UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PARTICIPAÇÃO DOS TRANSPORTADORES ABC NA DESTOXIFICAÇÃO DE ACARICIDAS NO CARRAPATO *Rhipicephalus (Boophilus) microplus*

PAULA CRISTIANE POHL

Porto Alegre

2012

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PARTICIPAÇÃO DOS TRANSPORTADORES ABC NA DESTOXIFICAÇÃO DE ACARICIDAS NO CARRAPATO *Rhipicephalus (Boophilus) microplus*

PAULA CRISTIANE POHL

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

Orientador:

Dra. Aoi Masuda (Centro de Biotecnologia- UFRGS)

Co-orientadores:

Dr. Itabajara da Silva Vaz Jr (Centro de Biotecnologia- UFRGS)

Dra. Sirlei Daffre (Departamento de Parasitologia- USP)

Porto Alegre, agosto de 2012.

INSTITUIÇÕES E FONTES FINANCIADORAS

Instituições:

Universidade Federal do Rio Grande do Sul (UFRGS), Centro de Biotecnologia, Avenida Bento Gonçalves, 9500, Prédio 43421, Porto Alegre, RS, 91501-970, Brasil.

Universidade de São Paulo (USP), Instituto de Ciências Biomédicas, Departamento de Prasitologia, Avenida Professor Lineu Prestes, 1374, São Paulo, SP, 05508-900, Brasil.

Fontes Financiadoras:

Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), consórcio Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular, Brasil.

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

Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Brasil.

Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERGS), Brasil.

“A mente que se abre a uma nova idéia

jamais voltará ao seu tamanho original”

Albert Einstein

Ao meu marido e aos meus pais,

pelo amor, apoio e compreensão.

AGRADECIMENTOS

À Dra. **Aoi Masuda,** pela oportunidade de iniciar minha carreira científica ainda como graduanda em seu laboratório, por me orientar e acreditar no meu potencial e por me ensinar com serenidade e com paciência a boa conduta que um profissional deve ter no laboratório, à elaboração dos experimentos e à interpretação dos resultados.

Ao Dr. **Itabajara da Silva Vaz Jr,** pela orientação e valiosa contribuição com seus conhecimentos para a execução dos trabalhos. Obrigada pelas enumuras discuções de resultados que tivemos, obrigada por confiar em mim e me apoiar quando uma ideia nova surgia, obrigada por suas palavras de otimismo quando eu não acreditava. Espero que nossa amizade e parceria continuem sempre produtivas e duradouras, mesmo que os objetivos mudem um pouco.

Agradeço aos dois por me apoiar e compreender que a vida longe do meu marido seria muito difícil e que não havia outra solução, senão me mudar para São Paulo. **MUITO OBRIGADA!**

À Dra. **Sirlei Daffre,** pelo imenso carinho com o qual me recebeu em seu laboratório e deu suporte a realização da minha tese de doutorado. Obrigada por colaborar com sua experiência no engrandecimento deste trabalho. Espero que a parceria que iniciamos agora dê fruto a bons resultados.

Aos Dr. **Pedro L. Oliveira** e Dr. **Flávio Lara,** pelas oportunidades e contribuições ao trabalho.

À Dra. **Andréa Fogaça,** pela serenidade e imensa inteligência com que trata os problemas. Obrigada por seu exemplo, carinho e amizade.

Ao Dr. **Guilherme Klafke,** por sua enorme colaboração no trabalho e por sua amizade.

Ao **Dr. João Ricardo Martins e ao seu grupo de pesquisa**, pela colaboração no trabalho com sua grande experiência no controle do carrapato.

Aos amigos que fiz no laboratório da UFRGS e que levo no coração para o restante da vida, **Adriana, Luís, Daiane, Melina, Lucas, Ana Carolina, Caroline, Ana Paula, Maria Lúcia, Leonara, Alexandra, Sandra, Clotilde e Alexandre**. Obrigada pelas inúmeras vezes que me auxiliaram e pela amizade.

Aos amigos que fiz no laboratório da USP que me acolheram e me fizeram sentir em casa assim que cheguei: **Maria Fernanda, Thaís, Eliane, Camila, Carlos** e **Suzana**. E

àqueles que chegaram depois e já são velhos amigos: **Sandra, Janaína, Rafael, Larissa** e **Mário.** Obrigada pelo companheirismo na rotina diária de trabalho, obrigada pelos momentos felizes de descontração e brincadeira e mais do que tudo, obrigada pelo apoio, carinho e amizade.

Aos secretários do PPGBCM **Luciano** e **Silvia,** que prestativamente me auxiliaram nas minhas dúvidas e cumprimento das minhas obrigações.

As queridas **Rosaura, Nilma, Renata** e **Suzana,** que são muito mais que amigas ou companheiras de "Confraria do Champagne", são minhas confidentes, são minhas irmãs, são minha família em São Paulo. Obrigada pelas risadas, encontros, viagens, festinhas, cineminhas, conversas, comidinhas, bebidinhas, chimas, receitinhas, dicas e tudo mais de sincero que nossa amizade possui. E aos seus maridos, **Márcio, Maurício, Mauro** e **Rodrigo,** pelo companheirismo e amizade.

Aos meus sogros **Maria Luisa** e **Adriano,** por apoiarem a mim e ao Daniel em nossas decisões.

À minha irmã **Clarise e sua família,** pelo exemplo de determinação que sempre foram para mim, pelo opoio e carinho e pelas inúmeras vezes em que me acolheram em sua casa. Obrigada!

À minha irmã **Elci e sua família,** pela amizade, pelo amor e pela enorme alegria e perceverança transmitida.

Aos meus pais, **Reneu** e **Lili,** pelos ensinamentos de toda a vida, pelos conselhos e pelos exemplos que fizeram de mim o que sou hoje. Por me apoiarem e compreenderem minhas decisões. Por aceitarem a distancia que hoje nos separa de um abraço diário, mas que jamais diminue nosso amor. **MUITO OBRIGADA!**

Ao meu marido **Daniel,** que merece mais do que todos em minha volta, o reconhecimento por sua dedicação, compreensão e paciência comigo. Por me apoiar e me incentivar sempre para que eu alcançasse meus objetivos. Pelas dificuldades, experiências e alegrias partilhadas que nos fizeram crescer juntos. E acima de tudo, obrigada por me amar e cuidar de mim!

SUMÁRIO

LISTA DE ABREVIATURAS

ABC: *ATP-binding cassette*, cassete de ligação de ATP

ABM: *after blood meal*, após alimentação sanguínea

AIT: *adult immersion test*, teste de imersão de adultos

BCRP: *breast cancer resistance protein*, proteína de resistência do câncer de mama

BME26: linhagem de células de *R. microplus* sensível a drogas

BME26-IVM: linhagem de células de *R. microplus* resistente à ivermectina

CsA: *cyclosporin A*, ciclosporina A

DDT: *dichlorodiphenyltrichloroethane*, dicloro-difenil-tricloroetano

DMSO: *dimethyl sulfoxide*, dimetilsulfóxido

dsRNA: *double-stranded ribonucleic acid*, ácido ribonuceico dupla-fita

FAO: *Food and Agriculture Organization*, Organização para a Alimentação e Agricultura

GABA: *gamma- aminobutyric acid*, ácido gama-amino-butírico

GluCl: *glutamate-gated chloride ion channel*, canal de cloro controlado por glutamato

GST: *glutathione s-transferase*, glutationa s-transferase

IVM: *ivermectin*, ivermectina

LC_{50}: *lethal concentration for 50%*, concentração letal para 50%

LPT: *larvae packet test*, teste de pacote de larvas

MDR: *multidrug resistance*, resistência a múltiplas drogas

ML: *macrocyclic lactone*, lactonas macrocíclicas

mRNA: *messenger ribonucleic acid*, ácido ribonucleico mensageiro

MRP: *multidrug resistance protein*, proteína de resistência a múltiplas drogas

MSP1: *major surface protein 1*, proteína principal de superfície 1

MTT: *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide*

NBDs: *nucleotide-binding domains*, domínios de ligação de nucleotídeo

OP: *organophosphate*. organofosfato

PCR: *polymerase chain reaction*, reação em cadeia da polimerase

P-gp: *P-glicoprotein*, glicoproteína-P

qPCR: *quantitative polymerase chain reaction*,reação em cadeia da polimerase quantitativa

RNAi: *ribonucleic acid interference*, ácido ribonucleico de interferência

RR: *resistance ratio*, razão de resistência

SF: *synergism factor*, fator de sinergismo

Sn-Pp IX: *tin-protoporphyrin IX*, estanho-protoporfirina IX

SP: *synthetic pyrethroid*, piretróide sintético

TMDs: *transmembrane domains*, domínios transmembrana

Zn-Pp IX: *zinc-protoprophyrin IX*, zinco-protoporfirina

RESUMO

A resistência aos acaricidas é um dos maiores desafios para o controle adequado do carrapato *Rhipicephalus (Boophilus) microplus*. Entender os mecanismos de resistência aos acaricidas pode ser fundamental para prolongar sua eficiência no controle desse parasita. Transportadores ABC são reconhecidos em um grande número de organismos, pela sua participação na destoxificação de drogas. Eles são proteínas transmembrana, responsáveis por remover da célula compostos tóxicos, endógenos ou exógenos. Desta forma, protegem os organismos e são associados à resistência a drogas em vários nematódeos, artrópodes parasitas e células cancerígenas. No presente trabalho, determinamos a participação de transportadores ABC na destoxificação de compostos tóxicos, com ênfase ao acaricida ivermectina, no carrapato *R. microplus*. O tratamento com inibidores de transportadores ABC aumentou a toxicidade da ivermectina em larvas e fêmeas adultas de populações de campo resistentes a este acaricida. Inibidores de transportadores ABC também aumentaram a toxicidade de abamectina, moxidectina e clorpirifós, em uma população multirresistente a acaricidas, indicando que estes transportadores são responsáveis pela destoxificação de um grande número de acaricidas estruturalmente não relacionados. Níveis de transcrição significativamente maiores do gene *RmABCB10* foram identificados no intestino de fêmeas de populações resistentes à ivermectina e amitraz, comparado à população suscetível, importante indicativo da participação deste transportador ABC na resistência a acaricidas. A toxicidade da ivermectina foi também significativamente aumentada, em uma população de células embrionárias de carrapato resistentes a este acaricida, quando estas foram co-incubadas com um inibidor de transportadores ABC. Além disso, os níveis de transcrição do gene

RmABCB10, foram também induzidos nesta mesma população, comparado às células parentais suscetíveis à ivermectina, indicando que mecanismos semelhantes são selecionados *in vivo* e *in vitro,* e confirmando a participação de transportadores ABC na resistência à ivermectina. Mostramos ainda, que transportadores ABC responsáveis pelo sequestro e, consequente, destoxificação da molécula heme para o interior dos hemossomos, presentes no intestino do carrapato, são importantes na destoxificação de acaricidas para o interior desta mesma organela. Sugerindo ser este um importante mecanismo de defesa contra os acaricidas no carrapato. O silenciamento gênico do *RmABCB10* por RNAi reduziu a destoxificação do heme nos hemossomos e aumentou a toxicidade da ivermectina em uma população resistente, indicando que o mesmo transportador usado para destoxificar heme é responsável pela destoxificação de acaricidas no intestino. Em conjunto, estes resultados revelam a participação de transportadores ABC na destoxificação de compostos endógenos e exógenos no carrapato e sua implicação como um mecanismo de resistência à ivermectina e outros acaricidas. Os dados aqui apresentados representam uma via de destoxificação de acaricidas até então desconhecida. E podem servir como alvo para o desenvolvimento de métodos de diagnóstico e monitoramento da resistência e para o desenvolvimento de drogas e vacinas, contribuindo para o controle do carrapato.

Palavras chaves: *R. microplus,* transportadores ABC, destoxificação de acaricidas, controle do carrapato.

ABSTRACT

Acaricide resistance is one of the biggest challenges in the control of the tick *Rhipicephalus (Boophilus) microplus*. Understanding the mechanisms of drug resistance in the cattle tick is critical to prolong the efficacy of acaricides to control this parasite. ABC transporters are recognized in a large number of organisms. They are membrane-integrated proteins responsible for pumping toxic compounds, either exogenous or endogenous, out of the cells, protecting these. In this sense, ABC transporters have been associated with drug resistance in several nematodes, parasitic arthropods and cancer cells. The present study reports on the participation of ABC transporters in the detoxification of toxic compounds, particularly ivermectin, in the tick *R. microplus*. ABC transporter inhibitors increased ivermectin toxicity in larvae and females of ivermectin-resistant populations. ABC transporter inhibitors also increased the toxicity of abamectin, moxidectin and chlorpyriphos in a multidrug resistant population, suggesting that ABC transporters are responsible for the detoxification of a large number of structurally unrelated acaricides. Increased transcription levels of *RmABCB10* found in midgut of females from ivermectin- and amitraz-resistant populations, compared to a susceptible tick population, indicated the participation of this ABC transporter in acaricide resistance. Increased ivermectin toxicity in a tick cell line resistant to this acaricide was observed when the cells were co-incubated with an ABC transporter inhibitor. Moreover, transcription levels of *RmABCB10* were also increased in this cell line, compared to the parental susceptible cell line, suggesting that similar mechanisms are selected *in vivo* and *in vitro* and confirming the participation of ABC transporters in ivermectin resistance. We even showed that ABC transporters responsible for the sequestration and hence detoxification of the heme into the hemosomes

present in the midgut of the tick, were also important for the detoxification of acaricides in the same organelle, suggesting this is an important defense mechanisms of the tick against acaricide. The down-regulation of *RmABCB10* by RNAi reduced heme detoxification in the hemosomes and increased ivermectin toxicity in resistant females, showing that the same ABC transporter is used to detoxify heme and acaricides in the midgut. Together, these results provide evidence of the participation of ABC transporters in the detoxification of endogenous and exogenous compounds in the tick, and indicate the role of these transporters as a mechanism of resistance to ivermectin and other acaricides. The data reported herein shed light on a new acaricide detoxification mechanism that may be useful in the development of assays to monitor drug resistance and design new anti-tick drugs and vaccines, contributing to the development of novel cattle tick control strategies.

Keywords: *R. microplus,* ABC transportes, acaracide detoxification, tick control.

APRESENTAÇÃO

No último século, a indústria de antiparasitários desenvolveu novas e efetivas drogas para o tratamento e o controle de um grande número de doenças economicamente importantes, o que resultou na diminuição das perdas na produtividade pecuária.

O fácil acesso e a aplicação das drogas, combinada com o imenso progresso nos conhecimentos epidemiológicos de parasitas, levou a um período de relativo sucesso no controle de endoparasitas e ectoparasitas (CASIDA & QUISTAD, 1998; FAO, 2004). No entanto, a falsa suposição de que o controle de parasitas é facilmente atingido pela aplicação, exclusivamente, de compostos químicos, levou a seleção da resistência nestes parasitas, dificultando seu controle. Atualmente, a resistência às drogas é considerada o maior obstáculo no controle eficiente de parasitas (FAO, 2004).

Sob o ponto de vista da pecuária bovina, o carrapato bovino é considerado um dos parasitas de maior impacto econômico. E como agravante, este parasita apresenta resistência à maioria dos produtos químicos utilizados no seu controle (GUERRERO *et al*., 2012a). Em países como o Brasil, onde a pecuária contribui expressivamente para a economia nacional (CNPC, 2010; IBGE, 2010), a melhoria dos meios de controle do carrapato constitui um objetivo nacional eminente.

A investigação dos mecanismos envolvidos no desenvolvimento da resistência às drogas pode ajudar neste sentido, uma vez que permite a criação de técnicas mais sensíveis de diagnóstico da resistência e a caracterização de alvos para o desenvolvimento de novas drogas (PERRY *et al*., 2011; ROSARIO-CRUZ *et al*., 2009a).

Os transportadores ABC (*ATP-binding cassette*) são reconhecidos em um grande número de parasitas pela sua participação na destoxificação de drogas. Eles são proteínas

transmembrana, que utilizam a energia derivada da hidrólise do ATP, para remover das células compostos tóxicos, endógenos ou exógenos. Desta forma, protegem e são responsáveis pela resistência a drogas em muitos organismos.

Mediante tal problema, a presente tese apresentará os resultados, cujo objetivo foi caracterizar o papel de transportadores ABC na destoxificação de acaricidas no carrapato bovino.

Na introdução, serão apresentados os aspectos gerais da biologia do carrapato, os impactos econômicos do parasitismo, os métodos empregados para seu controle e o problema da resistência aos acaricidas. Serão abordados também, os mecanismos de resistência aos acaricidas, com ênfase no papel dos transportadores ABC, e métodos alternativos para seu estudo.

A seção de resultados se divide em três tópicos apresentados sob a forma de manuscritos. O primeiro tópico expõe a importância dos transportadores ABC na destoxificação da ivermectina em populações de carrapato de campo, resistentes a este acaricida. Destaca ainda, o papel destes transportados como um mecanismo de resistência a múltiplas drogas. O segundo tópico mostra o estabelecimento de uma linhagem de células de carrapato resistentes à ivermectina e discute a importância da cultura de células, como uma ferramenta de análise dos mecanismos de resistência às drogas.

Por fim, o terceiro tópico trata do papel dos transportadores ABC na destoxificação da molécula heme, proveniente da degradação da hemoglobina e de acaricidas no intestino do carrapato. Após esta seção, segue uma discussão geral.

1. INTRODUÇÃO

1.1. Biologia do carrapato *Rhipicephalus (Boophilus) microplus*

O carrapato *Rhipicephalus (Boophilus) microplus*, comumente conhecido como carrapato do boi ou carrapato bovino, pertence ao filo Arthropoda, classe Arachnida, ordem Acarina e família Ixodidae. Em 2003, os carrapatos do gênero *Boophilus* foram reclassificados, com base em análises moleculares e morfológicas, como pertencentes ao gênero *Rhipicephalus*, subgênero *Boophilus*, sendo adotada, então, a presente nomenclatura (MURRELL & BARKER, 2003).

O carrapato *R. microplus* é uma das espécies de carrapato de maior distribuição mundial. É encontrado em grande parte das zonas tropicais e subtropicais da Ásia, nordeste da Austrália, Madagascar, sudeste da África até a linha do Equador, grande extensão das Américas Central e do Sul, México e Caribe (ESTRADA-PENÃ *et al*., 2006). No Brasil, o carrapato bovino é encontrado em todo o país, sua frequência varia de acordo com as condições climáticas e a raça de bovinos criados (GONZALES, 1995).

O *R. microplus* é um ectoparasita hematófago de um único hospedeiro, em geral, o bovino. Seu ciclo de vida é dividido em duas fases: a fase de vida livre e a fase de vida parasitária.

A duração da fase de vida livre depende das condições de temperatura e umidade. De modo geral, na região Sudeste do Brasil, a fase de vida livre varia de 40 a 50 dias, na primavera-verão, e 70 a 120 dias no outono-inverno (PEREIRA & LABRUNA, 2008). Essa fase inicia quando a fêmea, completamente ingurgitada (teleógina), se desprende do hospedeiro e cai ao solo, onde realiza a postura dos ovos e morre. A postura pode chegar a mais de 3.000 ovos por fêmea. Aproximadamente, 20 dias após o término da postura,

inicia-se a eclosão dos ovos, quando as condições são ideais para o desenvolvimento do embrião, ou seja, à temperatura de 28 ºC e umidade relativa superior a 70% (GONZALES, 1995). Após um período de aproximadamente sete dias, as neolarvas se transformam em larvas infectantes, que migram para as extremidades apicais da vegetação, a espera do hospedeiro (**Figura 1**).

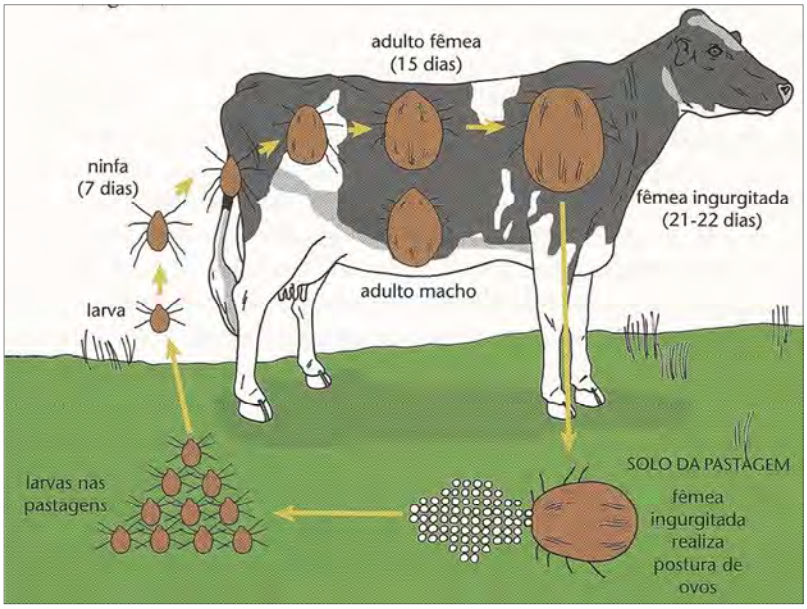


Figura 1 - Representação esquemática do ciclo de vida do carrapato *R. microplus*. Fonte: PEREIRA & LABRUNA, 2008.

A fase de vida parasitária do *R. microplus* se desenvolve sobre o bovino e tem duração média de 21 dias. Passa pelos estágios de larva, ninfa e adulto (**Figura 1**). A fêmea se alimenta a taxas muito baixas, até o acasalamento. Após a cópula, ela inicia a rápida fase alimentar, que culmina com o seu ingurgitamento e queda ao solo, dando início à fase de vida livre (PEREIRA & LABRUNA, 2008). Os machos, por sua vez,

permanecem no hospedeiro e sobrevivem até duas vezes mais que as fêmeas (ROBERTS, 1968).

É na fase de vida parasitária, que o carrapato se alimenta e transmite agentes patogênicos ao hospedeiro, determinando, desta forma, os prejuízos econômicos decorrentes deste parasitismo (CORDOVÉS, 1996).

1.2. Impactos econômicos causados pelo carrapato

As infestações por *R. microplus* são conhecidas pelo seu efeito adverso na produtividade pecuária (JONSSON, 2006). Durante a fase parasitária, um único carrapato suga de 2 a 3 ml de sangue (GONZALES, 1995), o que, dependendo da intensidade da infestação parasitária, reflete em anemia, perdas na produção de leite e carne (SUTHERST *et al*., 1983). Além disso, o carrapato causa lesões e reações inflamatórias nos pontos de fixação, que danificam o couro do animal (PEREIRA *et al*., 2005).

O *R. microplus* também é vetor de patógenos que causam doenças, como a babesiose (*Babesia bovis* e *Babesia bigemina*) e a anaplasmose (*Anaplasma marginale*) (JONSSON *et al*., 2008; NARI, 1995). Essas doenças acometem os bovinos e são responsáveis por altos índices de mortalidade, cujas medidas de prevenção e ocorrência de surtos são de grande impacto econômico na pecuária (YOUNG & MORZARIA, 1986). Além dos prejuízos diretos causados pela infestação de carrapatos, existem as despesas com a aquisição dos produtos e as instalações de equipamentos necessários para a aplicação de acaricidas nos rebanhos (FAO, 2004).

Estima-se que, anualmente, as perdas decorrentes da infestação pelo *R. microplus* atinjam a U$ 2 bilhões no Brasil (GRISI *et al*., 2002). Sabe-se que o efetivo nacional de bovinos, em 2010, chegou a 209,5 milhões de cabeças, representando o maior rebanho comercial de bovinos do mundo (CNPC, 2010; IBGE, 2010). Portanto, o controle do

carrapato é fundamental para a manutenção da sanitariedade do rebanho nacional e para o desenvolvimento da bovinocultura. Uma atividade de grande importância para o país.

