F. S.; RODRIGUES, R. O.; SOUZA, U. A.; WEBSTER, A.; LEITE, R. C.; GONZALES, J. C.; KLAFKE, G. M.; MARTINS, J. R. Does *Rhipicephalus microplus* tick infestation increase the risk for myiasis caused by *Cochliomyia hominivorax* in cattle? **Preventive Veterinary Medicine**, v. 113, n. 1, p. 59-62, Jan. 2014.

REDONDO, M.; FRAGOSO, H.; ORTÍZ, M.; MONTERO, C.; LONA. J.; MEDELLÍN. J. A.; FRÍA, R.; HERNÁNDEZ, V.; FRANCO, R.; MACHADO, H.; RODRÍGUEZ, M.; De La FUENTE, J. Integrated control of acaricide-resistant *Boophilus microplus* populations on grazing cattle in Mexico using vaccination with Gavac™ and amidine treatments. **Experimental and Applied Acarology**, v. 23, p. 841-849, 1999.

RODRIGUEZ, M.; PENICHET, M. L.; MOURIS, A. E.; LABARTA, V.; LUACES, L.; RUBIERA, R.; CORDOVÁ, C.; SÁNCHEZ, P. A.; RAMOS, E.; SOTO, A.; CANALES, M.; PALENZUELA, D.; TRIGUERO, A.; LLEONART, R.; HERRERA, L.; De La FUENTE, J. Control of *Boophilus microplus* populations in grazing cattle vaccinated with a recombinant Bm86 antigen preparation. **Veterinary Parasitology**, v. 57, p. 339-349, 1995.

RODRIGUEZ-VALLE, M.; MOOLHUIJZEN, P.; PIPER, E. K.; WEISS, O.; VANCE. M.; BELLGARD, M.; LEW-TABOR, A. *Rhipicephalus microplus* lipocalins (LRMs): Genomic identification and analysis of the bovine immune response using *in silico* predicted B and T cell epitopes. **International Journal for Parasitology**, v. 43, p. 739-752, 2013.

RODRIGUES, V. S.; GARCIA, M, V.; CRUZ, B. C.; MACIEL, W. G.; ZIMMERMANN, N. P.; KOLLER, W. W.; BARROS, J. C.; ANDREOTTI, R. Life cycle and parasitic competence of *Dermacentor nitens* Neumann, 1897 (Acari: Ixodidae) on different animal species. **Ticks and Tick-borne Disease**, v. 8, n. 3, p. 379-384, Mar. 2018.

RODRIGUEZ, V. E.; MÉNDEZ, L.; VALDEZ, M.; REDONDO, M.; ESPINOSA, C. M.; VARGAS, M.; CRUZ, R. L. L.; BARRIOS, H. P.; SEOANE, G.; RAMIREZ, E. S.; BOUE, O.; VIGIL, J. L.; MACHADO, H.; NORDELO, C. B.; PIÑEIRO, M. J. Integrated control of *Boophilus microplus* ticks in Cuba based on vaccination with the anti-tick vaccine Gavac™. **Experimental and Applied Acarology**, v. 34, n. 3-4, p. 375-382, 2004.

RODRIGUEZ-VIVAS, R. I.; ALONSO-DÍAZ, M. A.; RODRÍGUEZ-AREVALO, F.; FRAGOSO-SANCHEZ, H.; SANTAMARIA, V. M.; ROSARIO-CRUZ, R. Prevalence and potential risk factors for organophosphate and pyrethroid resistance in *Boophilus microplus* ticks on cattle ranches from the State of Yucatan, Mexico. **Veterinary Parasitology**, v. 1, n. 36, p. 335-342, 2006.

RODRIGUES, D. S.; LEITE, R. C. Impacto econômico de *Rhipicephalus* (*Boophilus*) *microplus*: estimativa de redução de produção de leite. **Arquivos Brasileiros de Medicina Veterinaria e Zootecnia**, v. 65, n. 5, p. 1570-1572, 2013.

RODRIGUEZ-VIVAS, R. I.; APANASKEVICH, D. A.; OJEDA-CHI, M. M.; TRINIDAD-MARTÍNEZ, I.; REYES-NOVELO, E.; ESTEVE-GASSENT, M. D.; PÉREZ De LEÓN, A. A. Ticks collected from humans, domestic animals, and wildlife in Yucatan, Mexico. **Veterinary Parasitology**, v. 215, p. 106-113, Jan. 2016.

RODRIGUEZ-VIVAS, R. I.; ARIETA-ROMÁN, R. J.; PÉREZ-COGOLLO, L. C.; ROSADO-AGUILAR, J. A.; RAMÍREZ-CRUZ, G. T.; BASTO-ESTRELLA, G. Uso de lactonas macrociclicas para el control de la garrapata *Rhipicephalus* (*Boophilus*) *microplus* en el ganado bovino. **Archivos de Medicina Veterinaria**, v. 42. n. 3, p. 115-123, 2010.