1.3. Controle do carrapato

1.3.1. Controle químico

Até o final do século dezenove, os meios disponíveis para controle de carrapatos eram limitados. Inicialmente, na Austrália, África e EUA, eram utilizados produtos rudimentares como óleo de semente de algodão, óleo de peixe, petróleo bruto, querosene, extrato de tabaco, enxofre, sabão, entre outros (GEORGE, 2000). Historicamente, o uso de acaricidas no controle do carrapato, iniciou-se de forma sistemática com a utilização de compostos arsenicais no ano de 1896 na Austrália (ANGUS, 1996).

Com a descoberta das propriedades inseticidas do DDT, em 1939, e o subsequente desenvolvimento de pesticidas orgânicos, a situação melhorou positivamente para os criadores de gado do mundo todo. As principais categorias de inseticidas, como os organoclorados, organofosfatos, amidinas e piretróides, resultaram em significantes produtos para o controle do carrapato (GRAF *et al*., 2004). Atualmente, existem no mercado nacional, produtos derivados de sete classes distintas: organofosforados (ex. clorpirifós, coumafós, etion), piretróides (ex. flumetrina, cipermetrina, deltametrina), amidinas (ex. amitraz), lactonas macrocíclicas (ex. ivermectina, abamectina, doramectina, moxidectina), fenilpirazóis (ex. fibronil), inibidores da síntese de quitina (ex. fluazuron) e espinosinas (ex. espinosade).

1.3.2. Métodos alternativos de controle

É inevitável afirmar, que o uso de acaricidas foi um dos fatores preponderantes no desenvolvimento da pecuária bovina em várias regiões. E é, até hoje, o método mais eficiente. Porém, não foram poupados esforços para desenvolver métodos alternativos de controle, para a aplicação individual ou de forma integrada ao uso de acaricidas. Estes métodos incluem o uso de raças bovinas resistentes ao carrapato (PIPER *et al*., 2010; REGINATO *et al*., 2008), uso de vegetações com função repelente aos carrapatos (FERNANDEZ-RUVALCABA *et al*., 2004), uso de microrganismos patogênicos, como os fungos *Metarhizium anisopliae* e *Beauveria bassiana* (CAMPOS et al., 2010; LEEMON & JONSSON, 2012; PERINOTTO *et al*., 2012) e uso de compostos naturais com função acaricida (BORGES *et al*., 2011; GIGLIOTI *et al*., 2011; RIBEIRO *et al*., 2011).

Um dos métodos mais promissores, como alternativa ao uso de acaricidas, é o controle através de vacinação (WILLADSEN, 2004; GUERRERO *et al*., 2012b). Em 1994 e 1995, foram lançadas duas vacinas comerciais contra o carrapato, a TickGard™, desenvolvida na Austrália (WILLADSEN *et al*., 1995), e a GAVAC, desenvolvida em Cuba (RODRÍGUEZ *et al*., 1995). Ambas formuladas com o antígeno Bm86, uma glicoproteína de membrana presente no intestino do carrapato (WILLADSEN *et al*., 1989). Os testes de estábulo realizados com essas vacinas apresentaram resultados variando de 50 a 90% de eficiência em condições de baixo desafio parasitário, mostrando-se promissora sua utilização no campo (RODRÍGUEZ *et al*., 1995; WILLADSEN *et al*, 1995). Porém, a eficácia da vacina é comercialmente insignificante em situações, cujas condições climáticas permitem a multiplicação do carrapato ao longo de todo o ano e que as raças bovinas são sensíveis aos carrapatos (como o caso da maior parte do Brasil) (LABRUNA, 2008).

De fato, as vacinas atualmente disponíveis não asseguraram o grau de proteção necessário para suprimir o uso de acaricidas, apesar de reduzirem o número de aplicações e diminuir os prejuízos econômicos (DE LA FUENTE *et al*., 2007; VALLE *et al*., 2004; WILLADSEN *et al.*, 1996). Entretanto, elas foram o primeiro passo para o desenvolvimento de outras vacinas com maior eficácia. Vários grupos de pesquisa no Brasil e no exterior estão investigando novos imunógenos derivados de diferentes tecidos de *R. microplus*, para serem utilizados numa futura nova vacina (ALMAZÁN *et al*., 2012; LEAL *et al.*, 2006; MARITZ-OLIVIER *et al*., 2012; PARIZI *et al*., 2011; PECONICK *et al*., 2008; WILLADSEN, 2006).

1.4. Resistência aos acaricidas

1.4.1. Situação atual da resistência no Brasil e no mundo

Atualmente, a resistência do *R. microplus* aos acaricidas existe mundialmente, principalmente, nos lugares onde é realizado seu controle químico (FAO, 2004). Das sete classes disponíveis no mercado, somente o fluazuron e o espinosade não possuem relatos de resistência em *R. microplus*.

Os organofosforados são o grupo mais antigo de acaricidas ainda comercializados para bovinos. Foram introduzidos no mercado em torno de 1955, para substituir os organoclorados (ANDREOTTI, 2010). No Brasil, a resistência aos organofosfosforados apareceu primeiramente, em 1963, no Rio Grande do Sul (ANDREOTTI, 2010). Nos anos 80, foram introduzidos os piretróides, que, por possuírem o mesmo modo de ação do DDT, logo enfrentaram problemas com o surgimento de resistência cruzada (GRAF *et al*, 2004).

A resistência a piretróides foi relatada no final de 1980, no Rio de Janeiro e no Rio Grande do Sul (ANDREOTTI, 2010). As amidinas foram introduzidas nos anos 70,

mas utilizadas com maior intensidade, a partir dos anos 80, quando a resistência aos piretróides já estava difundida (GRAF *et al*, 2004). No Brasil, o amitraz (amidina mais empregada) é um dos acaricidas mais utilizados no controle do carrapato bovino. O primeiro relato de resistência a este composto ocorreu em 1999 (FURLONG, 1999).

Nos dias de hoje, populações de carrapatos resistentes a organofosforados e piretróides estão disseminadas por todo o globo (FAO, 2004). A resistência ao amitraz também foi detectada em muitos países, como a Austrália (KUNZ & KEMP, 1994), Colômbia (BENAVIDES *et al*., 2000) e México (SOBERANES *et al*., 2002). No Brasil, a resistência aos organofosforados, piretróides e amitraz tem ocorrência generalizada nos estados do Rio Grande do Sul (CAMILLO *et al*., 2009; DOS SANTOS *et al*., 2009), Mato Grosso do Sul (ANDREOTTI *et al*., 2011) e São Paulo (MENDES *et al*., 2011).

As lactonas macrocíclicas, que têm como principal representante a ivermectina, surgiram no início da década de 80 e produziram grande revolução no mercado mundial dos antiparasitários por possuírem amplo espectro de ação, sendo eficazes para o controle de endoparasitas e ectoparasitas. A resistência às lactonas macrocíclicas foi primeiramente descrita no Brasil nos estados do Rio Grande do Sul (MARTINS & FURLONG, 2001) e São Paulo (KLAFKE *et al*., 2006). E mais recentemente, foram descritos casos no México (PEREZ-COGOLLO *et al*., 2010) e no Uruguai (CASTRO-JANER *et al*., 2011).

O fibronil começou a ser usado no controle do carrapato nos anos 90 e recentemente a resistência a este composto também já foi registrada no Brasil (CASTRO-JANER *et al*., 2010a) e no Uruguai (CASTRO-JANER *et al*., 2010b).

1.4.2. Desenvolvimento da resistência

Resistência pode ser funcionalmente definida como a habilidade que um organismo possui de sobreviver a doses de uma droga que é letal para um organismo

suscetível (WILSON, 2001). Ela é consequência do uso da droga, já que promove a seleção artificial de uma população, alterando a frequência de alelos na mesma. Ou seja, antes da administração de um acaricida novo, os alelos que conferem a resistência são raros. Eles ocorrem em cerca de um a cada um milhão ou mais de indivíduos (ROUSH & MCKENZIE, 1987). Portanto, a grande maioria dos indivíduos de uma população de carrapatos é suscetível. Quando o acaricida é usado, os indivíduos resistentes apresentam uma vantagem seletiva: sobrevivem ao tratamento, reproduzem e dão origem a uma população de carrapatos também resistente.

À medida que novas aplicações do produto são efetuadas, a parcela suscetível da população diminui e ocorre a predominância de indivíduos resistentes (**Figura 2**) (KLAFKE, 2008). Consequentemente, esse aumento do número de resistentes é refletido na diminuição da eficácia do produto e cada tratamento sucessivo é um processo de seleção.

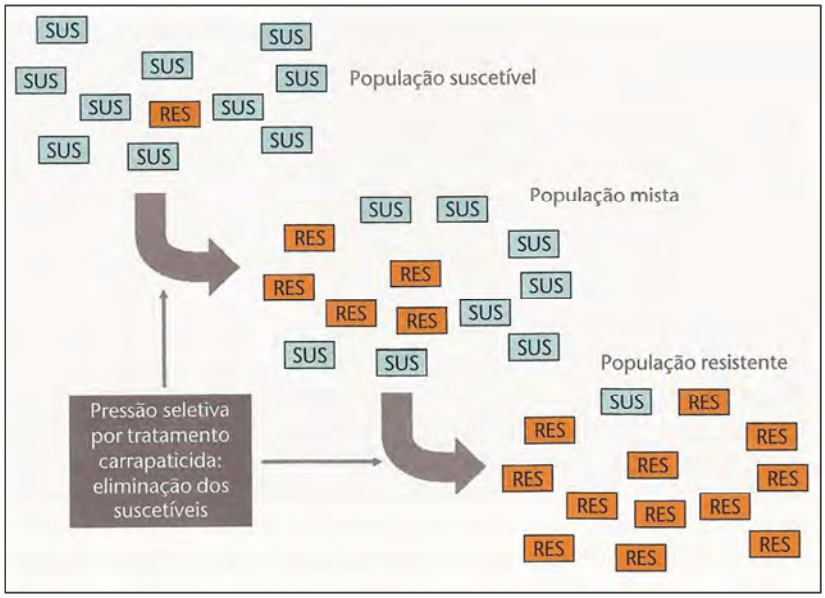


Figura 2- Fluxograma de desenvolvimento de resistência aos acaricidas. Fonte: (KLAFKE, 2008).

Um estudo recente mostrou como a evolução da resistência aos acaricidas pode ser rápida. Acompanhando o grau de resistência e a frequência para o alelo que confere resistência aos piretróides em 11 fazendas, os pesquisadores verificaram que, entre 8 e 24 meses após o uso contínuo de piretróides, a resistência aumentou de 2 para 125 vezes. E a frequência para o alelo que confere resistência, que variava entre 5 e 46%, no início do experimento, passou para 66 a 95% de frequência, no final do experimento (RODRIGUES-VIVAS *et al.*, 2011).

O grau de dominância dos alelos envolvidos, a frequência e a qualidade dos tratamentos acaricidas, são determinantes para o período de tempo até o desenvolvimento da resistência (KUNZ & KEMP, 1994). A diluição inapropriada dos produtos, falhas na mistura, a pulverização de quantidade insuficiente do produto nos animais, a dosagem incorreta de medicamentos injetáveis ou orais e o desrespeito ao intervalo entre tratamentos são erros que podem elevar a taxa em que a resistência se desenvolve em uma população de carrapatos (GEORGE, 2000).

Não existe no país, atualmente, um programa oficial de controle do carrapato. Por esta razão, os critérios para a aplicação dos acaricidas são definidos exclusivamente pelos produtores. Em geral, informações sobre a epidemiologia do parasita são negligenciadas. Acaricidas são escolhidos por critérios que não a eficácia, e a sua aplicação é inadequada, desafiando o sucesso do tratamento (LABRUNA, 2008). Aliado a isso, o curto período de tempo entre as gerações do carrapato favorece a rápida seleção de populações resistentes (KUNZ & KEMP, 1994).

Além disso, a resistência de um determinado organismo pode se manifestar para dois ou mais compostos químicos distintos, o que chamamos de resistência cruzada. Este fenômeno ocorre quando um mesmo mecanismo confere resistência a mais de um produto. O caso clássico é a resistência cruzada dos piretróides com o DDT (NOLAN *et al.*, 1977),

que se deve a mutações no sítio alvo que ambas as drogas compartilham, o canal de sódio controlado por voltagem (FFRENCH-CONSTANT *et al*., 2004).

Esta situação representa uma dificuldade adicional ao controle do carrapato, porque limita, ainda mais, o uso das drogas disponíveis para seu controle. Populações de carrapatos resistentes a múltiplas drogas têm sido descritas (BENAVIDES *et al.*, 2000; FERNÁNDEZ-SALAS *et al*., 2012; ORTIZ *et al*., 1995), mas pouco se conhece sobre os mecanismos moleculares envolvidos neste fenômeno.

É evidente que a seleção da resistência foi o principal motivador para o desenvolvimento de novas drogas para o controle do carrapato (GRAF *et al*., 2004). No entanto, não se pode esperar que um novo princípio ativo seja descoberto cada vez que a resistência for selecionada. Pois, sabe-se que os custos de desenvolvimento de novos produtos são bastante elevados e aspectos sociopolíticos associados a resíduos nos alimentos e toxicidade ambiental, bem como, a descoberta de novas moléculas e alvos devem ser considerados (GRAF *et al*., 2004; KLAFKE, 2008).

1.4.3. Diagnóstico da resistência

O diagnóstico da resistência é fundamental para identificar o problema, elaborar estratégias para o controle e prevenir a disseminação de carrapatos resistentes. Segundo a FAO (2004), um teste eficiente para o diagnóstico da resistência deve detectar o problema num estágio primário de seu desenvolvimento, antes de se tornar um problema maior de controle no campo.

Entretanto, poucos métodos de detecção da resistência foram desenvolvidos, e apesar de sua relativa baixa sensibilidade de detecção de resistência, os bioensaios permanecem os métodos de escolha para avaliar a suscetibilidade de populações de carrapato (FAO, 2004). Os bioensaios são baseados na resposta toxicológica, após a

exposição ao acaricida, e podem utilizar fêmeas adultas, como o teste de imersão de adultos (DRUMMOND *et al*., 1973) e larvas, como o teste de pacote de larvas (STONE & HAYDOC, 1962) e o teste de imersão de larvas (SHAW, 1966). O teste de pacote de larvas é utilizado como padrão da FAO para o diagnóstico de resistência (FAO, 2004). Os bioensaios são testes trabalhosos e os resultados levam de 4 a 6 semanas para serem concluídos. Além disso, nem sempre se ajustam a cada princípio químico a ser testado (FAO, 2004; SABATINI *et al*., 2001).

Assim, identificar os genes relacionados à resistência a cada princípio químico, pode auxiliar no desenvolvimento de métodos de diagnósticos mais sensíveis, bem como na melhoria das estratégias de controle (PERRY *et al*., 2011; ROSARIO-CRUZ *et al*., 2009a). Esforços nesse sentido têm sido realizados para o desenvolvimento de diagnósticos moleculares que possam detectar a resistência em seu estágio inicial. Já existem, em carrapatos, métodos para o diagnóstico molecular da resistência. Através de uma PCR alelo-específica, é possível determinar a presença de mutações no canal de sódio (resistência a piretróides), obtendo-se a frequência de indivíduos resistentes em uma população (GUERRERO *et al*., 2001).

1.5. Mecanismos de resistência aos acaricidas

A resistência é uma adaptação evolutiva, o que implica na existência de mecanismos comportamentais e fisiológicos que permitem a sobrevivência dos indivíduos resistentes. São descritos quatro mecanismos de resistência: resistência comportamental, redução da penetração da droga, insensibilidade do sítio alvo e aumento da destoxificação da droga (DESPRÉS *et al*., 2007; PERRY et al., 2011; WILSON, 2001).

A resistência comportamental ocorre quando o organismo é capaz de evitar a droga. Um comportamento mais frequente em insetos fitófagos (DESPRÉS *et al*., 2007). A

resistência pela redução na penetração da droga é proveniente de mudanças no organismo que reduzem a penetração da droga pela cutícula, sistema respiratório e/ou intestino (WILSON, 2001). Não se conhece a importância desses dois primeiros tipos de resistência no *R. microplus*, porém, os dois últimos mecanismos são considerados os mais relevantes e serão discutidos detalhadamente.

Na **tabela 1** estão resumidos os mecanismos de resistência, até agora descritos, em *R. microplus*.

Tabela 1- Mecanismos de resistência descritos para *R. microplus*.

Acaricida	**Mecanismo de resistência**		
	Alterações do sítio alvo	**Aumento de destoxificação**	
		Resistência metabólica	**Aumento de transporte**
Organofosforados	Acetilcolinesterase[1]	Esterases [2]/ P450[3]	Transportador ABC[4]
Piretróides	Canal de sódio[5]	Esterases [2]/ P450[6]	NI
Amitraz	NI	GST[7]	Transportador ABC[8]
Lactonas Macrocíclicas	NI	NI	Transportador ABC[4,9]

1- HE *et al*., 1999; 2- BAFFI *et al*., 2008; 3- LI *et al*., 2003 4- POHL *et al*, submetido à publicação; 5- PRUETT & POUND, 2006; 6- MILLER *et al*., 1999; 7- LI *et al*., 2004; 8- LARA *et al*., submetido à publicação; 9- POHL *et al.*, 2011; NI- não identificado.

1.5.1. Alterações genéticas responsáveis pela resistência às drogas

As principais alterações, em nível molecular, responsáveis pela resistência às drogas, podem ser devidas ao aumento da expressão gênica, aumento de atividade de enzimas ou transportadores e mutações em genes alvos.

O aumento da expressão de um gene pode ocorrer pela amplificação gênica, quando há aumento do número de cópias de um gene que codifica uma enzima ou um transportador, pelo aumento da estabilidade do RNA mensageiro (BASS & FIELD, 2011;

MÉNEZ *et al*., 2012), ou pela presença de mutações na região promotora dos genes, de forma a aumentar os níveis de expressão do mesmo (LI *et al*., 2007).

O aumento da atividade ocorre devido a mutações na região codificadora de genes, que modificam a afinidade de enzimas ou de transportadores pelo seu substrato, tornando o processo mais eficiente (CLAUDIANOS *et al*., 1999).

Além disso, mutações em genes alvos podem causar alterações nos aminoácidos da proteína codificada, que bloqueiam ou reduzem a ligação da droga ao seu sítio alvo, sem causar a perda da função primária dessa proteína (FFRENCH-CONSTANT *et al*., 2004; PERRY *et al*., 2011; WILSON, 2001).

1.5.2. Insensibilidade do sítio alvo

Uma vez dentro do parasita, a droga interage com sua molécula alvo e altera sua função (WILSON, 2001). A maioria dos acaricidas, atualmente disponíveis, atua sobre o sistema nervoso em sítios de sinapse ou axônios, como por exemplo, na acetilcolinesterase, nos canais de sódio controlados por voltagem, nos canais de cloro controlados por glutamato ou acido gama-aminobutírico (GABA), nos receptores de acetilcolina e nos receptores de octopamina (CASIDA, 2009). Ao atingir seu alvo, a droga causa, de maneira geral, um estímulo exacerbado ou interrupção do impulso nervoso e, consequentemente, a morte do organismo. Quando o sítio de interação da proteína alvo com a droga é alterado, a ligação da droga com o seu alvo diminui, ou é bloqueada, resultando em resistência no organismo (WILSON, 2001).

No carrapato *R. microplus* e em outros artrópodes, este tipo de mecanismo é bem conhecido para a resistência aos piretróides, que têm como sítio alvo o canal de sódio controlado por voltagem. A primeira descrição deste mecanismo ocorreu em 1999, por HE e colaboradores. Atualmente, são descritas três mutações distintas no gene que codifica a

proteína do canal de sódio, que conferem graus de resistência diferentes (HE *et al*., 1999; JONSSON *et al*., 2010; MORGAN *et al*, 2009), e têm ocorrências distintas entre populações de carrapato do México, Austrália, Brasil, Argentina e África do Sul (DOMINGUES *et al*., 2012; GUERRERO *et al*., 2012a; ROSARIO-CRUZ *et al*., 2009b).

Diversos estudos, sobre o carrapato *R. microplus*, têm tentado co-relacionar a resistência aos organofosforados à insensibilidade do seu sítio alvo, a acetilcolinesterase, um mecanismo comumente descrito em insetos (FOURNIER *et al*., 1989; WEILL *et al*., 2002). Estudos de cinética bioquímica e inibição mostram a insensibilidade a organofosforados de acetilcolinesterases provenientes de populações de carrapato resistentes (PRUETT, 2002; WRIGHT & AHRENS, 1988). Porém, nenhuma mutação específica foi, até então, identificada (TEMEYER *et al*., 2009; GUERRERO *et al*., 2012a). Contudo, é observado um aumento de atividade (PRUETT & POUND, 2006; MILLER *et al*., 2008) e da expressão da acetilcolinesterase em populações de carrapato resistentes (BAFFI *et al*., 2008; TEMEYER *et al*., 2012). Isso indica que a amplificação gênica, ou mutações na região promotora, podem ser responsáveis pelo aumento de expressão dessa enzima, podendo ser o mecanismo de resistência para os organofosforados no carrapato (GUERRERO *et al*., 2012).

1.5.3. Aumento da destoxificação da droga

A destoxificação de uma droga abrange três fases: a fase I envolve reações de oxidação e hidrólise, catalisadas pelas enzimas monooxigenases citocromo P450 e esterases, respectivamente. A fase II corresponde à conjugação da droga com glutationa reduzida (GSH), catalisada por glutationa S-transferases (GSTs) (PERRY *et al*., 2011). Em conjunto, estas fases são responsáveis por deixar a droga mais hidrofílica e menos tóxica. Alterações nas enzimas responsáveis por estas fases levam à resistência conhecida como

resistência metabólica (PERRY *et al*., 2011). A fase III corresponde à excreção e/ou sequestro da droga diretamente (também chamada de fase 0), ou dos metabólitos gerados nas duas fases anteriores (ISHIKAWA, 1992; SZAKÁCS *et al*., 2008). Esta etapa é mediada pelo transporte ativo da droga através de transportadores ABC (LAGE, 2003; LESLIE *et al*., 2005; LESPINE *et al*., 2008; SZAKÁCS *et al*., 2008).

1.5.3.1. Aumento da metabolização da droga

As enzimas monooxigenases citocromo P450, esterases e GSTs são coletivamente conhecidas como enzimas de destoxificação (LI *et al*., 2007; PERRY *et al*., 2011). São responsáveis pela metabolização de compostos endógenos e exógenos, fazendo parte de vários processos metabólicos (FREITAS *et al*., 2005; LI *et al*., 2007).

Boa parte dos acaricidas atualmente em uso são ésteres e, consequentemente, são sujeitos à hidrólise por esterases (DEVONSHIRE, 1991). Esterases são hidrolases que promovem a quebra de compostos orgânicos através da adição de água, atuando sobre as ligações éster de compostos endógenos ou exógenos (YAN *et al*., 2009). O aumento da atividade de esterases foi relacionado à resistência a organofosforados e piretróides em várias populações de carrapato em todo o mundo (ABDULLAH *et al*., 2012; BAFFI *et al*., 2008; VILLARINO *et al*., 2003). No entanto, não explica a resistência a estas drogas em outras populações de carrapato (ROSARIO- CRUZ *et al*., 2009b).

As monooxigenases citocromo P450 são enzimas que utilizam NADPH e O_2 para adicionar um grupo funcional hidrofílico à droga, tornando-a menos tóxica (WILSON, 2001). Podem realizar a oxidação de um grande número de substratos diferentes, em geral, hidrofóbicos ou pouco solúveis em água (SCOTT & WEN, 2001). A resistência aos piretróides (MILLER *et al*., 1999) e organofosforados (LI *et al*., 2003) no carrapato *R. microplus* é atribuída à atividade de monooxigenases.

GSTs catalisam a conjugação do grupo tiol da glutationa reduzida (GSH) a uma variedade de moléculas orgânicas, tornando-as mais solúveis em água e facilitando sua excreção (ENAYATI *et al*., 2005). A função de GSTs na resistência a acaricidas no carrapato *R. microplus* é menos conhecidas. DA SILVA VAZ e colaboradores (2004) observaram que vários acaricidas (organofosforados, piretróides, amitraz, ivermectina e flumetrina) inibem a atividade de uma GST recombinante, indicando a interação dela com os acaricidas. LI *et al*. (2004) observaram efeito sinérgico do dietil-maleato (inibidor de GST) em uma de três populações de carrapato resistentes ao amitraz.

1.5.3.2. Excreção e sequestro da droga

Uma etapa importante na destoxificação de uma droga é sua eliminação da célula e/ou sequestro para o interior de vesículas, seja da droga diretamente (não modificada), ou dos compostos gerados nas fases I e II (SZAKÁCS *et al*., 2008). Esta etapa é pouco considerada no estudo da resistência às drogas em artrópodes, mas é intensivamente estudada em células de vertebrados. Uma grande família de proteínas transmembrana, os transportadores ABC, são implicados nesta função e serão discutidos particularmente.

A **Figura 3** resume os mecanismos de resistência que podem existir em um organismo e que foram anteriormente discutidos.

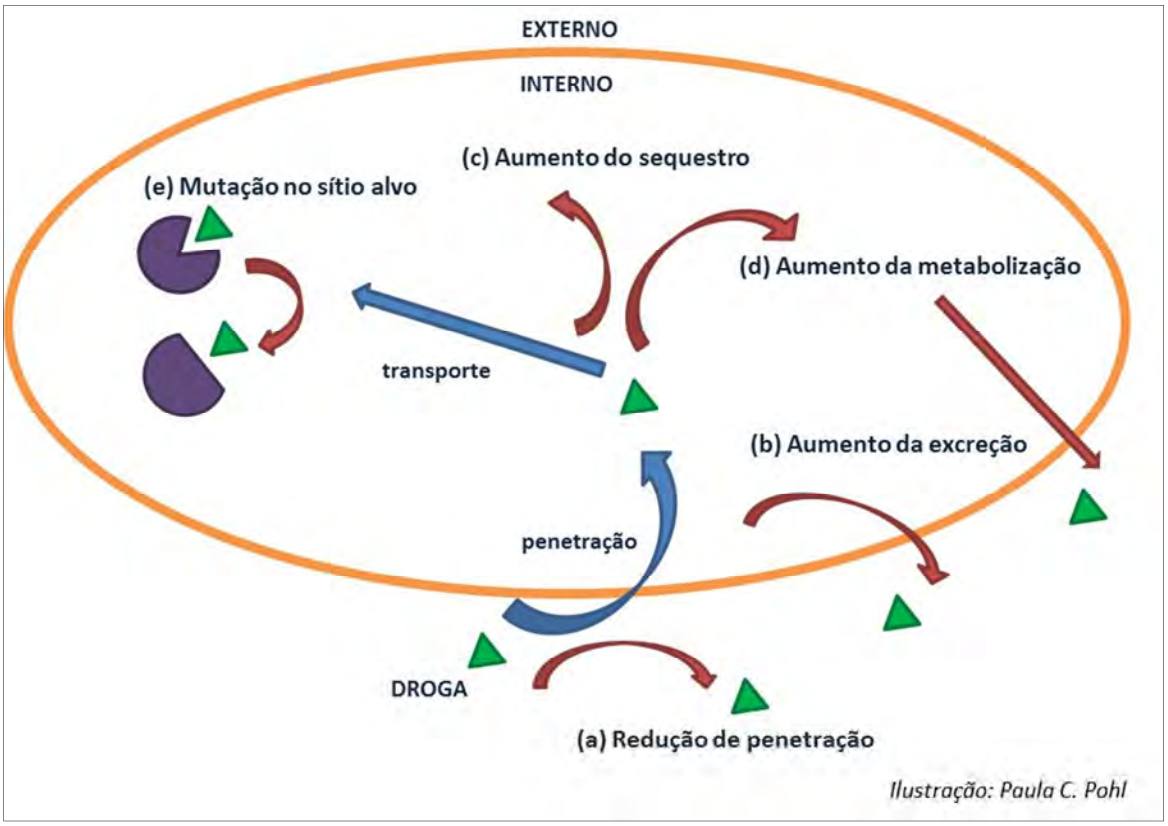


Figura 3- Principais etapas da passagem (setas azuis) e destoxificação de drogas (setas vermelhas) pelo organismo. A droga (triângulos verdes) pode ter sua entrada no organismo diminuída pela redução da penetração **(a)**. Após a penetração da droga, ela pode ser excretada **(b)** e/ou sequestrada **(c),** antes ou após sua metabolização **(d).** Mutações no sítio alvo da droga podem reduzir ou impedir seu efeito **(e).** A resistência por aumento de destoxificação de drogas é determinada pelas etapas **(b)**, **(c)** e/ou **(d)** e a resistência por insensibilidade do sítio alvo é determinada pela etapa **(e)**.

1.6. Transportadores ABC

Os transportadores ABC (*ATP-binding cassete*) pertencem a uma das maiores famílias de proteínas transmembrana e são identificados tanto em células procarióticas quanto eucarióticas (HIGGINS & LINTON, 2003; LAGE, 2003). Há, por exemplo, 69 transportadores ABC em *Escherichia coli* (5% do seu genoma codificante), 58 em *Caenorhabditis*, 51 em *Drosophila* e 48 em *Homo sapiens* (LINTON, 2007). Os transportadores ABC foram primeiramente identificados em bactérias nos anos 70, em que a maioria dos princípios fundamentais relacionados à sua estrutura e função, foram descritos (HIGGINS, 2001). No entanto, somente em 1986 se reconheceu que as subunidades de ligação do ATP, presentes em várias proteínas transportadoras de bactérias até então identificadas, definiam uma grande família de proteínas (HIGGINS *et al*., 1986). Neste mesmo ano, foi descoberto o primeiro transportador ABC eucariótico, a glicoproteína-P (P-gp) de humanos (GROS *et al*., 1986). Porém, o nome transportador ABC foi assumido somente em 1990 (HYDE *et al.*, 1990), consolidando a importância desta família de proteínas evolutivamente relacionada e diversa.

É conhecido hoje, que os transportadores ABC participam do transporte de uma ampla variedade de substratos através de membranas extras e intracelulares, participando, desta maneira, de uma grande variedade de processos fisiológicos (OSWALD *et al*., 2006).

1.6.1. Estrutura e classificação

Tipicamente, um transportador ABC possui dois domínios transmembrana (*transmembrane domains*- TMDs), que consistem em seis alfa-hélices, que atravessam e ancoram a proteína na membrana biológica, formando um poro através do qual o substrato se liga e é transportado. E dois domínios ligadores de ATP (*nucleotide-binding domains*-NBDs), constituídos por uma estrutura alfa/beta voltada para a face citoplasmática da

membrana, que forma um compartimento molecular onde a energia do ATP é liberada (HIGGINS & LINTON, 2003; SCHMITT & TAMBÉ, 2002). O domínio NBD contém dois motivos conservados, *Walker A* e *Walker B,* que são envolvidos com a ligação e a hidrólise do ATP e estão presentes em todas as proteínas ligadoras de ATP. Este domínio apresenta, ainda, um terceiro motivo altamente conservado chamado de *ABC signature*, típico apenas dos membros da família ABC. Outros quatro motivos conservados (*Q-loop, D-loop, H-loop e aromatic*), que também participam da ligação do ATP, são encontrados no domínio NBD (HIGGINS & LINTON, 2003; LINTON, 2007; SCHNEIDER & HUNKE, 1998) (**Figura 4**).

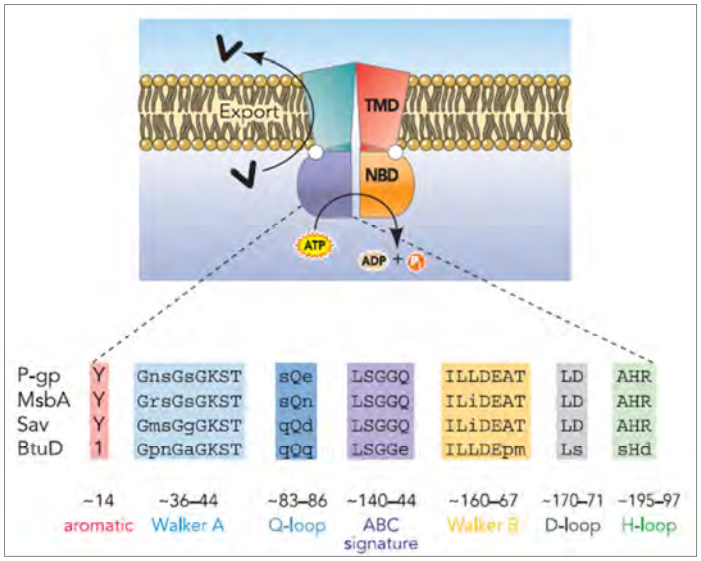


Figura 4- Estrutura mínima de um transportador ABC. Dois domínios TMD ligam e transportam o substrato por meio da energia liberada na hidrólise do ATP, realizada pelos dois domínios NBDs. Em destaque, a sequência de aminoácidos conservados do domínio NBD de diferentes transportadores ABC. P-gp, glicoproteína-P de *Homo sapiens*; MsbA, transportador de lipídeo A e toxinas de *E. coli*; Sav, transportador de drogas de *Staphlococcus aureus*; BtuD, transportador de vitamina B12 de *E. coli*. Fonte: LINTON, 2007.

Os quatro domínios (2 TMDs e 2 NBDs) podem ser formados por diferentes polipeptídeos. O que é comum para transportadores ABC de bactérias, como é o caso do transportador de vitamina B12, BtuCD, de *E. coli* (LINTON & HIGGINS, 1998). Transportadores ABC de eucariotos são normalmente classificados como *half transporters* ou *full transporters*. Nos *full transporters*, os quatro domínios são formados por um mesmo polipeptídeo, enquanto que nos *half transporters*, cada polipeptídeo compõe somente um TMD e um NBD, necessitando de dois polipeptídeos para formar uma estrutura biologicamente ativa, podendo ser homodímeros ou heterodímeros (LAGE, 2003).

Os domínios TMDs são pouco conservados entre os diferentes transportadores ABC. Entretanto, os NBDs são altamente conservados e permitem a classificação destas proteínas em subfamílias. De acordo com o sistema adotado pelo *HUGO Gene Nomenclature Committee* (HGNC, 2012), os transportadores ABC se dividem em cinco subfamílias: ABCA, ABCB, ABCC, ABCD e ABCG. Existem, ainda, duas subfamílias adicionais, ABCE e ABCF. Estas não possuem os domínios transmembrana, portanto não apresentam função de transporte, mas são envolvidas em processos celulares, como reparo de DNA e regulação da expressão gênica (DASSA, 2003; OSWALD *et al*., 2006). Os membros recebem nomes, de acordo com a subfamília a que pertencem, como ABCC1, subfamília C, membro 1. Também podem receber pseudônimos que abreviam suas funções. Neste caso, o ABCC1 também é conhecido como MRP1 (*multidrug resistance protein 1*) (DEAN *et al*., 2001; DASSA, 2003).

Os transportadores ABC são também classificados como importadores ou exportadores. Uma vez que o ATP é encontrado no citosol, um importador é definido como o transportador ABC que transporta moléculas para o interior do citosol. Em contraste, um exportador é o transportador ABC que realiza o transporte de moléculas para fora do

citosol, mesmo que a localização final seja uma organela intracelular. Enquanto os transportadores ABC eucarióticos coordenam aparentemente somente o efluxo de moléculas, transportadores ABC bacterianos são divididos em importadores e exportadores (DASSA, 2003).

1.6.2. Mecanismo de ação e interação com o substrato

Os detalhes do mecanismo pelo qual os transportadores ABC movem um soluto através de membranas celulares ainda não estão bem claros. Estudos bioquímicos, farmacológicos e a disponibilização de algumas estruturas tridimensionais de transportadores ABC, permitiram porém, algumas especulações sobre o transporte de substratos (BORTHS *et al.*, 2002; MARTIN *et al.*, 2001; YAO *et al.*, 1994). Essencialmente, os transportadores precisam alternar estados de alta e baixa afinidade com o substrato. Sabe-se que o ciclo de transporte é iniciado quando o substrato se liga com os TMDs na face intracelular da membrana. A ligação do substrato com os TMDs induz mudanças conformacionais que são transmitidas aos domínios NBDs, que interagem mais intrinsicamente e aumentam a afinidade pelo ATP. A ligação com o ATP, por sua vez, induz nova mudança conformacional nos domínios TMDs, que sofrem uma reorientação, permitindo uma ligação de menor afinidade com o substrato e fazendo com que ele se desligue. O ATP é hidrolisado, ADP e Pi são liberados e o transportador retorna ao estado conformacional original (HIGGINS & LINTON, 2003; KREWULAK & VOGEL, 2008; LINTON, 2007) (**Figura 5**).

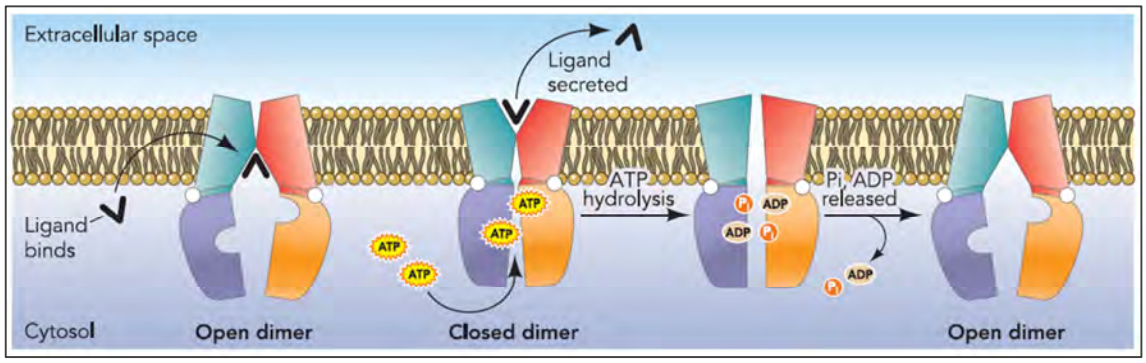


Figura 5- Mecanismo de ação de um transportador ABC. Fonte: LINTON, 2007.

A interação molecular dos transportadores ABC com seu substrato também é pouco conhecida. A variedade de substratos transportados por diferentes transportadores é enorme. Inclui aminoácidos, açúcares, íons inorgânicos, lipídios, polissacarídeos, peptídeos, proteínas e drogas (HIGGINS, 2001; HOLLAND & BLIGHT, 1999). Muitos transportadores ABC interagem com um único substrato, outros, entretanto, apresentam uma diversidade ampla de substratos, incluindo moléculas quimicamente muito diferentes, como é o caso dos transportadores de múltiplas drogas, ou *multidrug transporters*, como são conhecidos em inglês (HIGGINS, 2007). Tal heterogeneidade dificulta a elucidação das bases moleculares do reconhecimento de um substrato por essas proteínas.

Acredita-se que a ligação do substrato com os domínios TMDs ocorre através de pontes de hidrogênio e interações iônicas mais fracas, como forças de Van der Walls. A dissociação do complexo transportador-substrato é dependente do número e a força das ligações entre os dois, ditando se uma molécula será transportada ou funcionará como um inibidor (SEELIG, 1998). Por exemplo, é sugerido que o inibidor de transportadores ABC, ciclosporina A (CsA), apresenta alta afinidade por transportadores ABC de algumas classes, formando um grande número de ligações hidrofóbicas com estes, o que resulta em uma baixa taxa de dissociação entre eles e caracteriza uma inibição competitiva (SEELIG

& LANDWOJTOWICZ, 2000). Pesquisas também mostram que existem alguns resíduos nos domínios TMDs que são mais críticos na interação transportador-substrato (AMBUDKAR *et al*., 1999). Por exemplo, mutações nos resíduos 939 e 941 do segmento 11 da P-gp bloqueiam a formação de pontes de hidrogênio e prejudicam a ligação da proteína com seu substrato (KAJIJI *et al*., 1993).

1.6.3. Funções biológicas

A diversidade de substratos dos transportadores ABC se reflete na diversidade de papéis fisiológicos nos quais eles participam. Os transportadores ABC apresentam funções muito conservadas durante a evolução, e que estão relacionadas à nutrição e patogênese em bactérias, formação de esporos em fungos, transdução de sinal, secreção de proteínas e apresentação de antígenos em mamíferos (DASSA, 2003; PONTE-SUCRE, 2007).

No homem, as mutações em transportadores ABC são responsáveis por diversas doenças genéticas como a fibrose cística, causada por uma mutação no gene *ABCC7*/*CFTR* (*cystic fibrosis transmembrane conductance regulator*), que é responsável pela homeostase de íons (principalmente Cl^-) nas superfícies epiteliais dos tratos respiratórios, intestinais e reprodutivos (HANRAHAN & WIOLAND, 2004). E a adrenoleucodistrofia ligada ao X (X-ALD), que é causada por uma mutação no gene *ABCD1/ALDP*, que codifica um transportador ABC peroxissomal, causando o acúmulo de ácidos graxos de cadeia longa no plasma e tecidos e, consequente, redução da oxidação dos mesmos (MORITA *et al*., 2011).

1.6.3.1- Transporte de drogas e outras moléculas com potencial tóxico

Os transportadores ABC são reconhecidos por sua habilidade em modular a absorção, distribuição e excreção de toxinas nos organismos, influenciando, desta forma, na disposição destas nos mesmos (LESLIE *et al*., 2005; SZAKÁCS *et al*., 2008).

Coletivamente, eles são capazes de transportar uma vasta e quimicamente diversa quantidade de toxinas endógenas e exógenas, incluindo moléculas tóxicas derivadas da dieta como a aflotoxina B1, carcinógenos ambientais, como nitrosaminas derivadas do tabaco, drogas usadas nos tratamentos de doenças e infecções, produtos da peroxidação lipídica e moléculas com potencial redox (LESLIE *et al*., 2005).

A homeostase do heme (ferro-protoporfirina IX) dentro das células é estritamente regulada, uma vez que, quando livre, esta molécula apresenta grande potencial de gerar espécies reativas de oxigênio que podem causar danos à célula. O envolvimento dos transportadores ABC no transporte de heme é bem caracterizado em bactérias (WANDERSMAN & DELEPELAIRE, 2004; KREWULAK & VOGEL, 2008), em que o heme é importante para suprir as necessidades de ferro destes organismos. Em eucariotos, a importância dos transportadores ABCB6, ABCB7, ABCB10 e ABCG2, no metabolismo do heme é sugerida. No entanto, nenhuma evidência de sua participação no transporte de heme, diretamente, foi descrita (KRISHNAMURTHY *et al*., 2007; KRISHNAMURTHY & SCHUETZ, 2011; LYNCH *et al*., 2009).

O transporte de drogas pelos transportadores ABC tem importância fundamental na defesa dos organismos. No homem, diversos transportadores ABC são encontrados em tecidos importantes na absorção de drogas (como os pulmões e o intestino) e eliminação de drogas (como os rins e o fígado). Estão presentes também, nas barreiras hematoencefálica, hematotesticular e hematoplacentária (LESLIE *et al*., 2005). Porém, esta capacidade de transportar drogas, das mais diferentes classes, implica no desenvolvimento de resistência nos organismos que é, sem dúvida, a função mais descrita na literatura.

1.6.3.2- Resistência a drogas

O papel dos transportadores ABC na resistência às drogas é descrito em bactérias, fungos, endoparasitas e ectoparasitas (KERBOEUF *et al*., 2003; KOENDERINK *et al*., 2010; LAGE, 2003; LESPINE *et al.*, 2008; POELARENDS *et al*., 2002). Além disso, em humanos, estes transportadores estão associados à quimiorresistência nos tratamentos de câncer (NATARAJAN *et al*., 2012; WU *et al*., 2011). Este quadro dificulta enormemente o tratamento dessas doenças, infecções e parasitoses, principalmente, porque os transportadores ABC são relacionados à resistência a múltiplas drogas, limitando o número de drogas disponíveis para o tratamento delas. (LAGE, 2003; LESLIE *et al*., 2005).

Em um organismo sensível, o número de transportadores ABC nas células é reduzido e o efluxo da droga não é suficiente para limitar sua eficácia (**Figura 6 A**). No entanto, em um organismo resistente, o número e/ou a atividade destes transportadores aumenta e a droga é rapidamente eliminada das células ou exportada para o interior de vesículas (sequestro), reduzindo a concentração da mesma no organismo e prejudicando sua eficácia (LESPINE *et al*., 2008) (**Figura 6 B**).

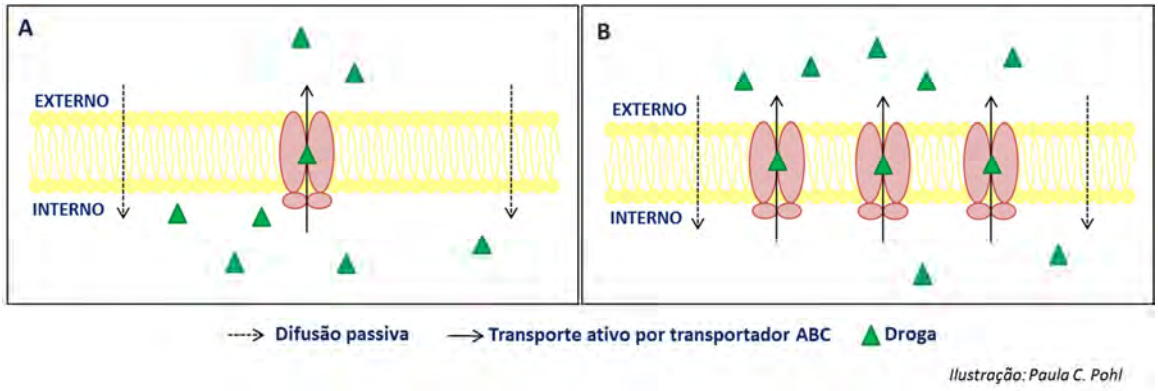


Figura 6- Papel dos transportadores ABC na destoxificação de drogas. (**A**) Em células sensíveis, os transportadores ABC eliminam a droga contra o gradiente de concentração. (**B**) Quando superexpressos, como ocorre em uma célula resistente, os transportadores ABC eliminam a droga, eficientemente, diminuindo sua eficácia e protegendo o organismo.

O aumento de expressão dos transportadores ABC é o principal mecanismo molecular associado ao desenvolvimento de resistência. Em vários tipos de câncer, incluindo leucemia mielóide aguda, câncer de mama e câncer do colo do útero, o aumento de expressão da P-gp (ABCB1) é associado à resistência a diversas drogas, como alcaloides de vinca, etoposide, colchicina, taxanos, camptotecina, entre outros (LAGE, 2003; WU *et al*., 2011). Outros transportadores ABC que também demonstram conferir resistência a múltiplas drogas quando superexpressos em mamíferos são o ABCC1 (MRP1) e ABCC2 (MRP2), para etoposide, cisplatina, antraciclinas, alcaloides de vinca, saquinavir, ironotecano, e o ABCG2 (BCRP) que confere resistência à maioria dos inibidores de topoisomerase I e II, topodecano, docetaxel, saquinavir, doxorubicina, entre outros (DEELEY *et al*., 2006; LAGE, 2003; WU *et al*., 2011). Em *Candida albicans*, o aumento da expressão do transportador ABC *Cdr1p* é observado em populações resistentes

ao fluconazol. Principal droga utilizada no tratamento da candidíase (HOLMES *et al*., 2008; SANGLARD *et al*., 1995). O aumento da expressão deste transportador confere, também, resistência a drogas estruturalmente não relacionadas como terbinafina, brefildin A, cerulenina e nigericina (NIIMI *et al*., 2004). Em nematódeos parasitas, como *Haemonchus contortus* e *Onchocerca volvulus*, e de vida livre, como *Caenorhabditis elegans*, o aumento de expressão de transportadores ABC se relaciona com resistência à ivermectina e moxidectina (HUANG & PRICHARD, 1999; JAMES & DAVEY, 2009; XU *et al*., 1998).