RODRIGUEZ-VIVAS, R. I.; LI, A. Y.; OJEDA-CHI, M. M.; TRINIDAD-MARTINEZ, I.; ROSADO-AGUILAR, J. A.; MILLER, R. J.; PÉREZ DE LEÓN, A. A. *In vitro* and *in vivo* evaluation of cypermethrin, amitraz, andpiperonyl butoxide mixtures for the control of resistant *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) in the Mexican tropics. **Veterinary Parasitology**, v. 197, p. 288-296, 2013.

RODRIGUEZ-VIVAS, R. I.; PÉREZ-COGOLLO, L. C.; ROSADO-AGUILAR, J. A.; OJEDA-CHI, M. M.; TRINIDAD-MARTINEZ, I.; MILLER, R. J.; LI, A. Y.; PÉREZ De LEÓN, A.; GUERRERO, F.; KLAFKE, G. *Rhipicephalus* (*Boophilus*) *microplus* resistant to acaricides and ivermectin in cattle farms of Mexico. **Brazilian Journal of Veterinary Parasitology**, v. 23, n. 2, p.113-122, Apr.-Jun. 2014.

RODRIGUEZ-VIVAS, R. I.; RIVAS, A. L.; CHOWELL, G.; FRAGOSO, S. H.; ROSARIO, C. R.; GARCÍA, Z.; SMITH, S. D.; WILLIAMS, J. J., SCHWAGER, S. J. Spatial distribution of acaricide profiles (*Boophilus microplus* strains susceptible or resistant to acaricides) in south eastern Mexico. **Veterinary Parasitology**, v. 146, n. 1-2, p. 158-169, May. 2007.

RODRÍGUEZ-VIVAS, R. I.; GRISI, L.; PÉREZ DE LEÓN, A. A.; VILLELA, H. S.; TORRES-ACOSTA, J. F. de J.; FRAGOSO, H. S.; ROMERO, S. D.; ROSARIO, C. R.; SALDIERNA, F.; GARCÍA C. D. Potential economic impact assessment for cattle parasites in Mexico. Review. **Revista Mexicana Ciencia Pecuaria**, v. 8, n. 1, p. 61-74, 2017.

ROSADO-AGUILAR, J. A.; RODRIGUEZ-VIVAS, R. I.; GARCIA-VAZQUEZ, Z.; FRAGOSO-SANCHEZ H.; ORTIZ-NAJERA, A.; ROSARIO-CRUZ R. Development of amitraz resistance in field populations of *Boophilus microplus* (Acari: Ixodidae) undergoing typical amitraz exposure in the Mexican tropics. **Veterinary Parasitology**, v. 152, p. 349-353, 2008.

ROSARIO-CRUZ, R.; GUERRERO, F. D.; MILLER, R. J.; RODRIGUEZ-VIVAS, R. I.; TIJERINA, M.; DOMINGUEZ-GARCIA, D. I.; HERNANDEZ-ORTIZ, R.; CORNEL, A. J.; MCABEE, R. D.; ALONSO-DIAZ, M. A. Molecular survey of pyrethroid resistance mechanisms in Mexican field populations of *Rhipicephalus* (*Boophilus*) *microplus*. **Parasitology Research**, v. 105, p. 1145-1153, 2009.

SANAVRIA, A.; PRATA, M. C. A. Metodologia para colonização do *Amblyomma cajenennse* (Fabricius, 1787) (Acari:Ixodidae) em laboratório. **Revista Brasileira de Parasitologia Veterinaria**, v. 5, n. 2, p. 87-90, 1996.

SÁNCHEZ-MONTES, S.; RÍOS-MUÑOZ, C. A.; ESPINOSA-MARTÍNEZ, D. V.; GUZMÁN-CORNEJO, C.; BERZUNZA-CRUZ, M.; BECKER, I. First report of "Candidatus *Rickettsia amblyommii*" in west coast of Mexico. , v. 7, n. 6, p. 1139-1145, 2016.

SOBERANES, C. N.; SANTAMARIA, M. V.; FRAGOSO, H. S.; GARCÍA, Z. V. Primer caso de resistencias al amitraz en la garrapata del ganado *Boophilus microplus* en México. **Tecnica Pecuaria México**, v. 40, n. 1, p. 81-92, 2002.

SONENSHINE, D. E.; ROE, R. M. **Biology of ticks**, 2nd edn. Oxford University Press. 2014.

SZABÓ, M. P. J.; MIKAI, L. S.; ROSA, P. C. S.; BECHARA, G. H. Differences in the acquired resistance of dogs, hamsters and guinea pigs to repeated infestations with adult ticks *Rhipicephalus sanguineus* (Acari: Ixodidae). **Brazilian of Journal Veterinary Research Animal Science**, v. 32, p. 43-50, 1995.