Curiosamente, em *Plasmodium falciparum*, o agente causador da malária, mutações em diferentes posições do gene que codifica o transportador ABC PfMDR1, são atribuídas à diminuição de sensibilidade do parasita a drogas como a cloroquina e amodiaquina, enquanto que o aumento de expressão desse gene não é observado em populações resistentes (KOENDERINK *et al*., 2010). Isso demonstra que o aumento de expressão de genes de transportadores ABC não é o único mecanismo molecular responsável por conferir resistência. Semelhantemente, foi observado que uma mutação na região transmembrana (posição 482) do transportador ABCG2 humano aumenta a resistência à antraciclina, indicando que a mutação em um único resíduo de aminoácido é capaz de alterar a especificidade aos substratos (HONJO *et al*., 2001).

Em ectoparasitas, a função de transportadores ABC na resistência a drogas é também identificada, apesar de ser menos descrita. No copépodo *Lepeophtheirus salmonis*, um ectoparasita de salmões, a expressão de transportadores ABC foi induzida quando estes foram tratados com emamectina (TRIBBLE *et al*, 2007). No piolho *Pediculus humanus humanus*, o gene codificador do transportador ABC PhABCC4 foi induzido após a seleção de resistência à ivermectina (YOON *et al*., 2011). Nos mosquitos *Culex pipiens* e *Aedes caspius*, a toxicidade de diferentes inseticidas foi aumentada quando as larvas foram

tratadas com verapamil, um inibidor de transportadores ABC (BUSS *et al*., 2002; PORRETTA *et al*., 2008).

1.7. Ivermectina: características e mecanismos de resistência

O grupo das lactonas macrocíclicas, dividido em avermectinas (ivermectina, abamectina e doramectina) e milbemicinas (moxidectina), são compostos estruturalmente relacionados e derivados da fermentação dos actinomicetos *Streptomyces avermetilis* e *Streptomyces hygroscopicus*, respectivamente (BURG *et al*., 1979; TAKIGUCHI *et al*., 1980). São moléculas grandes, hidrofóbicas e caracterizadas pela presença de um anel macrocíclico (LESPINE *et al*., 2006).

As lactonas macrocíclicas estão entre as classes de drogas antiparasitárias de maior sucesso. Elas são amplamente utilizadas no controle de endoparasitas e ectoparasitas, incluindo o *R. microplus* (BENZ *et al*.,1989; FOX, 2006). Em 1981, a ivermectina foi a primeira lactona macrocíclica a ser comercializada e, dentro de um período de dois anos, tornou-se uma das drogas líderes no mercado de antiparasitários no mundo todo. Em 1985, ela foi comercializada para o controle de pragas agrícolas e, em 1986, foi aprovada para uso em humanos (OMURA, 2008). Em bovinos, a ivermectina é usada para o controle de endoparasitas como *Ostertagia ostertagi* e *Cooperia oncophora* (EDMONDS *et al*., 2010) e ectoparasitas como o piolho *Haematopinus eurysternus* e os ácaros *Sarcoptes scabiei* var. *bovis* e *R. microplus* (BENZ *et al*., 1989; KLAFKE *et al*., 2006; SOLL *et al*., 1992).

A eficácia da ivermectina é atribuída à sua ligação aos canais de cloro controlados por glutamato, presentes nas células musculares e nervosas dos invertebrados. A abertura destes canais, promovida pela ligação da ivermectina, resulta em um lento e irreversível aumento da condutância aos íons cloreto na membrana, levando ao bloqueio da

neurotransmissão, paralisia das funções motoras periféricas e, em última análise, causando a morte do parasita (FOX, 2006; OMURA, 2008). Em altas concentrações, a ivermectina pode também se ligar aos canais de cloro controlados por GABA em invertebrados e vertebrados. Como em mamíferos, eles estão presentes em neurônios que se encontram protegidos pela barreira hematoencefálica, a droga é segura para uso nestes (OMURA, 2008).

Como resultado da massiva utilização da ivermectina, a seleção de resistência é descrita em um grande número de parasitas, incluindo os nematódeos *O. volvulus* (TOWNSON *et al*., 1994), *Cooperia spp* (COLES *et al*., 2001) e *H. contortus* (COLES *et al*., 2005; KAPLAN, 2004) e os ácaros *Tetranychus urticae* (KWON *et al*., 2010), *S. scabiei* (CURRIE *et al*., 2004) e *R. microplus* (MARTINS & FURLONG, 2001; KLAFKE *et al.*, 2006). Os mecanismos de resistência a essa droga foram estudados nestas e em outras espécies, e foram atribuídos à insensibilidade do sítio alvo, canal de cloro controlado por glutamato, em *C. elegans* (GLENDINNING *et al*., 2011) e *T. urticae* (KWON *et al*., 2010) e à atividade das enzimas de destoxificação, principalmente P450, em *P. h. humanus* (YOON *et al*., 2011) e *Leptinotarsa decemlineata* (ARGENTINE *et al*., 1992). Contudo, o mecanismo predominantemente descrito, é a destoxificação mediada por transportadores ABC, em diversas espécies de nematódeos como *C. elegans* (JAMES & DAVEY, 2009), *H. concortus* (BARTLEY *et al*., 2009; LIFSCHITZ *et al*., 2010a), *Trichostrongylus colubriformis* (BARTLEY *et al*., 2009; DICKER *et al.*, 2011) *O. vulvulus* (BOURGUINAT *et al*., 2008), *O. ostertagi* (LIFSCHITZ *et al*., 2010b) e artrópodes como *S. scabiei* (MOUNSEY *et al*., 2010), *P. h. humanus* (YOON *et al*., 2011), *C. pipiens* (BUSS *et al*., 2002) e *Chironomus riparius* (PODSIADLOWSKI *et al*., 1998). No carrapato, porém, a resistência à ivermectina e outras lactonas macrocíclicas é pouco

estudada, e os mecanismos moleculares de resistência a esta droga são desconhecidos, apesar da sua importância para o controle desse parasita.

1.8. Alternativas ao uso de animais para o estudo do carrapato

O carrapato *R. microplus,* por ser uma espécie com alta especificidade por seu hospedeiro (PEREIRA & LABRUNA, 2008), requer bovinos para sua manutenção em laboratório e para a realização de experimentos. Algo que se torna complexo e custoso (GONSIOROSKI *et al*., 2012). Neste sentido, duas alternativas ao uso de animais têm sido empregadas e têm provido resultados em pesquisas que abordam diferentes tópicos de estudo. São elas: a alimentação *in vitro* do carrapato e o uso de linhagens celulares.

1.8.1. Alimentação *in vitro*

A alimentação de carrapatos *in vitro,* ou artificial, como também é chamada, foi padronizada em várias espécies de carrapato, como *Rhipicephalus appendiculatus* (WALADDE *et al*., 1993), *Dermacentor variabilis* (MACALUSO *et al*., 2001), *Ixodes ricinus* (KRÖBER & GUERIN, 2007a), *Dermacentor nitens* (RANGEL *et al*., 2008), *Amblyomma cajennense* (ABEL *et al.,* 2008) e também *R. microplus* (GONSIOROSKI *et al*., 2012), em que é empregada para o estudo de diferentes aspectos fisiológicos do carrapato. Por exemplo, no carrapato *I. ricinus,* a alimentação *in vitro* foi aplicada para avaliar o efeito de diferentes concentrações dos acaricidas fibronil e ivermectina, na taxa de mortalidade destes carrapatos (KRÖBER & GUERIN, 2007a) e de anticorpos contra proteínas do carrapato, no tempo de alimentação e ingurgitamento das fêmeas. No carrapato *R. microplus*, a alimentação *in vitro* foi empregada para analisar o efeito de inibidores de enzimas, anticorpos e acaricidas, sobre diferentes aspectos fisiológicos do

carrapato, como mortalidade, ingurgitamento, postura e eclosão dos ovos (FABRES *et al*., 2010; GONSIOROSKI *et al*., 2012; POHL *et al*., 2011).

A principal vantagem da alimentação *in vitro*, é a possibilidade de quantificar os efeitos da administração de doses precisas de uma substância na fisiologia do carrapato, sob condições controladas, além de reduzir os custos com aquisição de animais (KRÖBER & GUERIN, 2007b).

1.8.2. Cultura de células

Outra ferramenta útil é a cultura de células. O desenvolvimento de sistemas de cultivo *in vitro* a partir de tecidos de hospedeiros e vetores, principalmente linhagens contínuas, contribuiu de maneira significativa para a pesquisa sobre carrapatos (BELL-SAKYI *et al*., 2007). Ao longo dos últimos 30 anos, após o estabelecimento da primeira linhagem de células do carrapato *R. appendiculatus* (VARMA *et al*., 1975), mais de 40 linhagens celulares, de 13 espécies de carrapatos, foram também estabelecidas e utilizadas em estudos de interação vetor-patógeno, biologia do carrapato, genômica, proteômica e manipulação genética (BELL-SAKYI *et al*., 2007). A linhagem BME26, proveniente de tecidos embrionários do carrapato *R. microplus* (ESTEVES *et al*., 2008), tem sido utilizada para analisar diversos processos biológicos que ocorrem no carrapato, como a resposta imune a patógenos (ESTEVES *et al*., 2009) e o metabolismo de carboidratos (DE ABREU *et al*., 2009).

2. OBJETIVOS

Diversos relatos da literatura suportam a importância dos transportadores ABC na defesa da célula contra moléculas tóxicas, sejam elas provenientes do metabolismo do organismo (como a molécula de heme) ou provenientes do ambiente (como os acaricidas). Contudo, a participação dos transportadores ABC na destoxificação de moléculas no carrapato *R. microplus* nunca foi explorada. Uma vez que, determinar o papel dos transportadores ABC no carrapato pode ter valiosa importância no seu controle, o presente trabalho possui os objetivos que se seguem:

2.1. Objetivo geral

Caracterização da importância fisiológica dos transportadores ABC na destoxificação de compostos tóxicos, com ênfase ao acaricida ivermectina, no carrapato *R. microplus.*

2.2. Objetivos específicos

a- Determinar a participação de transportadores ABC na destoxificação da ivermectina e outros acaricidas em populações de carrapato sensíveis e resistentes aos acaricidas;

b- Estabelecer uma linhagem de células de carrapato resistentes a ivermectina, determinando a participação de transportadores ABC no desenvolvimento da resistência *in vitro*;

c- Caracterizar a participação de transportadores ABC na destoxificação de acaricidas e da molécula de heme no intestino do carrapato.

3. RESULTADOS

3.1. TRANSPORTADORES ABC NA RESISTÊNCIA À IVERMECTINA E OUTROS ACARICIDAS NO CARRAPATO

ARTIGO I

ABC transporter efflux pumps: a defense mechanism against ivermectin in *Rhipicephalus (Boophilus) microplus*.

Paula C. Pohl[a], Guilherme M. Klafke[b], Danielle D. Carvalho[c], João Ricardo Martins[d], Sirlei Daffre[b], Itabajara da Silva Vaz Jr[a,e,#], Aoi Masuda[a,f]

[a]Centro de Biotecnologia , Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; [b]Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brazil; [c]Departamento de Análises Especiais, SD&W Modelagem e Soluções Estratégicas Ltda, São Paulo, Brazil; [d]Instituto de Pesquisas Veterinárias Desidério Finamor- FEPAGRO, Eldorado do Sul, Brazil; [e]Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; [f]Departamento de Biologia Molecular e Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

International Journal for Parasitology 41 (2011) 1323- 1333.

Abstract

ATP-binding cassette (ABC) transporters are efflux transporters found in all organisms. These proteins are responsible for pumping xenobiotic and endogenous metabolites through extra- and intracellular membranes, thereby reducing cellular concentrations of toxic compounds. ABC transporters have been associated with drug resistance in several nematodes and parasitic arthropods. Here, the ability of ABC transporter inhibitors to enhance ivermectin (IVM) sensitivity was tested in larvae and adult females of *Rhipicephalus (Boophilus) microplus*. Larvae of susceptible and IVM-resistant tick populations were pre-exposed to sublethal doses of the ABC transporter inhibitors Cyclosporin A (CsA) and MK571 and subsequently treated with IVM in a Larval Packet Test (LPT). ABC transporter inhibition by both drugs significantly reduced the LC_{50} values of four IVM-resistant populations, but IVM sensitivity of a susceptible population remained unchanged. IVM sensitivity in adults was assessed through an artificial feeding assay. The addition of CsA to blood meal substantially affected IVM toxicity in adult female ticks from a resistant population, by reducing oviposition and egg viability, though it did not alter IVM toxicity in susceptible females. Three partial nucleotide sequences with similarity to ABC transporters were retrieved from the *DFCI Boophilus microplus Gene Index*. Their transcriptional levels in the midgut of resistant and susceptible females were determined by qPCR, showing that one of these sequences was significantly up regulated in IVM-resistant females and suggesting its participation in IVM detoxification. This work reports for the first time the participation of ABC transporters in IVM resistance in *R. microplus*.

Keywords: ivermectin; resistance; ATP-binding cassette transporter; acaricide detoxification; tick control

1. Introduction

The selection of pesticide resistance in arthropod populations is one of the main obstacles to the chemical control of important vector species (Rosario-Cruz et al., 2009). In the last 20 years, almost one century after the first report of arthropod resistance (Melander, 1914), an increase in new cases of resistance has been reported for various parasite species (FAO, 2004).

The cattle tick *Rhipicephalus (Boophilus) microplus* (Acari: Ixodide) is one of the most important ectoparasites in tropical and subtropical areas of the world, being responsible for severe economic losses to cattle production manifested as blood loss and hide damage (Jonsson, 2006; Seixas et al, in press). Moreover, it is the most important vector of cattle disease agents like *Babesia* spp. and *Anaplasma* spp (Jonsson et al., 2008; Kocan et al., 2000). In several countries its resistance to most acaricides has been confirmed, which represents a worldwide drawback for successful tick control (Castro-Janer et al., 2010; Dos Santos et al., 2009; Klafke et al., 2006; Martins and Furlong, 2001). In such scenario, defining molecular mechanisms behind acaricide resistance in *R. microplus* is crucial for parasite control efforts, since more detailed knowledge on this problem could become a fresh framework for the development of new acaricides, fostering the progress of faster and more sensitive resistance detection methods (Rosario-Cruz et al., 2009).

Macrocyclic lactones (MLs) are among the most successful classes of anti-parasitic drugs. They are widely used for the control of endo- and ecto-parasites, including *R. microplus* (revised by Geary, 2005 and Fox, 2006). MLs activate glutamate-gated (GluCl) and/or gamma-aminobutyric acid-gated chloride ion channels in nerve and muscle cells in arthropods and nematodes, leading to paralysis of peripheral motor function, inhibition of feeding and reproduction and, ultimately, death (Fox, 2006; Wolstenholme

and Rogers, 2005). Despite the positive results MLs have afforded in terms of parasite control, the selective pressure caused by the massive application of these drugs in the past few years has triggered the development of resistance in a number of these parasites, including the nematodes *Onchocerca volvulus* (Townson et al., 1994), *Cooperia spp* (Coles et al., 2001) and *Haemonchus contortus* (Coles et al., 2005; Kaplan, 2004), as well as the mites *Tetranychus urticae* (Kwon et al., 2010) and *Sarcoptes scabiei* (Currie et al., 2004). Currently, IVM is one of the ML most used for tick control (Mendes et al., 2011; Perez-Cogollo et al., 2010). As a result, cattle tick populations resistant to IVM have been reported in Brazil since 2001 (Klafke et al., 2006; Martins and Furlong, 2001) and, more recently, in Mexico (Perez-Cogollo et al., 2010) and Uruguay (Castro-Janer et al., 2011).

At present, the molecular basis of resistance to MLs is not well understood. Insensitivity of the GluCl receptor, which prevents drug binding to its target site, has been associated with IVM resistance in some nematodes and arthropods (Dent et al, 2000; Kwon et al., 2010; McCavera et al., 2009). In arthropods, ML resistance is also associated with an increase in oxidative metabolism (Argentine et al., 1992; Scott, 1989) and a decrease in drug penetration (Scott, 1989). Although multiple factors can influence drug resistance, the final concentration of a drug in the parasite is a key determinant for it efficacy, and is dictated by drug absorption, distribution and elimination parameters. Recently, it has become evident from molecular, biochemical and pharmacokinetic studies that the most important molecules involved in all these processes are ABC transporter proteins (Bourguinat et al., 2011; Lespine et al., 2008).

ABC transporters comprise a superfamily of membrane-integrated proteins expressed in all organisms, from bacteria to humans. They are essential to several physiological processes, since they are responsible for the translocation of a wide variety of substrates, including amino acids, metal ions, peptides, metabolites, and toxins (Holland

and Blight, 1999). One of their most studied functions is cellular defense (Holland and Blight, 1999; Leslie et al., 2005), when these transporters actively pump a broad range of structurally and chemically different compounds (as multidrug transporters) out of the cell against their concentration gradients in an ATP-dependent process, mediating multidrug resistance (MDR) mechanisms, some of which have been well characterized in cancer cells and pathogens (revised by Higgins, 2007 and Lage, 2003).

Most ABC transporters share a fundamental structure composed of four domains: two multiple membrane-spanning domains that are poorly conserved between orthologues from different organisms and two well conserved nucleotide-binding domains (NBDs). In humans, 49 ABC transporters have been identified, based on sequence homology and protein organization, and are divided into seven subfamilies, designated ABCA to ABCG (Dean et al., 2001). Only members of the families ABCB (ABCB1, P-glycoprotein, P-gp), ABCC (ABCC1-5, multidrug resistance-associated proteins, MRPs) and ABCG (ABCG2, *breast cancer resistance protein*, BCRP) appear to be associated with MDR (Leslie et al., 2005). The overexpression of these genes has been associated with drug resistance in cancer cells and constitutes one of the molecular mechanisms responsible for treatment failure (Kuo, 2007). Also, overexpression of human ABC transporter homologues has been associated with drug resistance in other organisms. IVM resistance has been associated with the overexpression of the genes *mrp-1* and *pgp-1* in the nematode *Caenorhabditis elegans* (James and Davey, 2009) and with overexpression of *pgp-1* in the mite *S. scabiei* (Mounsey et al., 2010).

Taking into account the importance of ABC transporters, the aim of the present study was to investigate their involvement in IVM resistance in *R. microplus*. Inhibitors of ABC transporters were used to assess their potential to enhance the susceptibility of resistant tick populations to IVM in two life stages: larvae, analyzed through LPT, and

adult females, by an artificial capillary feeding assay. To establish the possible molecular mechanisms of IVM resistance, we also determined the transcriptional levels of three ABC transporter genes belonging to the ABCB and ABCC subfamilies. The results reported here show the first evidence of the participation of ABC transporters in acaricide detoxification by *R. microplus*.

2. Materials and methods

2.1. Cattle

Six-month-old Hereford steers were obtained from a tick-free area and housed in individual tick-proof pens on slatted floors at the Faculdade de Veterinária from the Universidade Federal do Rio Grande do Sul, Brazil. The animals were infested with 15-day-old tick larvae (Parizi et al., 2011). Twenty-one days post-infestation, partially engorged female ticks were manually removed from cattle, and detached fully engorged female ticks were collected from the floor. All experiments were conducted following the guidelines of the Ethics Committee on Animal Experimentation of the same university.

2.2. Tick strains

The Porto Alegre tick strain (POA), obtained by experimental infestation on bovines, was used as susceptible control. This strain was originally collected in the district of Porto Alegre, state of Rio Grande do Sul (Brazil), in a farm without history of acaricide use and was established in our laboratory, been maintained under standard laboratory conditions in the absence of acaricide exposure for multiple generations. This strain has been used as susceptible reference strain to cypermetrhin, deltamethrin and flumethrin (Martins et al., 1995), ivermectin (Klafke et al., 2006) and fipronil (Castro-Janer et al,

2010). Engorged female ticks of field populations were collected from cattle on farms located in the municipality of Jacareí, State of São Paulo, Brazil (JUA population) and in the municipalities of Pântano Grande, São Gabriel and Alegrete, State of Rio Grande do Sul, Brazil (PNO, SGA and CAV populations, respectively). In all farms, MLs were used for tick control. Detached engorged females were maintained at 27–28 °C and 80–90% relative humidity. Following oviposition, eggs were transferred to 3-mL glass vials which were then plugged with a cotton cap. Larval hatching occurred approximately 30 days after collection of engorged females. Bioassays were performed with 14- to 21-day larvae.

2.3. In vitro selection of resistant larvae and colony maintenance

Twenty engorged females of the JUA strain (generations F1 and F2) were placed in a 100-mL plastic container with 20 mL of ethanol 1% solution containing IVM at 100 ppm (technical grade ivermectin, Sigma–Aldrich, USA), as this concentration allowed the oviposition of treated females. Ticks were kept in solution for 30 min at room temperature with gentle agitation. Afterwards, females were dried on paper towels and glued dorsally onto double-sided sticky tape in a Petri dish for oviposition. After 14 days, egg masses were transferred to glass tubes for larval hatching. Larvae (F2 and F3 generations) from treated adults (F1 and F2 generations) were used to infest bovines for colony maintenance. Larvae from generation F1 and F3 were used for resistance assessment.

2.4. Larvae bioassays

2.4.1. Larval Packet Test (LPT) with pre-exposure to a single concentration of ABC transporter inhibitors

The LPT was used to investigate IVM resistance of *R. microplus* following FAO protocol (1984). Briefly, technical grade IVM was diluted at 1% in a 2:1 (v/v)

trichloroethylene and olive oil vehicle (IVM stock solution). Then, the IVM stock solution was diluted to seven concentrations, from 0.03% to 0.3%, in the same vehicle. A volume of 0.67 mL of each IVM dilution or vehicle alone (control) was applied on a filter paper (Whatman 541, 850 mm x 750 mm; FAO, 1984). Trichloroethylene was evaporated for 24 hours. Previously to the LPT, larvae of the POA susceptible reference strain and field populations were exposed to ABC transporter inhibitors by immersion. The inhibitors CsA and MK571 (Sigma–Aldrich, USA) were diluted to 5 µM (predetermined sublethal dose, data not shown) in 1% ethanol containing 0.02% Triton-X 100. Approximately 100 larvae were transferred to microcentrifuge tubes containing 0.5 mL of inhibitor solution or control solution (diluent alone). After a 10-min immersion in each solution, larvae were dried and placed in filter papers impregnated with IVM. The larval packets were sealed and incubated at 27–28 °C and 80-90% relative humidity for 24 h, and mortality was determined. Only larvae capable of locomotion were considered alive. Each experiment was performed at least three times.

2.4.2. LPT with a single dose of IVM and pre-exposure to increasing concentrations of ABC transporter inhibitors

Larvae of the POA susceptible reference strain and JUA F3 strain were exposed by immersion to four concentrations of CsA and MK571 (5, 15, 30 and 45 µM) or diluent alone, as described above. Larvae were transferred to filter papers impregnated with vehicle alone or IVM 0.15% (as this concentration allowed the survival of at least 20% of the susceptible larval strain in order to observe the effects of the inhibitors). The packets were sealed and incubated at 27–28 °C and 80–90% relative humidity for 24 h, and mortality was determined as described above. Each experiment was replicated at least three times.

2.4.3. LPT data analysis

Mortality data was submitted to probit analysis and a chi-square test was used to test the hypothesis of parallelism and equality ($p \leq 0.05$) with POLO PLUS software (LeOra software, 2004) to estimate the lethal concentrations for 50% (LC_{50}) with its confidence intervals of 95% (CI 95%). Resistance ratios (RR) were calculated in relation to POA reference strain based on LC_{50} values. To estimate the toxicity caused by the addition of the ABC transporter inhibitors, synergism factors (SF) were calculated based on LC_{50} values from control conditions in each population. Significance of each comparison was determined only if no overlap was observed in the confidence intervals.

2.5. Administration of IVM and ABC transporters inhibitors by artificial feeding

Partially-engorged tick females (between 25 and 70 mg) from POA and JUA strains were removed from experimentally infested animals and clustered in seven groups with 25 ticks each. Females were fixed on expandable polystyrene plates with double-sided sticky tape. Blood from non-infested bovines was collected in tubes with sodium citrate and administered to females with microhematocrit capillary tubes (Fabres et al., 2010). Blood treatments were (i) blood control (blood alone), (ii) CsA control (50 µL of blood with 1 µM CsA followed by blood alone until female repletion), (iii) MK571 control (50 µL of blood with 1 µM MK571 followed by blood alone until female repletion), (iv) IVM (50 µL of blood alone followed by blood with 0.02 µg mL^{-1} IVM until female repletion), (v) IVM + CsA (50 µL of blood with 1µM CsA followed by blood with 0.02 µg mL^{-1} IVM until female repletion), (vi) IVM + MK571 (50 µL of blood with 1µM MK571 followed by blood with 0.02 µg mL^{-1} IVM until female repletion) and (vii) IVM + CsA and MK571 (50 µL of blood with 1µM CsA and MK571 followed by blood with 0.02 µg mL^{-1} IVM until female repletion). Females were fed for about 28 h, weighted, reared in separate vials and

maintained at 27–28 °C and 80–90% relative humidity. The weight of eggs and larvae was determined separately for each female.

2.6. Effects of IVM and ABC transporter inhibitors on tick engorgement and reproduction

The effects of the administration of IVM and ABC transporter inhibitors on tick engorgement and reproduction were investigated by measuring the engorgement ratio (body weight after feeding/ initial body weight), mortality (percentage of dead females), index of fertility (egg weight/ female body weight after feeding) and egg viability (percentage of egg mass eclosion). Statistical analyses of the engorgement ratio and fertility index were performed using one-way ANOVA followed by Tukey's test. Tick mortality and egg viability were compared by chi-square test followed by Tukey's test. *P* values ≤ 0.05 were considered statistically significant. Females that did not engorge were removed from the statistical analysis and females which did not lay eggs were considered dead. Egg samples of each group were collected from individual 17-day-old egg masses and examined microscopically using a light inverted microscope (Axiovert25, Zeizz, Germany) and photographed with a digital camera (P700, Nikon, Japan).

2.7. ABC transporter sequence analysis

Three partial nucleotide sequences encoding ABC transporter proteins were retrieved from the *DFCI Boophilus microplus Gene Index* (http://compbio.dfci.harvard.edu/index.html) (Accession Nos. TC16417, EST782137 and TC20085) (Guerrero et al., 2005; Wang et al. 2007). Putative *R. microplus* ABC transporter genes were compared with orthologous from other eukaryotic genomes using Blastx at the NCBI Web site (http://www.ncbi.nlm.nih.gov/). Predicted amino acid

sequences of ABC transporters were analyzed for the presence of the ABC signature, Walker A and Walker B conserved motifs using Conserved Domain search program (Marchler-Bauer et al., 2011) from NCBI Web site and multiple sequence alignments were performed using MUSCLE algorithm (Edgar, 2004) with the default settings in MEGA software version 5 (Tamura et al., unpublished results). The sequence used for multiple alignments were *Sarcoptes scabiei* ABCC1 (AAZ75675.2), *Caenorhabditis elegans* MRP-1 (NP 741702.1) and *Mus musculus* ABCB10 (NP 062425.1).

2.8. Relative quantification of ABC transporters mRNA

After oviposition, females of the POA susceptible and JUA IVM-resistant strains fed on blood plus IVM or with blood alone were dissected and the midguts were collected. To evaluate the mRNA expression of ABC transporters, midgut total RNA was extracted using TRIzol® reagent (Invitrogen, USA) following the manufacturer's recommendations. RNA quantity and quality were estimated spectrophotometrically at 260/ 280 nm in NanoDrop 1000 instrument (Thermo Fisher Scientific, USA). One microgram of total RNA was treated with DNase I (Invitrogen, USA) and reverse-transcribed using the High-capacity cDNA Reverse Transcription kit with random primers, according to the manufacturer's recommendations (Applied Biosystems, USA). Quantification of ABC transporters mRNA was carried out by quantitative PCR (qPCR) with Quantimix Easy Sybr Green amplification kit (Biotools, Spain) in Mastercycler® ep realplex real-time PCR instrument (Eppendorf, Germany). The specific primers designed for gene amplification are described in Table 1. Gene amplification of β-Actin protein (Da Silva Vaz Jr et al., 2005) was used for normalization (Mercado-Curiel et al., 2011; Schuijt et al., 2011). Cycling parameters were 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 15 s annealing at 60 °C and extension at 72 °C for 20 s. To confirm primer specificity to

producing a single amplification product, the dissociation curve analysis was performed using instrument default parameters and representative products from qPCR were subjected to electrophoresis analysis. Primer efficiency was measured with 6-fold serially diluted cDNA in triplicate, and for the analyses 300 ng cDNA were added to each reaction. The relative amount of each ABC transporter mRNA produced per unit of β-Actin was calculated for each sample. Each analysis was conducted in triplicate. The relative expression ratio of ABC transporter genes in each experiment was calculated according to the mathematical model described by Pfaffl (2001) in Relative Expression Software Tool (REST-MCS©, version 2) (Pfaffl et al., 2002). One-way ANOVA followed by Tukey's test were used to compare three independent experiments.

Table 1- Primers used in the relative quantification of *Rhipicephalus (Boophilus) microplus* ATP-binding cassette (ABC) transporter mRNAs by quantitative PCR (qPCR).

Gene	NCBI accession number	Primer	Sequence 5'-3'	Tm	Amplicon size (bp)
β-actin	AY255624.1	Sense	GAG GAA GTA CTC CGT CTG GAT CGG CG	63.7	205
		Antisense	CCG TAG GGT GGC GTT GCC GG	66.0	
RmABCB10	JN098446	Sense	GCC GCA GTT GTC ACT TGT TGG TTT G	61.3	95
		Antisense	ACG TCC GCT GCC ACT TGC CTC	64.9	
RmABCC1	JN098447	Sense	GAC ACC ATT CAC CGA GAG TTC AGT AGC AC	61.9	120
		Antisense	GCC CTG CTC CAC TAT TTC GCC ACC	64.2	
RmABCC2	JN098448	Sense	CGC GGG ACC TTC TGA AGC	58.9	84
		Antisense	GGT AGC TCG GTA TAG GGC TAG ACG	59.8	

3. Results

3.1 Effects of ABC transporter inhibitors on IVM toxicity of larvae

To examine the role of ABC transporter proteins in the defense against IVM in *R. microplus*, we conducted a LPT using larvae previously exposed to a sublethal dose (5 μM) of CsA or MK571, ABC transporter inhibitors of subfamilies B and C, respectively. Figure 1 shows mortality data plots subjected to the probit regression analysis against logarithmic IVM concentrations from the susceptible strain (POA) and four IVM-resistant populations (SGA, CAV, PNO, JUA). Mortality rate increased in larvae exposed to CsA or MK571 in the four IVM-resistant populations, but it did not change in exposed larvae of the POA susceptible strain (Figure 1). Table 2 reports the LC_{50} and RR_{50} values calculated from probit analysis for each tick population. IVM toxicity in larvae of IVM-resistant populations significantly increased in CsA and/or MK571 exposed larvae, as no overlap was observed between CI 95% values of IVM alone and IVM + CsA or IVM + MK571 (indicated with asterisk) (Table 2). Additionally, treatments with sublethal doses of ABC transporter inhibitors significantly reduced the RR_{50} in strains SGA, PNO and JUA F3 (Table 2). These results suggest the participation of ABC transporter proteins from subfamilies B and C in the detoxification of the acaricide IVM in larvae from resistant *R. microplus* populations.

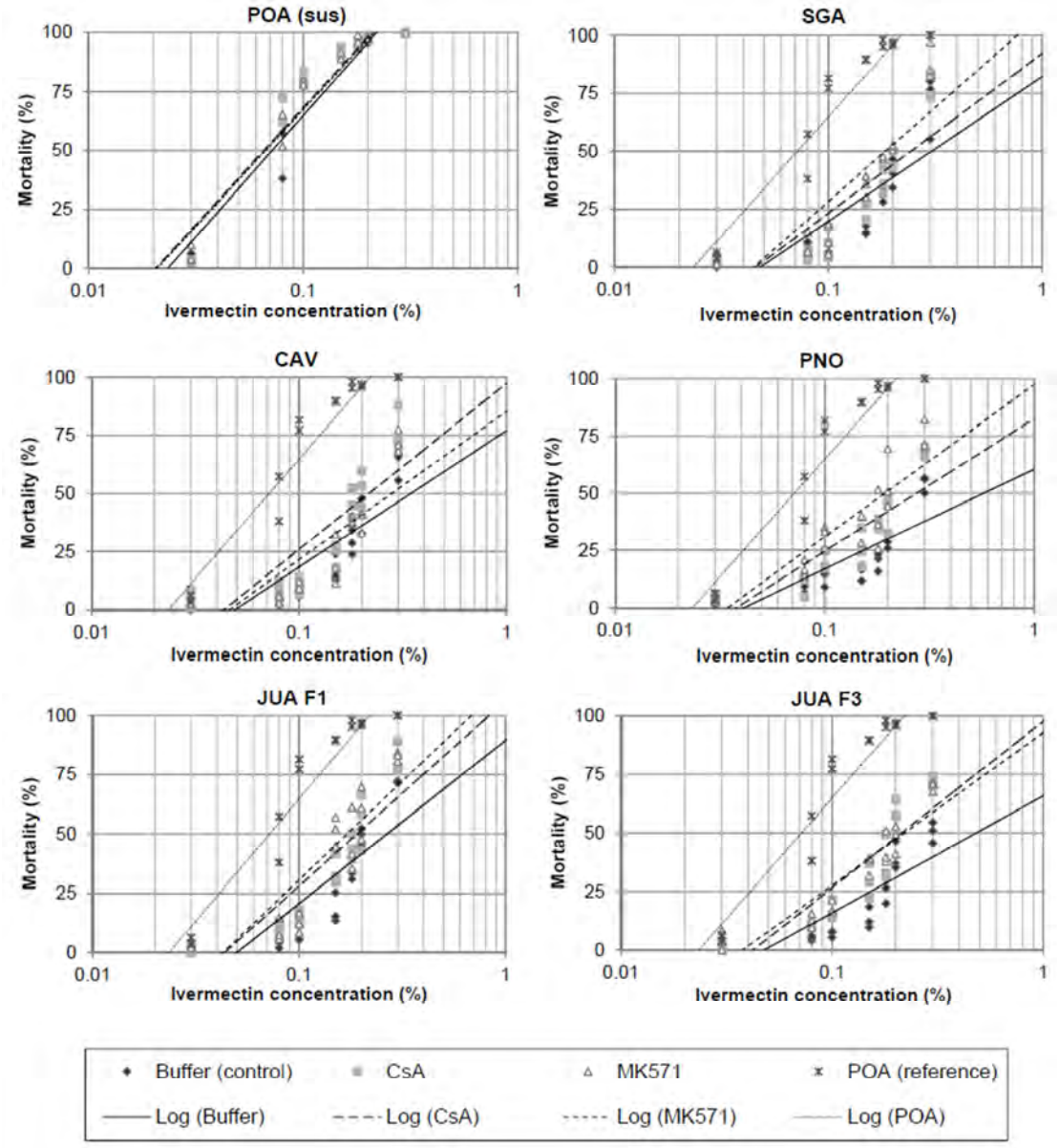


Fig. 1. Effect of ATP-binding cassette (ABC) transporter inhibitors cyclosporine (CsA) and MK571 (5µM) on mortality of susceptible (POA) and resistant (SGA, CAV, PNO and JUA) *Rhipicephalus (Boophilus) microplus* larvae to increasing ivermectina (IVM) concentrations determined with larvae packet test (LPT). Larvae were immersed in the inhibitors solutions or diluent alone (control) for 10 min, dried and transferred to filter papers impregnated with increasing IVM concentrations. Mortality was determined after 24 hours.

Table 2- Lethal concentrations and resistance ratios to ivermectina (IVM) alone or in association with inhibitors cyclosporine (CsA) or MK571 obtained by larvae packet test (LPT) with *Rhipicephalus (Boophilus) microplus* larvae from susceptible (POA) and resistant (SGA, CAV, PNO and JUA) populations.

Population	Drug association	*n*	slope (S.E.)	LC_{50} (CI 95%)	RR_{50} (CI 95%)	SF (CI 95%)
POA	IVM	3200	4.553 ± 0,235	0.077 (0.069 - 0.084)	-	-
	IVM + CsA	2670	4.478 ± 0.207	0.068 (0.062 - 0.074)	-	1.016 (0.987 - 1.086)
	IVM + MK571	2622	4.264 ± 0.194	0.069 (0.064 - 0.074)	-	1.084 (1.013 - 1.159)
SGA	IVM	2377	3.950 ± 0.317	0.238 (0.217 - 0.268)	3.104 (2.873 - 3.354)	-
	IVM + CsA	2318	4.505 ± 0.293	0.213 (0.202 - 0.226)	3.112 (2.898 - 3.342)	1.116 (1.043 - 1.195)
	IVM + MK571	2375	3.695 ± 0.166	0.182 (0.157 - 0.217) [a]	2.632 (2.450 - 2.829) [a]	1.309 (1.223 - 1.402)
CAV	IVM	2664	3.413 ± 0.194	0.255 (0.228 - 0.299)	3.325 (3.055 - 3.620)	-
	IVM + CsA	2385	4.270 ± 0.261	0.206 (0.192 - 0.221) [a]	3.003 (2.795 - 3.226)	1.240 (1.148 - 1.338)
	IVM + MK571	2450	3.109 ± 0.165	0.235 (0.199 - 0.306)	3.410 (3.131 - 3.714)	1.083 (0.990 - 1.184)
PNO	IVM	3435	2.878 ± 0.229	0.312 (0.277 - 0.372)	4.071 (3.680 - 4.504)	-
	IVM + CsA	3077	3.017 ± 0.218	0.235 (0.214 - 0.265) [a]	3.441 (3.171 - 3.733)	1.325 (1.196 - 1.467)
	IVM + MK571	3313	2.510 ± 0.144	0.194 (0.173 - 0.222) [a]	2.810 (2.600 - 3.038) [a]	1.606 (1.454 - 1.774)
JUA F1	IVM	2583	4.561 ± 0.239	0.221 (0.211 - 0.232)	2.887 (2.695 - 3.092)	-
	IVM + CsA	2616	4.080 ± 0.219	0.189 (0.178 - 0.202) [a]	2.769 (2.582 - 2.968)	1.180 (1.116 - 1.248)
	IVM + MK571	2667	3.543 ± 0.156	0.175 (0.157 - 0.198) [a]	2.534 (2.361 - 2.721)	1.265 (1.195 - 1.339)
JUA F3	IVM	2315	3.206 ± 0.218	0.288 (0.263 - 0.324)	3.753 (3.407 - 4.133)	-
	IVM + CsA	2233	2.959 ± 0.183	0.202 (0.183 - 0.229) [a]	2.953 (2.698 - 3.233) [a]	1.417 (1.273 - 1.578)
	IVM + MK571	2278	2.564 ± 0.190	0.210 (0.190 - 0.237) [a]	3.039 (2.749 - 3.359) [a]	1.368 (1.218 - 1.535)

LC: lethal concentration; CI: confidence interval; RR: resistance ratio; SF: synergism factor; [a] statistically different values ($p \leq 0.05$).

3.2. IVM-resistance selection

Engorged females from F1 and F2 generation of JUA IVM-resistant strain were treated with IVM at 100 ppm and the surviving larvae were used to infest an untreated head of cattle to produce the F2 and F3 generations, respectively. The LC_{50} and RR_{50} values of generations F1 and F3 were significantly different (Figure 1 and Table 2). Bioassay results produced RR_{50} (CI 95%) of 2.887 (2.695 - 3.092) for F1 larvae of the JUA strain and RR_{50} (CI 95%) of 3.753 (3.407 - 4.133) for F3 larvae compared with the susceptible POA strain. As the resistance ratio, the effects of ABC transporter inhibitors in the JUA strain led to an increase in the mortality in the F3 generation, when compared with the F1 generation.

3.3. Dose-dependent effect of ABC transporter inhibitors on the IVM toxicity in larvae

To investigate the effect of increasing concentrations of the ABC transporter inhibitors on larval mortality, larvae from the susceptible POA strain and from the IVM-resistant JUA strain were treated with 5, 15, 30 or 45 µM of CsA or MK571 and exposed to a single concentration of IVM (0.15%) or solvent alone on LPT (Figure 2). The treatment with increasing inhibitor concentrations promoted a dose-dependent effect on the mortality of the larvae from the IVM-resistant strain. The maximum effect was observed at 45 µM for both inhibitors, resulting in mortality rates of approximately 50%, whereas the mortality rate with IVM alone was around 20%. However, larval mortality did not change in the susceptible POA strain. The mortality of larvae was not affected by the inhibitors alone (data not shown).

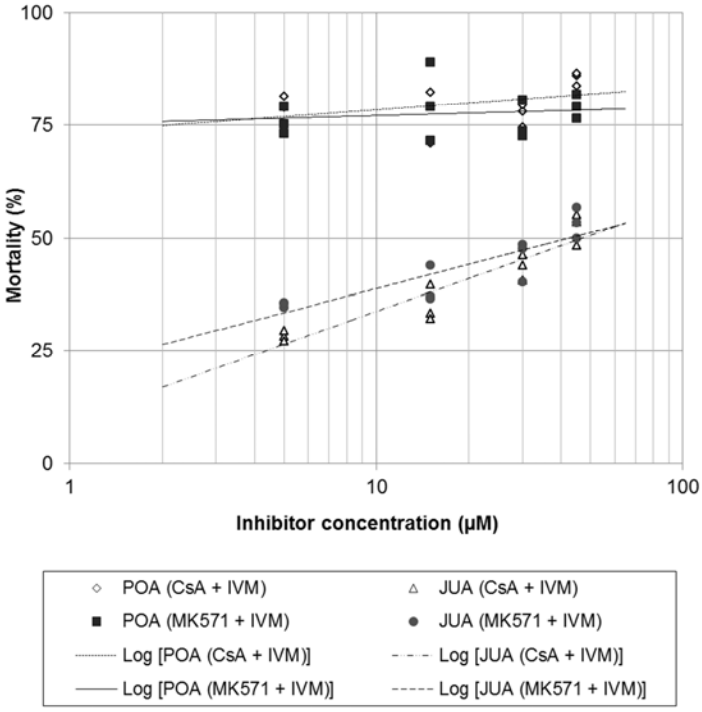


Fig. 2. Effect of increasing concentrations of CsA and MK571 on the mortality of susceptible (POA) and resistant (JUA) *R. microplus* larvae exposed to 0.15 % of IVM determined with LPT. Larvae were immersed in 5, 15, 30 or 45µM of inhibitor solutions or diluent alone for 10 min, dried and transferred to filter papers impregnated with 0.15% of IVM. Mortality was determined after 24 hours.

3.4. Effects of ABC transporter inhibitors on IVM toxicity to adult females

Ivermectin acts systemically and is distributed through the blood of host animals (Davey et al., 2010). Since ticks are exposed to ivermectin during host feeding, we tested the effect of the IVM by artificial capillary feeding partially-engorged females when ABC transporter inhibitors were added to blood meal. Results for tick engorgement ratio, mortality, fertility index and egg viability are presented in Table 3. The parameters analyzed in control groups fed on CsA and MK571 alone (Table 3; POA and JUA groups ii

and iii) were not significantly different from the control group fed on blood (Table 3; POA and JUA group i), indicating that these inhibitors individually did not affect female feeding and reproduction.

Addition of IVM to the blood meal of partially-engorged females from the POA strain affected engorgement and reproduction, as expected for a susceptible population. A significant decrease in tick engorgement ratio (1.21 ± 0.19) and fertility index (0.15 ± 0.09) was observed in the IVM-fed group (Table 3; POA group iv), when compared with the control group fed on blood alone, which had an engorgement ratio of 3.24 (± 0.97) and a fertility index of 0.35 (± 0.09) (Table 3; POA group i). However, no significant differences in engorgement ratio and fertility index were observed when the inhibitors were added to the IVM blood meal (Table 3; POA groups v, vi and vii) in comparison with the group fed on IVM alone (Table 3; POA group iv). In addition, after 17 days of incubation, eggs from females of the POA strain treated with IVM or IVM + inhibitors showed an atypical morphology, without embryo formation (Figure 3: D, E, F and G). As expected, egg viability was significantly reduced to ≤ 17.65 % (Table 3; POA groups iv, v, vi and vii), when compared to the control group, where egg viability was ≥ 71.43 % (Table 3; POA groups i, ii and iii) and the eggs demonstrated a normal morphology (Figure 3: A, B and C).

For the IVM-resistant population (JUA), the engorgement ratio (2.06 ± 0.64) and the index of fertility (0.29 ± 0.05) were not significantly different from the control group fed on blood alone, which showed engorgement ratio and fertility index of 2.88 (± 0.90) and 0.31 (± 0.15), respectively (Table 3; JUA group i and iv). Interestingly, when CsA was added to the IVM blood meal, a significant reduction in the fertility index (0.10 ± 0.12) was observed (Table 3; JUA groups v and vii) in comparison with the group fed on IVM alone (0.29 ± 0.05) (Table 3; JUA group iv). However, although the addition of CsA did

not significantly affect the engorgement ratio, mortality rate increased to 37.5% and 60% in the CsA-treated groups (Table 3; JUA group v and vii). However, the addition of MK571 (Table 3; JUA group vi) had no effect on any of the parameters analyzed (Table 3; JUA group i).

We observed a mortality rate of 8.7 and 15.7 in control groups (Table 3, POA and JUA group i). This was consequence of manipulation of the ticks and however it did not interfere in the analysis. Similar mortality rate was also observed by Fabres and coworkers (2010) in artificial capillary feeding experiments.

The eggs from IVM-treated females of the JUA strain (figure 3: K) showed a normal morphology, similar to the control groups (figure 3: H, I, J), differently from eggs from females treated with IVM plus CsA or CsA + MK571 (Figure 3: M and N), which showed an atypical morphology, without embryo formation (Figure 3: M and N). This result is similar to what was observed in eggs from IVM-treated females of the susceptible POA strain (figure 3, D). Egg viability of the groups treated with IVM plus CsA or CsA + MK571 was significantly reduced to 40% and 16.67%, respectively, when compared to control groups, which had egg viability $\geq$ 93.33%.