TAPIAS, V. M. C.; VACA, R. J. L. Carga de ixódidos en bovinos de predios lecheros, provincia cercado, Beni, Bolivia, 2009. **Agrociencias Amazonia**, v. 1, n. 2, p. 25-34, 2013.

TARRAGONA, E. L.; MANGOLD, A. J.; MASTROPAOLO, M.; GUGLIELMONE, A. A.; NAVA, S. Ecology and genetic variation of *Amblyomma tonelliae* in Argentina. **Medicine Veterinay Entomology**. v. 29, n. 3, p. 297-304, Sept. 2015.

THANGAMANI, S.; BENTE, D. Establishing protocols for tick containment at biosafety level 4. **Pathogens Disease**, v. 71, n. 2, p. 282-285, July. 2014.

HEIDMANN, M. J.; De SOUZA, A. A. D.; Do NASCIMENTO, C. G.; BRAGAGLIA, G. N.; LIMA, L. M. S.; TOMA, S. B.; ROSA, S. C.; de CASTRO, B. G. Eficácia acaricida da associação de cipermetrina, fenthion e clorpirifós em bovinos naturalmente infestados por *Rhipicephalus microplus* na Amazônia Matogrossense. **Veterinaria e Zootecnia**, v. 23, n. 4, p. 713-721, 2016.

TROUGHTON, D. R., LEVIN, M. L. Life cycles of seven Ixodid tick species (Acari: Ixodidae) under standardized laboratory conditions. **Journal Medicine Entomology**, v. 44, n. 5, p.732-740, Sept. 2007.

VOLTZIT, O. V. A review of Neotropical Amblyomma species (Acari:Ixodidae). **Acarina**, v. 15, n. 1, p. 3-134, 2007.

WALL R., SHEARER, D. **Veterinary entomology**. First Edition. Oklahoma. Springer Science+Business Media, LLC, Dordrecht., 1997.

WAMBURA, P. N.; GWAKISA, P. S.; SILAYO, R. S.; RUGAIMUKAMU, E. A. Breed associated resistance to tick infestation in *Bos indicus* and their crosses with *Bos taurus*. **Veterinay Parasitology**, v. 77, n. 1, p. 63-70, May. 1998.

WANG, H.; NUTTAL, P. A. Immunoglobulin-binding proteins in ticks: new target for vaccine development against a blood-feeding parasite. **Cells Molecular Life Scince**, v. 56, p. 286-295, 1999.

WHARTON, R. H.; ROULSTON, W. J. Resistance of Ticks to Chemicals. **Annais Reviews Entomology**, v. 15, p. 381-404. 1970.

WHARTON, R. H.; UTECH, K. B. W. The relation between engorgement and dropping of *Boophilus microplus* (Canestrini) (Ixodidae) to the assessment of the tick numbers on cattle. **Journal Australian Entomology Society**, v. 9, p. 171-182, 1970.

WILLADSEN, P.; RIDING, G. A.; McKENNA, R. V.; KEMP, D. H.; TELLAM, R. L.; NIELSEN, N. J.; LAHNSTEIN, J.; COBON, G. S.; GOUGH, J. M. Immunologic control of a parasitic arthropod Identification of a protective antigen from *Boophilus microplus*. **Journal of Immunology**, v. 143, n. 4, Aug. p. 1346-1351, 1989.

WILLADSEN, P. Vaccination against ectoparasites. **Parasitology**, v. 133, n. 2-3, p. 9-25, Jul. 2006.

WILLADSEN, P.; JONGEJAN, F. Immunology of the Tick-Host Interaction and the Control of Ticks and Tick-borne Diseases. **Parasitology Today**, v. 15, n. 7, p. 258-262, 1999.

WILLADSEN, P.; KEMP, D. H.; COBON, D. S.; WRIGHT, I. G. Succesful vaccination against *Boophilus microplus* and *Babesia bovis* using recombinant antigens. **Memorias do Instituto Oswaldo Cruz**, v. 87, n. 3, p. 289-297, 1992.

XAVIER, L. M. S.; ÁVILA, C. J. Patogenicidade de isolados de *Mearhizium anisopliae* (Mestch.) *Sokorin* e de *Beauveria bassiana* (Bals.) Vuillemin a *Scaptocoris Carvhalhoi* Becker (*Hemiptera, Cydnidae*). **Revista Brasileira de Entomologia**, v. 50, n. 4, p. 540-546, 2006.

YU, Z., ZHENG, H.; CHEN, Z.; ZENG, B.; MA, H.; ZELIU, J. The life cycle and biological characteristics of *Dermacentor silvarum* Olenev (Acari: Ixodidae) under field conditions. **Veterinary Parasitology**, v.168, p. 323-328. Mar. 2010.

ZSUZSANNA, D.; VERONIKA, C.; TOMPA, P.; SIMON, I. IU Pred: web server for the prediction of intrinsically unstructured regions of proteins based on estimated energy content. **Bioinformatics**, v. 21, n. 16, p. 3433-3434, Aug. 2005.