The inhibitor CsA, but not MK571, affected the reproduction of IVM-resistant JUA strain females fed on IVM, indicating that the inhibitor increases the detrimental effect of the acaricide and suggesting the participation of ABC transporters in IVM detoxification in adult females. The effects on the reproduction parameters (fertility index and egg viability) of IVM-resistant JUA strain females fed on CsA and IVM (Table 3, JUA group v) were similar to the effects obtained in susceptible POA strain females fed on IVM alone (Table 3, POA group iv), suggesting a partial reversal in the resistance phenotype in adult females using CsA by artificial capillary feeding.

Table 3- Effects of ATP-binding cassette (ABC) transporter inhibitors and ivermectina (IVM) on *Rhipicephalus (Boophilus) micprolus* partially engorged female ticks (artificial feeding).

	Engorgement ratio [A]	Mortality (%) [B]	Index of fertility [C]	Egg viability (%) [D]
POA (sus)				
(i) Blood (control)	3.24 (± 0.97) [a]	8.7 [a]	0.35 (± 0.09) [a]	71.43 [a]
(ii) CsA (control)	2.64 (± 0.83) [a]	9.5 [a]	0.36 (± 0.08) [a]	89.47 [a]
(iii) MK571 (control)	2.93 (± 0.78) [a]	4.5 [a]	0.34 (± 0.08) [a]	90.48 [a]
(iv) IVM	1.21 (± 0.19) [b]	15.0 [a]	0.15 (± 0.09) [b]	11.76 [b]
(v) IVM + CsA	1.38 (± 0.14) [b]	22.7 [a]	0.25 (± 0.08) [b]	11.76 [b]
(vi) IVM + MK571	1.33 (± 0.24) [b]	15.0 [a]	0.16 (± 0.08) [b]	17.65 [b]
(vii) IVM + CsA and MK571	1.34 (± 0.21) [b]	36.4 [a]	0.12 (± 0.05) [b]	7.14 [b]
JUA (res)				
(i) Blood (control)	2.88 (± 0.90) [a]	15.7 [a]	0.31 (± 0.15) [a]	93.75 [a]
(ii) CsA (control)	2.18 (± 0.87) [a]	0.0 [a]	0.25 (± 0.06) [a]	100 [a]
(iii) MK571 (control)	2.29 (± 0.60) [a]	0.0 [a]	0.35 (± 0.08) [a]	94.12 [a]
(iv) IVM	2.06 (± 0.64) [a]	0.0 [a]	0.29 (± 0.05) [a]	93.75 [a]
(v) IVM + CsA	1.95 (± 0.58) [a]	37.5 [b]	0.10 (± 0.12) [b]	40 [b]
(vi) IVM + MK571	2.23 (± 0.73) [a]	6.25 [a]	0.29 (± 0.10) [a]	93.33 [a]
(vii) IVM + CsA and MK571	1.75 (± 0.45) [a]	60.0 [b]	0.06 (± 0.08) [b]	16.67 [b]

[a] Average (± SD) of the engorgement ratio calculated as the final body weight after feeding by the initial body weight before feeding.
[b] Tick mortality expresses the percentage of dead females to total number of females in the group.
[c] Average (± SD) of the index of fertility calculated as the weight of eggs oviposited by the body weight of the female after feeding.
[d] Egg viability expresses the percentage of egg masses ecloded to total of egg masses oviposited
Different letters within the same columns refer to statistically different values ($p \leq 0.05$).

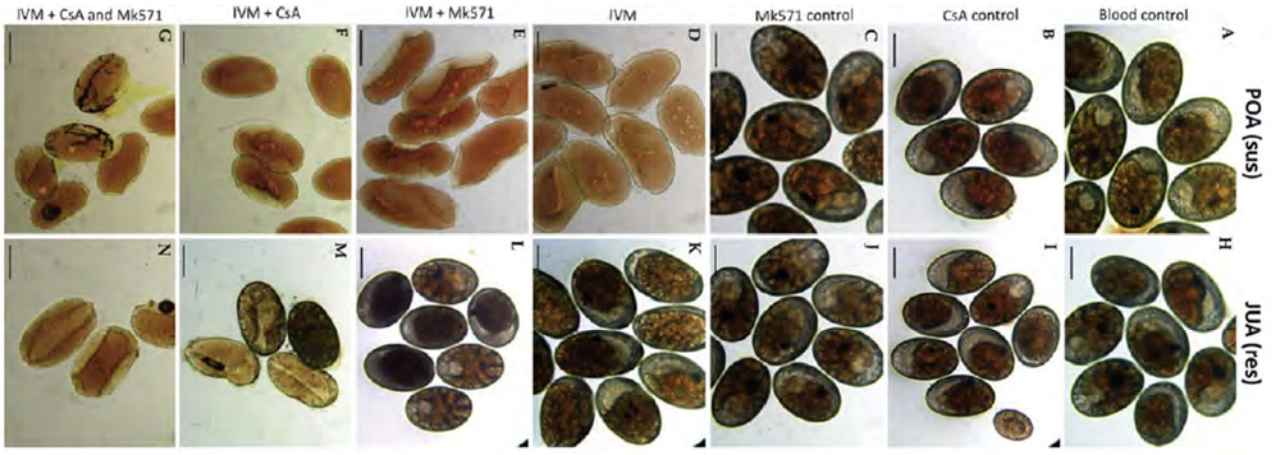


Fig. 3. Effect of IVM and ABC transporter inhibitors on egg development. Representative eggs (17 days after oviposition) from susceptible POA treated females (A-G) and IVM-resistant JUA treated females (H-N). (A and H) Blood control (blood alone), (B and I) CsA control (50 µL of blood with 1 µM CsA followed by blood alone until female repletion), (C and J) MK571 control (50 µL of blood with 1 µM MK571 followed by blood alone until female repletion), (D and K) IVM (50 µL of blood alone followed by blood with 0.02 µg mL^{-1} IVM until female repletion), (E and L) IVM + MK571 (50 µL of blood with 1 µM MK571 followed by blood with 0.02 µg mL^{-1} IVM until female repletion), (F and M) IVM + CsA (50 µL of blood with 1 µM CsA followed by blood with 0.02 µg mL^{-1} IVM until female repletion) and (G and N) IVM + CsA + MK571 (50 µL of blood with 1 µM CsA and MK571 followed by blood with 0.02 µg mL^{-1} IVM until female repletion). Bar= 0.1mm.

3.5. Sequence analysis of R. microplus ABC transporters

Three partial nucleotide sequences encoding ABC transporter proteins were retrieved from the *DFCI Boophilus microplus Gene Index*. Two of the *R. microplus* ABC transporter sequences share identity and similarity to ABCC1 from other organisms and were designated RmABCC1 and RmABCC2. They were deposited in GeneBank™ data base with accession numbers JN098447 and JN098448, respectively. RmABCC1 has 27% identity and 36% similarity with carboxy-terminal amino acid sequences of *S. scabiei* ABCC1 and *C. elegans* MRP-1 whereas RmABCC2 has 43% identity and 58% similarity with carboxy-terminal amino acid sequences of *S. scabiei* ABCC1 and *C. elegans* MRP-1. The third sequence shares identity and similarity to ABCB10 from other organisms and was designated RmABCB10. It was deposited in GeneBank™ data base with accession number JN098446. RmABCB10 has 56% identity and 75% similarity with carboxy-terminal amino acid sequence of *M. musculus* ABCB10. The conserved motifs ABC signature, Walker A and Walker B, characteristic of ABC transporters, are conserved in RmABCB10. In RmABCC1 and RmABCC2 only Walker B and signature motifs were found (Figure 4). Accession numbers for all ABC transporters described in this work are listed in materials and methods and in Figure 4.

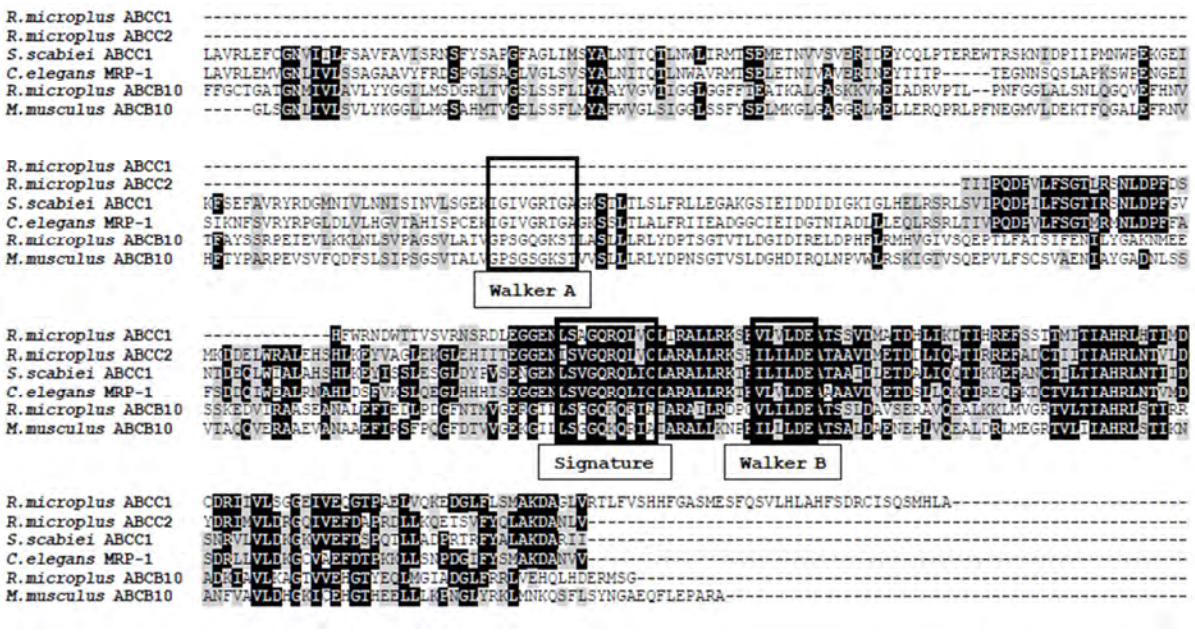


Fig. 4. Multiple sequence alignment of partial predicted amino acid sequence of *R. microplus* ABC transporters ABCB10 (JN098446), ABCC1 (JN098447), ABCC2 (JN098448) and carboxy- terminal amino acid sequences of *S. scabiei* ABCC1 (AAZ75675.2), *C. elegans* MRP-1 (NP741702.1) and *M. musculus* ABCB10 (NP062425.1). N-terminal extensions were included in the alignment but omitted in the figure for clarity's sake. Conserved amino acid sequences are indicated in bold and conserved motifs of the nucleotide-binding domain (NBD) are indicated by boxes. Alignments were done using MUSCLE program with the default settings in MEGA software version 5.

3.6. Transcription levels of ABC transporter genes

To investigate whether the transcription levels of ABC transporters correlate with changes in IVM sensitivity, midgut total RNA from JUA and POA strain females artificially fed on blood alone or blood with IVM were analyzed by qPCR. We observed a significant increase in the transcription level of *RmABCB10* in IVM-resistant females,

when compared to POA susceptible females (Figure 5). Additionally, *RmABCB10* transcription was up regulated in IVM-resistant females in response to IVM feeding. However, the expression levels of *RmABCC1* and *RmABCC2* in the JUA and POA populations were not significantly different.

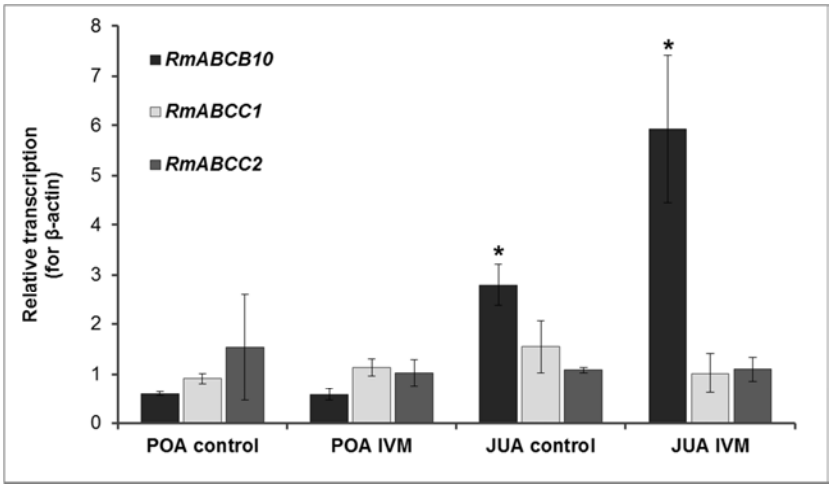


Fig. 5. Relative transcription levels of ABC transporter genes in the midgut of susceptible POA females and IVM-resistant JUA females after artificial feeding with blood alone (POA control and JUA control) or blood with IVM (POA IVM and JUA IVM). After egg oviposition, females were dissected and RNA was extracted from midgut. *RmABCB10*, *RmABCC1* and *RmABCC2* were amplified from cDNA using gene-specific primers. Data represent mean ± SD of three females from each group, normalized relative to β-actin transcript levels. Asterisks (*) denote significant differences as determined by one-way ANOVA followed by Tukey's test ($p \leq 0.05$).

4. Discussion

Despite accumulating data supporting the central importance of ABC transporter proteins in drug detoxification in several biological systems (Lage, 2003), in ticks this field of research remains to be explored. In this work we investigated the role of ABC transporter proteins in cellular defense against the acaricide IVM in the cattle tick. ABC transporter inhibitors have been used to increase drug bioavailability in pathogens and cancer cells, providing a possible strategy to increase drug efficacy (Lespine et al., 2008; Lifschitz et al., 2010; Yang et al., 2010). In addition, ABC transporter inhibitors have been useful to study the importance of ABC transporters in drug resistance in several organisms (Bartley et al., 2009; Buss et al., 2002). Here, we investigated the effect of two ABC transporter inhibitors on IVM sensitivity. The compounds used in this study, CsA and MK571, are known to interfere with the function of P-gp proteins (ABCB transporters) and with MRP proteins (ABCC transporters), respectively (Choi, 2005; Ponte-Sucre, 2007).We initially performed larvae bioassays with IVM and with ABC transporter inhibitors and observed a significant increase in IVM toxicity when larvae from IVM-resistant populations were previously exposed to sublethal CsA or MK571 concentrations (Figure 1 and Table 2). These results indicate the involvement of ABC transporter proteins from both ABCB and ABCC subfamilies in larval defense against IVM. However, ABC transporter inhibitors caused different effects for each population. For the CAV population, CsA had a greater effect, whereas MK571 had a more prominent effect on the other populations (Figure 1), as expected, since each population has a different resistance profile. Interestingly, a change in the effect of each inhibitor was observed between generations F1 and F3 of the JUA IVM-resistant strain after 2 generations of resistance selection (Figure 1 and Table 2). These results could reflect a difference in the drug selection pressure of each tick population that can generate different resistance genotypes. In fact, it has been

observed that the expression of different ABC transporter genes in *C. elegans* was dependent on nematode resistance to lower or higher levels of IVM (James and Davey, 2009). We also observed that larval treatment with increasing inhibitor concentrations promoted a dose-dependent effect on the mortality rate in the IVM-resistant JUA strain (Figure 2). Interestingly, the addition of ABC transporter inhibitors had no effect on larval mortality in the POA susceptible strain (Figures 1 and 2). It is known that MLs are potent substrates for ABC transporters (Lespine et al., 2006). When overexpressed, as observed in resistant organisms, ABC transporters protect cells from the entry of MLs, limiting their efficacy. With the use of inhibitors, ABC transporters function is reduced and MLs accumulate in the cells (Lespine et al., 2008). Therefore, the results obtained afford to conclude that higher inhibitor concentrations may promote the intracellular accumulation and the consequent exacerbation of the toxic effects of IVM in larvae that would otherwise be removed by ABC transporters (Figure 2). In the field, tick control with IVM can be achieved through blood meal ingestion, since IVM is distributed systemically in cattle in injectable or pour-on formulations (Davey et al., 2010; Gayrard et al., 1999). To assess the effects of ABC transporter inhibitors on IVM toxicity through the same route of IVM penetration in ticks fed on cattle blood, an *in vitro* feeding assay was employed. *In vitro* feeding assays offer a means to appropriately quantify the effect of precise doses of test products, reducing the cost and the number of cattle heads required for the experiments (Kröber and Guerin, 2007a). *In vitro* feeding assays have been used for various species of ticks, as *Rhipicephalus appendiculatus* (Waladde et al., 1993), *Dermacentor variabilis* (Macaluso et al., 2001) and *Ixodes ricinus* (Kröber and Guerin, 2007b). An approach to feed partially-engorged *R. microplus* females to repletion using microhaematocrit capillary tubes and to analyze the effects of administration of drugs on engorgement and reproduction was previously developed (Fabres et al., 2010). Herein, the same approach

was adopted, and the effects of IVM administration on partially-engorged females in the presence or absence of ABC transporter inhibitors in the blood meal were assessed by determining the engorgement ratio, mortality rate, fertility index and egg viability. As expected, the addition of IVM in the blood meal had a drastic effect on females from the susceptible POA strain, with a consequent reduction in all parameters analyzed (Table 3). Also, female feeding and reproduction in the JUA strain were not significantly affected, confirming its IVM resistance. However, when CsA (inhibitor of ABCB proteins) was added to the blood meal of JUA strain, a significant increase in IVM toxicity was observed, with a striking effect on fertility index and egg viability. Additionally, in the few eggs laid, embryos were absent (Figure 3). One of the effects of IVM intoxication on adult ticks is the reduction in reproductive performance of engorged females. In fact, Davey and coworkers (2005) showed that egg masses produced by females recovered from IVM-treated cattle weighed approximately 5-8 times less than egg masses produced by females recovered from untreated cattle. Unlike the results obtained in larvae bioassays, MK571 (inhibitor of ABCC proteins) did not increase IVM toxicity of adult females from the resistant JUA strain. One hypothesis is that different ABC transporter proteins may have different degrees of relevance in detoxication during larval or adult stages. Huang and Prichard (1999) observed that the expression levels of two ABCB genes in *Onchocerca volvulus* adults were higher than in larvae, and in the zebra mussel *Dreissena polymorpha* the expression of ABCB1 gene in larvae was higher than in embryo (Faria et al., 2011), suggesting that some ABC transporters can be more active in a specific life stage. Another hypothesis is that different pathways of IVM penetration, through the cuticle in the larvae bioassay and orally in adults, can induce the expression of different ABC transporter proteins. Indeed, Lincke and coworkers (1993) found two ABCB genes expressed exclusively in intestinal cells in *C. elegans*. They suggested that these ABC transporter

proteins constitute a protection mechanism to orally ingested toxins, with the intestine forming the first line of defense.

Despite the fact that ABC transporter inhibitors increase the IVM toxicity in IVM-resistant larvae and adults ticks, the IVM susceptibility was not restored to that of the susceptible ticks. This can reflect the participation of other resistance mechanisms like as insensitivity of the GluCl receptor and increase in metabolic detoxification mediated by esterases, cytochrome P450 monooxigenase and glutathione-S-transferase. The participation of these mechanisms in *R. microplus* IVM resistance is also being investigated (Klafke, personal communication).

Our results suggest that the detoxification by ABC transporters proteins is one mechanism of IVM resistance in *R. microplus*. To our knowledge, this is the first work reporting a decrease in acaricide toxicity by ABC transporter proteins in ticks. Considering that these transporters act on a wide spectrum of chemical compounds, their involvement in cell defense potentiates the risk of occurrence of cross-resistance. In mosquitoes *Culex pipiens* toxicity to three insecticides classes (cypermethrin, endosulfan, ivermectin), but not to chlorpyrifos, was increased when larvae were treated in conjunction with verapamil, an ABC transporter inhibitor (Buss et al., 2002). In *Aedes caspius* the treatment with the same inhibitor increased larvae toxicity to temephos and diflubenzuron (Porretta et al., 2008). More importantly, the inhibition of ABC transporters may represent a novel control strategy. Increasing tick susceptibility to acaricides could reduce the dose and frequency of application. Inhibitory agents could be used in synergy with the acaricide to enhance its efficiency. For a strategy to be feasible, specific inhibitors must be identified, which requires the identification and characterization of ABC transporters. Up to the present, ABC transporters have not been described in *R. microplus*. A search on the *DFCI Boophilus microplus Gene Index* allowed the identification of three partial nucleotide

sequences encoding ABC transporter proteins, and amino acid sequence homology was found with ABC transporter sequences of other organisms. Although their sequences are incomplete, characteristic NBD domain with the Walker A, Walker B and signature motifs were found. Walker A, Walker B and signature motifs are responsible for ATP binding and hydrolysis. The signature motif is unique to the ABC transporter family and has proven to be a useful tool in identifying putative new members of the family (Schneider and Hunke, 1998).

Over-expression of ABC transporters is usually associated with ML resistance, as reported in IVM-resistant *H. contortus* (Xu et al., 1998), in *C. elegans* selected for IVM resistance *in vitro* (James and Davey, 2009) and in IVM-exposed *S. scabiei* (Mounsey et al., 2010). Corroborating this hypothesis, we found that the transcription level of *RmABCB10* significantly increased in IVM-resistant, when compared with POA susceptible females. Besides, this gene was up regulated in IVM-resistant females in response to IVM feeding. This result is in agreement with artificial feeding results, where CsA, an inhibitor of subfamily B members of ABC transporters such as RmABCB10, increased IVM toxicity in JUA IVM-resistant population. These results suggest that RmABCB10 contribute to IVM resistance in *R. microplus.* Additionally, other ABC genes may be involved, and further investigation on this aspect is necessary.

This study demonstrates that compounds interfering with ABC transporters activity could enhance IVM efficacy in larvae and adults of tick resistant populations. These results support, to our knowledge for the first time, the notion that ABC transporters can play an important role in cellular defense against IVM in the cattle tick. This may suggest new perspectives for using compounds which selectively target ABC transporters and thus improve the efficacy of acaricide treatment.

Acknowledgements

We thank Dr Carlos Eduardo Cruz for help in the redaction of the manuscript. This work was supported by grants from CNPq - Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular, FINEP, CAPES, CNPq, FAPERGS and FAPESP (Brazil).

References

Argentine, J.A., Clark, J.M., Lin, H., 1992. Genetics and biochemical mechanisms of abamectin resistance in two isogenic strains of Colorado potato beetle. Pestic. Biochem. Physiol. 44, 191- 207.

Bartley, D.J., McAllister, H., Bartley, Y., Dupuy, J., Ménez, C., Alvinerie, M., Jackson, F., Lespine, A., 2009. P-glycoprotein interfering agents potentiate ivermectin susceptibility in ivermectin sensitive and resistant isolates of *Teladorsagia circumcincta* and *Haemonchus contortus*. Parasitology. 136, 1081-1088.

Bourguinat, C., Keller, K., Blagburn, B., Schenker, R., Geary, T.G., Prichard, R.K., 2011. Correlation between loss of efficacy of macrocyclic lactone heartworm anthelmintics and P-glycoprotein genotype. Vet. Parasitol. 176, 374-81.

Buss, D.S., McCaffery, A.R., Callaghan, A., 2002. Evidence for p-glycoprotein modification of insecticide toxicity in mosquitoes of the *Culex pipiens* complex. Med Vet Entomol. 16, 218-22.

Castro-Janer, E., Martins, J.R., Mendes, M.C., Namindome, A., Klafke, G.M., Schumaker, T.T., 2010. Diagnoses of fipronil resistance in Brazilian cattle ticks (*Rhipicephalus (Boophilus) microplus*) using in vitro larval bioassays. Vet. Parasitol. 173, 300-306.

Castro-Janer, E., Rifran, L., González, P., Niell, C., Piaggio, J., Gil, A., Schumaker, T.T.S. 2011. Determination of the susceptibility of *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) to ivermectin and fipronil by Larval Immersion Test (LIT) in Uruguay. Vet. Parasitol. In press. DOI. 10.1016/j.vetpar.2010.12.035.

Choi, C.H., 2005. ABC transporters as multidrug resistance mechanisms and the development of chemosensitizers for their reversal. Cancer Cell Int. 5, 30.

Coles, G.C., Watson, C.L., Anziani, O.S., 2001. Ivermectin-resistant *Cooperia* in cattle. Vet. Rec. 148, 283-4.

Coles, G.C., Rhodes, A.C., Wolstenholme, A.J., 2005. Rapid selection for ivermectin resistance in *Haemonchus contortus*. Vet. Parasitol. 129, 345-347.

Currie, B.J., Harumal, P., McKinnon, M., Walton, S.F., 2004. First documentation of in vivo and in vitro ivermectin resistance in *Sarcoptes scabiei*. Clin. Infect. Dis. 39, 8-12.

Da Silva Vaz Jr, I., Imamura, S., Nakajima, C., de Cardoso, F.C., Ferreira, C.A., Renard, G., Masuda, A., Ohashi, K., Onuma, M., 2005. Molecular cloning and sequence

analysis of cDNAs encoding for *Boophilus microplus, Haemaphysalis longicornis* and *Rhipicephalus appendiculatus* actins. Vet Parasitol. 127, 147-55.

Davey, R.B., Miller, J.A., George, J.E., Miller, R.J., 2005. Therapeutic and persistent efficacy of a single injection treatment of ivermectin and moxidectin against *Boophilus microplus* (Acari: Ixodidae) on infested cattle. Exp Appl Acarol. 35, 117-29.

Davey, R.B., Pound, J.M., Miller, J.A., Klavons, J.A., 2010. Therapeutic and persistent efficacy of a long-acting (LA) formulation of ivermectin against *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) and sera concentration through time in treated cattle. Vet Parasitol. 169, 149-56.

Dean, M., Rzhetsky, A., Allikmets, R., 2001. The human ATP-binding cassette (ABC) transporter superfamily. Genome Res. 11, 1156-66.

Dent, J.A., Smith, M.M., Vassilatis, D.K., Avery, L., 2000. The genetics of ivermectin resistance in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. U S A. 97, 2674-9.

Dos Santos, T.R., Pappen, F.G., da Rosa Farias, N.A., da Silva Vaz Junior, I. 2009. In vitro analysis of amitraz efficacy against *Rhipicephalus (Boophilus) microplus* (Canestrini, 1887) populations of southern region of Rio Grande do Sul state. Rev. Bras. Parasitol. Vet. 18, 54-7.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792-1797.

Fabres, A., De Andrade, C.P., Guizzo, M., Sorgine, M.H., Paiva-Silva, G., Masuda, A., da Silva Vaz Junior, I., Logullo, C., 2010. Effect of GSK-3 activity, enzymatic inhibition and gene silencing by RNAi on tick oviposition and egg hatching. Parasitology. 137, 1537-46.

FAO, 1984. Ticks and tick borne disease control. A practical field manual. Volume I. Tick control. Food and Agriculture Organization, Animal Production and Health Division, Rome, 299 pp.

FAO, 2004. Resistance Management and Integrated Parasite Control in Ruminants-guidelines, Executive Summary, Food and Agriculture Organization, Animal Production and Health Division, Rome, p.11.

Faria, M., Navarro, A., Luckenbach, T., Piña, B., Barata, C., 2011. Characterization of the multixenobiotic resistance (MXR) mechanism in embryos and larvae of the zebra mussel (*Dreissena polymorpha*) and studies on its role in tolerance to single and mixture combinations of toxicants. Aquat. Toxicol. 101, 78-87.

Fox, L.M., 2006. Ivermectin: uses and impact 20 years on. Curr Opin Infect Dis. 19, 588-93.

Gayrard, V., Alvinerie, M., Toutain, P.L., 1999. Comparison of pharmacokinetic profiles of doramectin and ivermectin pour-on formulations in cattle. Vet Parasitol. 81, 47-55.

Geary, T.G., 2005. Ivermectin 20 years on: maturation of a wonder drug. Trends Parasitol. 21, 530-2.

Guerrero, F.D., Miller, R.J., Rousseau, M.E., Sunkara. S., Quackenbush, J., Lee, Y., Nene, V., 2005. BmiGI: a database of cDNAs expressed in *Boophilus microplus*, the tropical/southern cattle tick. Insect. Biochem. Mol. Biol. 35, 585-95.

Higgins, C.F., 2007. Multiple molecular mechanisms for multidrug resistance transporters. Nature. 446, 749-57.

Holland, I.B., Blight, M.A., 1999. ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. J. Mol. Biol. 293, 381-99.

Huang, Y.J., Prichard, R.K., 1999. Identification and stage-specific expression of two putative P-glycoprotein coding genes in *Onchocerca volvulus*. Mol. Biochem. Parasitol. 102, 273-81.

James, C.E., Davey, M.W., 2009. Increased expression of ABC transport proteins is associated with ivermectin resistance in the model nematode *Caenorhabditis elegans*. Int. J. Parasitol. 39, 213-20.

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.

Jonsson, N.N., Bock, R.E., Jorgensen, W.K., 2008. Productivity and health effects of anaplasmosis and babesiosis on *Bos indicus* cattle and their crosses, and the effects of differing intensity of tick control in Australia. Vet. Parasitol. 155, 1-9.

Kaplan, R.M., 2004. Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol. 20, 477-81.

Klafke, G.M., Sabatini, G.A., de Albuquerque, T.A., Martins, J.R., Kemp, D.H., Miller, R.J., Schumaker, T.T., 2006. Larval immersion tests with ivermectin in populations of the cattle tick *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) from State of São Paulo, Brazil. Vet. Parasitol. 142, 386-90.

Kocan, K.M., Blouin, E.F., Barbet, A.F., 2000. Anaplasmosis control. Past, present, and future. Ann. N. Y. Acad. Sci. 916:501-9.

Kröber, T., Guerin, P.M., 2007(a). In vitro feeding assays for hard ticks. Trends Parasitol. 23, 445-9.

Kröber, T., Guerin, P.M., 2007(b). An in vitro feeding assay to test acaricides for control of hard ticks. Pest. Manag. Sci. 63, 17-22.

Kuo, M.T., 2007. Roles of multidrug resistance genes in breast cancer chemoresistance. Adv. Exp. Med. Biol. 608, 23-30.

Kwon, D.H., Yoon, K.S., Clark, J.M., Lee, S.H., 2010. A point mutation in a glutamate-gated chloride channel confers abamectin resistance in the two-spotted spider mite, *Tetranychus urticae* Koch. Insect. Mol. Biol. 19, 583-91.

Lage, H., 2003. ABC-transporters: implications on drug resistance from microorganisms to human cancers. Int. J. Antimicrob. Agents. 22, 188-99.

LeOra Software, 2004. Polo Plus Probit and Logit Analysis: User's Guide, Berkeley, p. 36.

Leslie, E.M., Deeley, R.G., Cole, S.P., 2005. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicol. Appl. Pharmacol. 204, 216-37.

Lespine, A., Dupuy, J., Orlowski, S., Nagy, T., Glavinas, H., Krajcsi, P., Alvinerie, M., 2006. Interaction of ivermectin with multidrug resistance proteins (MRP1, 2 and 3). Chem Biol Interact. 159, 169-79.

Lespine, A., Alvinerie, M., Vercruysse, J., Prichard, R.K., Geldhof, P., 2008. ABC transporter modulation: a strategy to enhance the activity of macrocyclic lactone anthelmintics. Trends Parasitol. 24, 293-8.

Lifschitz, A., Entrocasso, C., Alvarez, L., Lloberas, M., Ballent, M., Manazza, G., Virkel, G., Borda, B., Lanusse, C., 2010. Interference with P-glycoprotein improves ivermectin activity against adult resistant nematodes in sheep. Vet. Parasitol. 172, 291-8.

Lincke, C.R., Broeks, A., The, I., Plasterk, R.H., Borst, P., 1993. The expression of two P-glycoprotein (pgp) genes in transgenic *Caenorhabditis elegans* is confined to intestinal cells. EMBO J. 12, 1615-20.

Macaluso, K.R., Sonenshine, D.E., Ceraul, S.M., Azad, A.F., 2001. Infection and transovarial transmission of rickettsiae in *Dermacentor variabilis* ticks acquired by artificial feeding. Vector Borne Zoonotic Dis. 1, 45-53.

Marchler-Bauer, A., Lu, S., Anderson, J.B., Chitsaz, F., Derbyshire, M.K., Deweese-Scott, C., Fong, J.H., Geer, L.Y., Geer, R.C., Gonzales, N.R., Gwadz, M., Hurwitz, D.I., Jackson, J.D., Ke, Z., Lanczycki. C.J., Lu, F., Marchler, G.H., Mullokandov, M., Omelchenko, M.V., Robertson, C.L., Song, J.S., Thanki, N, Yamashita, R.A., Zhang, D., Zhang, N., Zheng, C., Bryant, S.H., 2011. CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Res. 39, 225-9.

Martins, J.R., Furlong, J., 2001. Avermectin resistance of the cattle tick *Boophilus microplus* in Brazil. Vet. Rec. 149, 64.

McCavera, S., Rogers, A.T., Yates, D.M., Woods, D.J., Wolstenholme, A.J., 2009. An ivermectin-sensitive glutamate-gated chloride channel from the parasitic nematode *Haemonchus contortus*. Mol. Pharmacol. 75, 1347-55.

Melander, A.L., 1914. Can insects become resistant to sprays? J. Econ. Entom. 7, 167–173.

Mendes, M.C., Lima, C.K., Nogueira, A.H., Yoshihara, E., Chiebao, D.P., Gabriel, F.H., Ueno, T.E., Namindome, A., Klafke, G.M., 2011. Resistance to cypermethrin, deltamethrin and chlorpyriphos in populations of *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) from small farms of the State of São Paulo, Brazil. Vet. Parasitol. 178: 383-8.

Mercado-Curiel R.F., Palmer, G.H., Guerrero, F.D., Brayton, K.A., 2011. Temporal characterisation of the organ-specific *Rhipicephalus microplus* transcriptional response to *Anaplasma marginale* infection. Int J Parasitol. 41: 851- 860.

Mounsey, K.E., Pasay, C.J., Arlian, L.G., Morgan, M.S., Holt, D.C., Currie, B.J., Walton, S.F., McCarthy, J.S., 2010. Increased transcription of Glutathione S-transferases in acaricide exposed scabies mites. Parasit. Vectors. 3, 1-9.

Parizi, L.F., Utiumi, K.U., Imamura, S., Onuma, M., Ohashi, K., Masuda, A., da Silva Vaz, I. Jr., 2011. Cross immunity with *Haemaphysalis longicornis* glutathione S-transferase reduces an experimental *Rhipicephalus (Boophilus) microplus* infestation. Exp Parasitol. 127, 113- 118.

Perez-Cogollo, L.C., Rodriguez-Vivas, R.I., Ramirez-Cruz, G.T., Miller, R.J., 2010. First report of the cattle tick *Rhipicephalus microplus* resistant to ivermectin in Mexico. Vet. Parasitol. 168, 165-9.

Pfaffl , M. W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, 2002-7.

Pfaffl, M. W., Horgan, G. W., Dempfle, L., 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30, 01-10.

Ponte-Sucre, A., 2007. Availability and applications of ATP-binding cassette (ABC) transporter blockers. Appl. Microbiol. Biotechnol. 76, 279-86.

Porretta, D., Gargani, M., Bellini, R., Medici, A., Punelli, F., Urbanelli, S., 2008. Defence mechanisms against insecticides temephos and diflubenzuron in the mosquito *Aedes caspius*: the P-glycoprotein efflux pumps. Med. Vet. Entomol., 22: 48-54.

Rosario-Cruz, R., Almazan, C., Miller, R.J., Dominguez-Garcia, D.I., Hernandez-Ortiz, R., de la Fuente, J., 2009. Genetic basis and impact of tick acaricide resistance. Front. Biosci. 14, 2657-65.

Schneider, E., Hunke, S., 1998. ATP-binding-cassette (ABC) transport systems: functional and structural aspects of the ATP-hydrolyzing subunits/domains. FEMS Microbiol Rev. 22, 1-20.

Schuijt, T.J., Narasimhan, S., Daffre, S., DePonte, K., Hovius, J.W., Van't Veer, C., van der Poll, T., Bakhtiari, K., Meijers, J.C., Boder, E.T., van Dam, A.P., Fikrig, E., 2011. Identification and characterization of *Ixodes scapularis* antigens that elicit tick immunity using yeast surface display. PLoS One, 6(1), e15926.

Scott, J.G., 1989. Cross-resistance to the biological insecticide abamectin in pyrethroid resistant strains of house flies. Pestic. Biochem. Physiol. 34, 27-31.

Seixas, A., Oliveira, P., Termignoni, C., Logullo, C., Masuda, A., da Silva Vaz, I. Jr., in press. *Rhipicephalus (Boophilus) microplus* embryo proteins as target for tick vaccine. Vet Immunol Immunopathol.

Townson, S., Tagboto, S.K., Castro, J., Lujan, A., Awadzi, K., Titanji, V.P., 1994. Comparison of the sensitivity of different geographical races of *Onchocerca volvulus* microfilariae to ivermectin: studies in vitro. Trans. R. Soc. Trop. Med. Hyg. 88, 101-6.

Waladde, S.M., Young, A.S., Ochieng, S.A., Mwaura, S.N., Mwakima, F.N., 1993. Transmission of *Theileria parva* to cattle by *Rhipicephalus appendiculatus* adults fed as nymphae in vitro on infected blood through an artificial membrane. Parasitology. 107, 249-56.

Wolstenholme, A.J., Rogers, A.T., 2005. Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics. Parasitology. 131, S85-95.

Xu, M., Molento, M., Blackhall, W., Ribeiro, P., Beech, R., Prichard, R., 1998. Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homolog. Mol Biochem Parasitol. 91, 327-35.

Yang, A.K., Zhou, Z.W., Wei, M.Q., Liu, J.P., Zhou, S.F., 2010. Modulators of multidrug resistance associated proteins in the management of anticancer and antimicrobial drug resistance and the treatment of inflammatory diseases. Curr. Top. Med. Chem. 10, 1732-56.

ARTIGO II

ABC transporters as a multidrug detoxification mechanism in *Rhipicephalus (Boophilus) microplus*

Paula C. Pohl[a], Guilherme M. Klafke[b], José Reck Júnior[b], João Ricardo Martins[b], Itabajara da Silva Vaz Jr[a,c,#], Aoi Masuda[a,d]

[a]Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; [b]Instituto de Pesquisas Veterinárias Desidério Finamor- FEPAGRO, Eldorado do Sul, Brazil; [c]Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; [d]Departamento de Biologia Molecular e Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

Aceito para publicação na revista Parasitology Research.

Abstract

ATP-binding cassette (ABC) transporters are responsible for pumping drugs across membranes, and are an important drug detoxification mechanism. Since ABC transporters act on a wide spectrum of chemical compounds, they have been associated with multidrug resistance phenotype in various parasites and cancer cells. Here we document the presence of a *Rhipicephalus (Boophilus) microplus* tick population (Jaguar) resistant to four acaricide classes (organophosphates (OP), synthetic pyrethroids (SP), amitraz and macrocyclic lactones (ML)) and reveal that the cattle tick has a multidrug detoxification mechanism based on ABC transporter proteins. Acaricide toxicity was assessed using the larval packet test (LPT) and mortality data were subjected to probit analysis using a susceptible strain (POA) as reference. Larvae were pre-exposed to sub-lethal doses of the ABC-transporter inhibitors, Cyclosporin A (CsA) and MK571, and subsequently treated with ivermectin, abamectin, moxidectin, chlorpyriphos, cypermethrin or amitraz in LPT. Results show that lethal concentrations 50 % (LC_{50}) of ivermectin, abamectin, moxidectin (MLs) and chlorpyriphos (OP) were significantly reduced in larvae exposed to CsA and MK571 inhibitors in the Jaguar resistant population, but LC_{50} did not change in POA susceptible strain larvae. LC_{50} of cypermetrin (SP) and amitraz remained unchanged in inhibitor-exposed larvae, compared to larvae from Jaguar and POA strains not exposed to inhibitor. These results suggest that ABC transporter proteins can protect ticks against a wide range of acaricides and have an important implication in drug resistance development as a multidrug detoxification mechanism.

Keywords: *Rhipicephalus (Boophilus) microplus*, multidrug resistance, detoxification, ATP-binding cassette transporters

1. Introduction

Rhipicephalus (Boophilus) microplus is one of the most widely distributed tick species and one of the most important parasites of cattle in terms of economic losses and damage to animal health (FAO 2004). Apart from blood loss and damage to hides, *R. microplus* is responsible for the transmission of the pathogens that cause babesiosis and anaplasmosis (Jonsson et al. 2008). The major problem in controlling this parasite is acaricide resistance, which has been confirmed for organophosphates (Patarroyo and Costa 1980), synthetic pyrethroids (Nolan et al. 1989), amitraz (Soberanes et al. 2002), ivermectin (Martins and Furlong, 2001) and fipronil (Castro-Janer et al. 2010). Multidrug resistance is also a common phenotype found in cattle tick populations. Double-resistant (organophosphate and synthetic pyrethroid) (Ortiz et al. 1995), triple-resistant (organophosphate, synthetic pyrethroid and amidine) (Benavides et al. 2000) and quadruple-resistant (organophosphate, synthetic pyrethroid, amidine and macrocyclic lactone) (Fernández-Salas et al. 2012) populations have been reported. This phenotype is also found in other pathogens, such as the nematodes *Teladorsagia circumcincta* (Martínez-Valladares et al., 2012), *Trichostrongylus colubriformis* and *Haemonchus contortus* (Kaminsky et al., 2011). In this sense, considering that the number of new drugs discovered and developed for that purpose has declined (FAO 2004), renewed efforts are required to seek novel target sites.

The molecular basis of acaricide resistance has been the focus of considerable studies (revised by Guerrero et al. 2012). Resistance is mediated via a range of alterations that include reduced drug penetration, mutations that diminish the binding of drug to target, and altered metabolism of drugs (Rosario-Cruz et al. 2009a; Perry et al. 2011). It is known that metabolism of drugs comprises phase I, II and III systems. In phase I, the reactions involved are oxidation, reduction or hydrolysis, catalyzed by cytochrome P450

and carboxylesterase enzymes (Perry et al. 2011). In phase II, after conjugation with specific metabolites, the drugs catalyzed by glutathione-S-transferases and UDT-glycosyltransferases are transformed into less toxic and more soluble end-products (Perry et al. 2011). In phase III, more recently recognized as an important stage in the detoxification of drugs (Ishikawa 1992), ATP-binding cassette (ABC) transporters play crucial roles in drug absorption, distribution and excretion (Leslie et al. 2005; Lespine et al. 2008; Pohl et al. 2011).

ABC transporters are membrane-integrated proteins found in all organisms (Lage 2003). These proteins actively pump a broad range of structurally and chemically different compounds (as multidrug transporters) out of the cells against their concentration gradients in an ATP-dependent process, thereby reducing cellular concentrations of toxic compounds and acting in cellular defense (Holland and Blight, 1999; Leslie et al. 2005). For this reason, ABC transporters have been associated with multidrug resistance (MDR) mechanisms, some of which have been well characterized in cancer cells and pathogens (revised by Lage 2003).

Enzymes active during phase I and II have been the object of greater attention in drug detoxification in *R. microplus* (Guerrero et al. 2002; Da Silva Vaz et al. 2004; Li et al. 2008; Rosario-Cruz et al. 2009b); however, recently we provided the first proof of the participation of ABC transporters in ivermectin detoxification in *R. microplus* (Pohl et al. 2011).

Considering that, these transporters play an active role in a wide spectrum of chemical compounds; this study investigates whether ABC transporters are involved in the detoxification of other classes of acaricides. The potential of inhibitors of ABC transporters to enhance the susceptibility of a multidrug resistant tick population to macrocyclic lactones (ML) (ivermectin, abamectin and moxidectin), organophosphate (OP)

(chlorpyriphos), synthetic pyrethroid (SP) (cypermethrin) and amitraz was assessed through the Larvae Packet Test (LPT).

The discovery of this novel detoxification mechanism with a wide substrate range can contribute to improve tools to detect and shed new light on resistance mechanisms of this important parasite.

2. Materials and methods

2.1. Ticks

The Porto Alegre (POA) strain, used as susceptible reference strain, was originally collected from a farm without history of acaricide use in the state of Rio Grande do Sul (Brazil) and was maintained under standard laboratory conditions with artificial infestations on calves at the Faculdade de Veterinária from the Universidade Federal do Rio Grande do Sul, Brazil. The field tick population Jaguar, used as a multi-acaricide resistant population, was originally collected from a farm where acaricides are intensively used, in the municipality of Eldorado do Sul, state of Rio Grande do Sul (Brazil). The tick population was maintained based on artificial infestations on calves at the Instituto de Pesquisas Veterinárias Desidério Finamor- FEPAGRO, Eldorado do Sul, Brazil. All experiments were conducted following the guidelines of the Ethics Committee on Animal Experimentation of UFRGS and FEPAGRO.

2.2. Preparation of ticks

Calves were infested with 15-day-old tick larvae. Twenty-one days post-infestation, detached fully engorged female ticks were collected from ground and maintained at 27- 28 °C and 80- 90% relative humidity. Following oviposition, eggs were transferred to 3-mL glass vials which were then plugged with a cotton cap. Larval hatching occurred

approximately 30 days after collection of engorged females. Bioassays were performed using 14- to 21-day-old larvae.

2.3. Bioassays

2.3.1. Acaricides

Technical grade acaricides used were: chlorpyriphos 99.9 % (Syngenta, Brazil), cypermethrin 95.5 % (Novartis, Brazil), ivermectin 99.9 % (Sigma Aldrich, USA), moxidectin (Fort Dodge, USA) and abamectin (Ourofino, Brazil).

2.3.2. Preparation of the packets to determine resistance to organophosphates, synthetic pyrethroids and macrocyclic lactones

The LPT was used for detection of ivermectin, abamectin, moxidectin, chlorpyriphos and cypermethrin resistance according to the FAO protocol (1984). Treatment solutions were prepared by serially dissolving the acaricides in a 2:1 (v/v) trichloroethylene and olive oil vehicle. A volume of 0.67 mL of each acaricide dilution or vehicle alone (control) was applied on a filter paper (Whatman 541, 850 mm x 750 mm; FAO, 1984). Trichloroethylene was evaporated for 24 hours. Ivermectin, abamectin and moxidectin 0.03, 0.08, 0.1, 0.15, 0.18, 0.2, 0.3%; cypermethrin 0.02, 0.03, 0.04, 0.08, 0.1, 0.2, 0.3% and chlorpyriphos 0.003, 0.006, 0.01, 0.02, 0.03, 0.04, 0.06% were used against the susceptible strain. Ivermectin 0.03, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.6, 1.0% ; abamectin and moxidectina 0.03, 0.08, 0.1, 0.15, 0.18, 0.2, 0.3% ; cypermethrin 0.3, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0, 4.0% and chlorpyriphos 0.3, 0.6, 0.8, 1.0, 2.0, 3.0, 4.0% were used against the resistant strain. The concentrations of amitraz used against the susceptible strain were 0.0000625, 0. 00025, 0.0005, 0.0039, 0.0078, 0.0156, 0.03%, and against the resistant strain were 0.0005, 0.001, 0.002, 0.0078, 0.0156, 0.0625, 0.125, 0.25, 0.5%.

2.3.3. Preparation of the packets to determine resistance to amitraz

A modified version of the LPT was conducted following the procedures described in Miller et al. (2002). Formulated amitraz (12.5 %) was used to prepare treatment solutions serially diluted in a 2:1 (v/v) trichloroethylene and olive oil vehicle. A volume of 0.67 mL of each acaricide dilution or vehicle alone (control) was applied on a nylon fabric (Type 2320, Cerex Advanced Fabrics, Pensacola, FL). Trichloroethylene was evaporated for 24 hours. The concentrations of amitraz used for susceptible strain were 0.0000625, 0. 00025, 0.0005, 0.0039, 0.0078, 0.0156, 0.03% and for resistant strain were 0.0005, 0.001, 0.002, 0.0078, 0.0156, 0.0625, 0.125, 0.25, 0.5%.

2.3.4. Larval Packet Test (LPT) with pre-exposure to a single concentration of ABC transporter inhibitors

Before LPT, larvae of the POA and Jaguar strains were exposed to ABC transporter inhibitors by immersion (Pohl et al. 2011). The inhibitors CsA and MK571 (Sigma–Aldrich, USA) were diluted to 15 µM (predetermined sublethal dose, data not shown) in 1% ethanol containing 0.02 % Triton-X 100. Approximately 100 larvae were transferred to microcentrifuge tubes containing 0.5 mL of inhibitor solution or control solution (diluent alone). After 10-min immersion in each solution, larvae were dried and placed on the filter paper pieces impregnated with the acaricides. The larval packets were sealed and incubated at 27- 28 ℃ and 80- 90 % relative humidity for 24 hours, and mortality was determined. Only larvae that were observed to move were considered alive. Each experiment was performed at least three times.

2.3.5. LPT data analysis

Mortality data was submitted to probit analysis and a chi-square test assessed the hypothesis of parallelism and equality ($p \leq 0.05$) using the POLO PLUS software (LeOra software 2004) to estimate the lethal concentrations for 50 % (LC_{50}) with its confidence intervals of 95 % (CI 95 %). Resistance ratios (RR) were calculated using the POA strain as reference, based on LC_{50} values. To estimate the toxicity caused by the addition of the ABC transporter inhibitors, synergism factors (SF) were calculated based on LC_{50} values from control conditions in each population. Significance of each comparison was determined only if no overlap was observed in the confidence intervals.

3. Results

RR to ivermectin, abamectin, moxidectin, cypermethrin, chlorpyriphos and amitraz were 3.44, 2.38, 3.99, 15.3, 267.3, 4.85, respectively (Table 1). These results indicate that the Jaguar tick population is resistant to OP, SP, ML and amitraz.

The role of ABC transporter proteins in the defense against acaricides was analyzed based on a LPT conducted using larvae previously exposed to a sublethal dose of CsA or MK571, ABC transporter inhibitors. Mortality data plots analyzed by probit regression against logarithmic acaricides concentrations are shown in Figure 1 (Jaguar resistant population) and Supporting Figure 1 (POA susceptible strain). The pre-exposure of the larvae from the Jaguar resistant tick population to the two ABC transporter inhibitors increased mortality rate to ivermectin, moxidectin, abamectin and chlorpyriphos, though with no effect on cypermethrin and amitraz toxicity (Figure 1). Compared to unexposed larvae, mortality rate of larvae of the POA susceptible strain exposed to inhibitor did not change after exposure to all acaricides tested (Table 1). Table 1 reports the LC_{50} and RR_{50} values calculated from probit analysis for each acaricide. The LC_{50} (CI 95 %) of

ivermectin, abamectin, moxidectin and chlorpyriphos in the Jaguar population, were significantly lower in CsA and MK571 exposed larvae, compared to control larvae (Table 1), indicating that these acaricides present higher toxicities. Additionally, treatments with ABC transporter inhibitors significantly ($p \leq 0.05$) reduced the RR_{50} for these acaricides in the Jaguar population (Table 1).

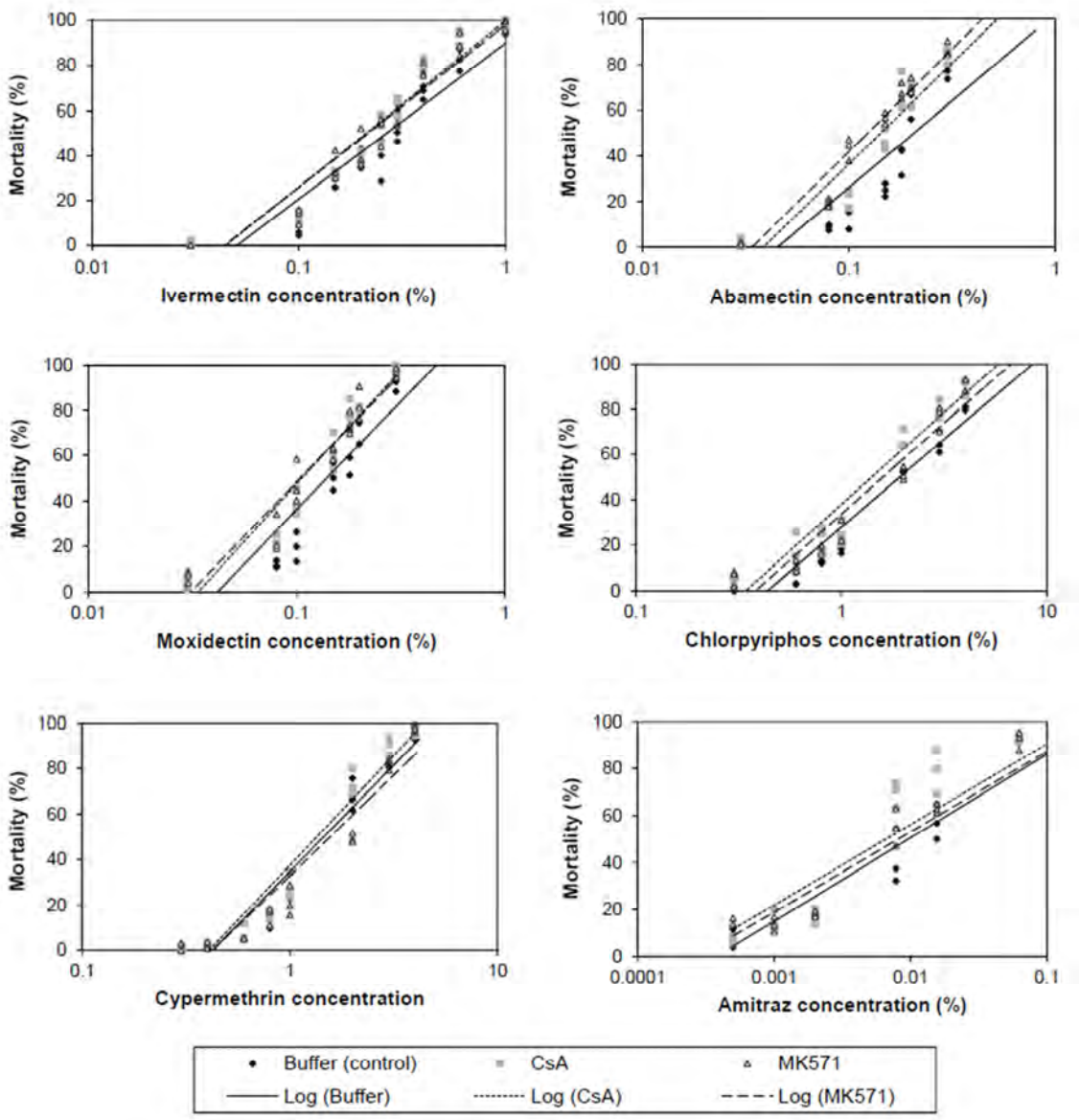
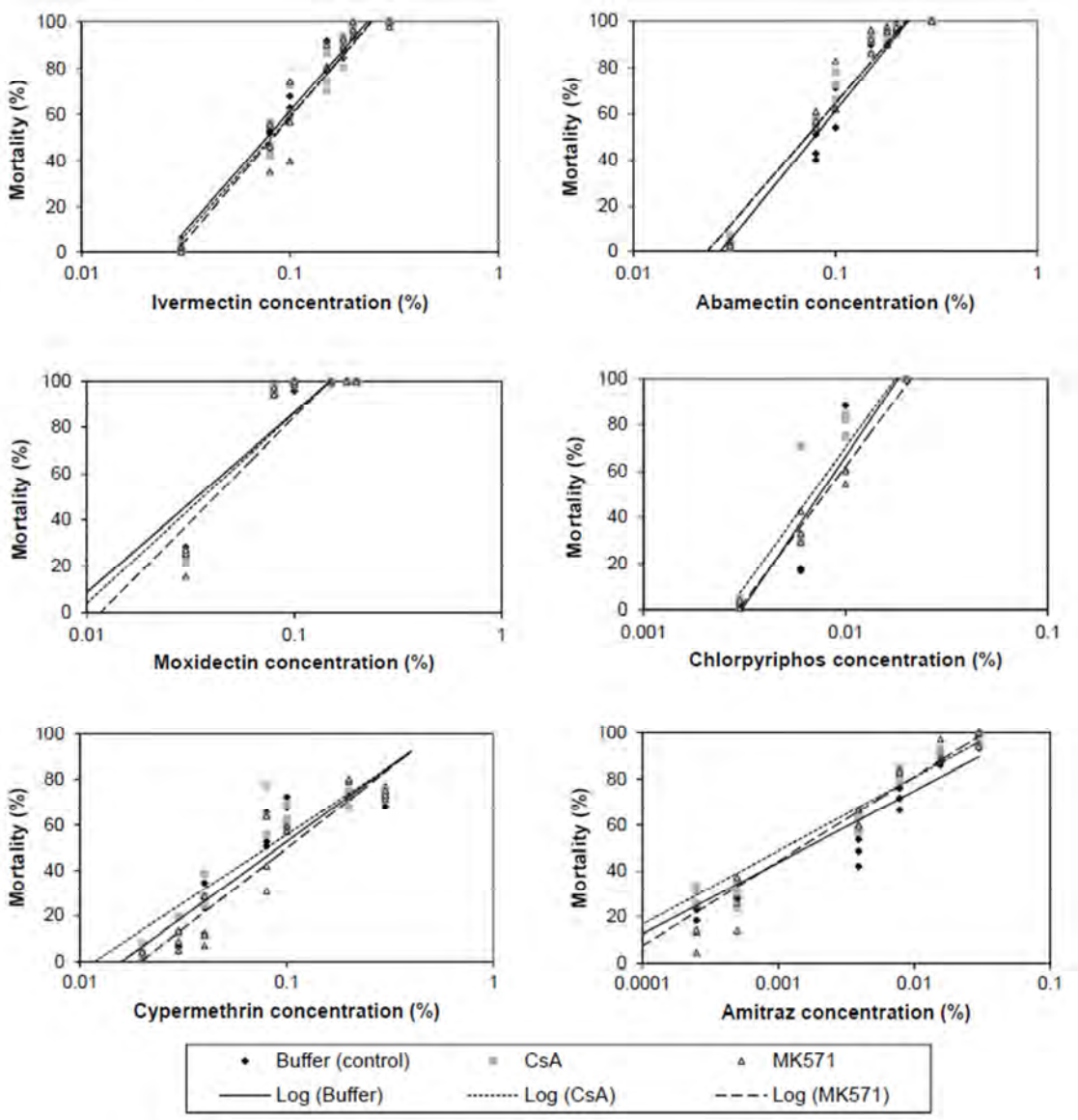


Fig. 1. Effect of CsA and MK571 (15µM) on mortality of resistant (Jaguar) *R. microplus* larvae to acaricides determined by LPT. Larvae were immersed in the inhibitors solutions or diluent alone (control) for 10 min, dried and transferred to filter paper impregnated with increasing acaricide concentrations. Mortality was determined after 24 hours.



Supporting Figure 1. Effect of CsA and MK571 (15µM) on mortality of susceptible (POA) *R. microplus* larvae to acaricides determined by LPT, as described in fig. 1.

Table 1. Lethal concentrations and resistance ratios to six acaricide alone or in association with inhibitors cyclosporine (CsA) or MK571 obtained by LPT with *R. microplus* larvae from susceptible (POA) and multidrug resistant (Jaguar) population.

Strain	Drug association	LC_{50} (CI 95%)	RR_{50} (CI 95%)	SF (CI 95%)
POA	IVM	0.082 (0.076 - 0.087)	-	-
	IVM + CsA	0.087 (0.079 - 0.094)	-	0.945 (0.881 - 1.013)
	IVM + MK571	0.088 (0.081 - 0.095)	-	0.928 (0.868 - 0.992)
Jaguar	IVM	0.281 (0.265 - 0.299)	3.44 (3.21 - 3.68)	-
	IVM + CsA	0.230 (0.218 - 0.242)*	2.66 (2.47 - 2.86)	1.222 (1.136 - 1.314)
	IVM + MK571	0.231 (0.216 - 0.246)*	2.62 (2.44 - 2.81)	1.218 (1.132 - 1.310)
POA	ABA	0.083 (0.079 - 0.086)	-	-
	ABA + CsA	0.076 (0.071 - 0.080)	-	1.090 (1.017 - 1.169)
	ABA + MK571	0.076 (0.071 - 0.081)	-	1.087 (1.011 - 1.167)
Jaguar	ABA	0.197 (0.175 - 0.229)	2.38 (2.23 - 2.54)	-
	ABA + CsA	0.151 (0.142 - 0.160)*	1.99 (1.86 - 2.13)	1.306 (1.229 - 1.389)
	ABA + MK571	0.131 (0.124 - 0.138)*	1.72 (1.60 - 1.85)	1.505 (1.412 - 1.604)
POA	MOX	0.038 (0.035 - 0.040)	-	-
	MOX + CsA	0.038 (0.034 - 0.042)	-	0.986 (0.901 - 1.079)
	MOX + MK571	0.040 (0.038 - 0.042)	-	0.936 (0.861 - 1.018)
Jaguar	MOX	0.150 (0.137 - 0.164)	3.99 (3.71 - 4.29)	-
	MOX + CsA	0.118 (0.108 - 0.128)*	3.11 (2.88 - 3.35)	1.266 (1.197 - 1.340)
	MOX + MK571	0.111 (0.100 - 0.122)*	2.77 (2.57 - 2.99)	1.347 (1.261 - 1.439)
POA	CYP	0.101 (0.083 - 0.125)	-	-
	CYP + CsA	0.086 (0.071 - 0.105)	-	1.182 (1.042 - 1.341)
	CYP + MK571	0.112 (0.095 - 0.134)	-	0.903 (0.808 - 1.009)
Jaguar	CYP	1.547 (1.470 - 1.631)	15.29 (13.92 - 16.79)	-
	CYP + CsA	1.413 (1.355 - 1.474)	16.51 (14.86 - 18.33)	1.095 (1.028 - 1.166)
	CYP + MK571	1.690 (1.568 - 1.829)	15.08 (13.78 - 16.51)	0.915 (0.855 - 0.981)
POA	CHLOR	0.0075 (0.0068 - 0.0082)	-	-
	CHLOR + CsA	0.0068 (0.006 - 0.0075)	-	1.110 (1.041 - 1.183)
	CHLOR + MK571	0.0079 (0.007 - 0.0088)	-	0.949 (0.885 - 1.019)
Jaguar	CHLOR	2.020 (1.916 - 2.134)	267.34 (249.53 - 286.42)	-
	CHLOR + CsA	1.481 (1.365 - 1.611)*	217.57 (201.46 - 234.98)	1.363 (1.257 - 1.479)
	CHLOR + MK571	1.645 (1.472 - 1.851)*	208.78 (192.69 - 226.21)	1.228 (1.133 - 1.331)
POA	AMZ	0.0017 (0.0012 - 0.0025)	-	-
	AMZ + CsA	0.0014 (0.0010 - 0.0018)	-	1.249 (0.981 - 1.590)
	AMZ + MK571	0.0018 (0.0013 - 0.0025)	-	0.926 (0.745 - 1.149)
Jaguar	AMZ	0.0084 (0.0068 - 0.0102)	4.85 (3.91 - 6.01)	-
	AMZ + CsA	0.0055 (0.0046 - 0.0065)	3.99 (3.23 - 4.95)	1.513 (1.261 - 1.817)
	AMZ + MK571	0.0070 (0.0058 - 0.0084)	3.89 (3.10 - 4.57)	1.187 (0.979 - 1.440)

S.E.: standard error; LC: lethal concentration; CI: confidence interval; RR: resistance ratio; SF: synergism factor; asterisk: statistically different values ($p \leq 0.05$). CsA: Cyclosporine A; IVM: ivermectin; ABA: abamectin; MOX: moxidectin; CYP: cypermethrin; CHLOR: chlorpyriphos; AMZ: amitraz

4. Discussion

Multidrug resistance against unrelated drugs that differ widely in molecular structure and target specificity have been described for several parasites. This phenotype implies serious consequences to animals and human health (Kaminsky et al. 2011; Martínez-Valladares et al. 2012). In the cattle tick, the presence of this phenotype highlights the need for a better understanding of the molecular mechanisms involved in multidrug resistance. Such more in-depth knowledge would be useful in efforts directed towards extending acaricide marketability.

In this study we described a *R. microplus* population resistant to four classes of chemical products used to control this parasite. We diagnosed resistance to OP, SP, ML and amitraz. Recently, Fernández-Salas and coworkers (2012) have also reported a tick population resistant to these four classes of acaricides in Mexico.

ABC transporters inhibitors may promote the intracellular accumulation of a drug, increasing its efficacy against the pathogen. So, they are useful to study the importance of ABC transporters in drug resistance (Buss et al. 2002; Lespine et al. 2008; Lifschitz et al. 2010; Tompkins et al. 2011). Using this approach, we recently showed the association of ABC transporters in ivermectin resistance in *R. microplus* (Pohl et al. 2011). Here, the association of ABC transporters in other MLs (abamectin and moxidectin) and OP (chlorpyriphos) was also demonstrated.

ABC transporters have been associated with resistance to a number of drugs in different organisms, and their capacity to transport a variety of chemically different drugs makes them an important molecular mechanism in multidrug resistance (Lage 2003). Although resistance conferred by ABC transporters plays a crucial role, it should be emphasized that resistance by detoxification may emerge through a number of different ways. Enzymes active during phase I and II metabolism may play parallel roles (Guerrero

et al. 2012) and additional studies are required, since detoxification systems are highly complex, show considerable variability, and genetic uniqueness (Ffrench-Constant et al. 2004).

Overexpression of ABC transporters is usually associated with ivermectin resistance, as reported for a ivermectin-resistant *H. contortus* strain (Xu et al. 1998), for *C. elegans* and in *Pediculus humanus humanus* selected for ivermectin resistance *in vitro* (James and Davey 2009; Yoon et al. 2011), and for ivermectin-exposed *S. scabiei* (Mounsey et al. 2010) and *Brugia malayi* (Tompkins et al. 2011).

In addition, ABC transporters have been reported to protect against or interact with a variety of pesticides. Bain and LeBlan (1996) showed that halophenoxy, organochlorine, organophosphate and synthetic pyrethroid interact with the human P-glycoprotein, an ABC transporter. Toxicity of *Culex pipiens* to cypermethrin, endosulfan and ivermectin (but not to chlorpyrifos), was higher in larvae treated in conjunction with verapamil, an ABC transporter inhibitor (Buss et al. 2002). The treatment with the same inhibitor increased toxicity in *Aedes caspius* larvae exposed to temephos and diflubenzuron (Porretta et al. 2008). Moreover, James and Davey (2009) showed cross-resistance of ivermectin-resistant *C. elegans* to moxidectin and levamisole, but not to pyrantel.

Our results suggest that ABC transporter proteins can protect ticks against a wide range of acaricides, and can have an important implication in drug resistance development as a multidrug detoxification mechanism. These findings indicate that planning vector control programs may become more complex; on the other hand, understanding and reversing the mechanisms involved in the development of resistance to currently used acaricides could provide a means to extend their usage and lifespan, and to improve the testing methods for monitoring tick resistance.

Acknowledgements

We thank the CNPq - Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular, FINEP, CAPES, CNPq and FAPERGS for supporting our work.

References

Bain LJ, LeBlanc GA (1996) Interaction of structurally diverse pesticides with the human MDR1 gene product P-glycoprotein. Toxicol Appl Pharmacol 141: 288- 298.

Benavides E, Rodríguez JL, Romero A (2000) Isolation and partial characterization of the Montecitos strain of *Boophilus microplus* (Canestrini, 1877) multiresistant to different acaricides. Ann NY Acad Sci 916: 668- 671.

Buss DS, McCaffery AR, Callaghan A (2002) Evidence for p-glycoprotein modification of insecticide toxicity in mosquitoes of the *Culex pipiens* complex. Med Vet Entomol 16: 218-22.

Castro-Janer E, Martins JR, Mendes MC, Namindome A, Klafke GM, Schumaker TT (2010) Diagnoses of fipronil resistance in Brazilian cattle ticks (*Rhipicephalus (Boophilus) microplus*) using in vitro larval bioassays. Vet Parasitol 173: 300-306.

Da Silva Vaz Jr I, Lermen TT, Michelon A, Ferreira CAS, de Freitas DRJ, Termignoni C, Masuda A (2004) Effect of acaricides on the activity of a *Boophilus microplus* glutathione S-transferase. Vet Parasitol 119: 237- 45.

FAO (1984) Ticks and tick borne disease control. A practical field manual. Volume I. Tick control. Food and Agriculture Organization, Animal Production and Health Division, Rome, 299 pp.

FAO (2004) Resistance Management and Integrated Parasite Control in Ruminants-guidelines, Executive Summary, Food and Agriculture Organization, Animal Production and Health Division, Rome, 11 pp.

Fernández-Salas A, Rodríguez-Vivas RI, Alonso-Díaz MA (2012) First report of a *Rhipicephalus microplus* tick population multi-resistant to acaricides and ivermectin in the Mexican tropics. Vet Parasitol 183: 338-42.

Ffrench-Constant RH, Daborn PJ, Le Goff G (2004) The genetics and genomics of insecticide resistance. Trends Genet 20: 163-70.

Guerrero FD, Pruett JH, Li AY (2002) Molecular and biochemical diagnosis of esterase-mediated pyrethroid resistance in a Mexican strain of *Boophilus microplus* (Acari: Ixodidae). Exp Appl Acarol 28: 257-64.

Guerrero FD, Lovis L, Martins JR (2012) Acaricide resistance mechanisms in *Rhipicephalus (Boophilus) microplus*. Rev Bras Parasitol Vet 21: 1-6.

Holland IB, Blight MA (1999) ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. J Mol Biol 293: 381-99.

Ishikawa T (1992) The ATP-dependent glutathione S-conjugate export pump. Trends Biochem Sci 17: 463-468.

James CE, Davey MW (2009) Increased expression of ABC transport proteins is associated with ivermectin resistance in the model nematode *Caenorhabditis elegans*. Int J Parasitol 39: 213-20.

Jonsson NN, Bock RE, Jorgensen WK (2008) Productivity and health effects of anaplasmosis and babesiosis on *Bos indicus* cattle and their crosses, and the effects of differing intensity of tick control in Australia. Vet Parasitol 155: 1-9.

Kaminsky R, Bapst B, Stein PA, Strehlau GA, Allan BA, Hosking BC, Rolfe PF, Sager H (2011). Differences in efficacy of monepantel, derquantel and abamectin against multi-resistant nematodes of sheep. Parasitol Res 109:19- 23.

Lage H (2003) ABC-transporters: implications on drug resistance from microorganisms to human cancers. Int J Antimicrob Agents 22: 188-99.

LeOra Software (2004) Polo Plus Probit and Logit Analysis: User's Guide, Berkeley, p. 36.

Leslie EM, Deeley RG, Cole SP (2005) Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicol Appl Pharmacol 204: 216-37.

Lespine A, Alvinerie M, Vercruysse J, Prichard RK, Geldhof P (2008) ABC transporter modulation: a strategy to enhance the activity of macrocyclic lactone anthelmintics. Trends Parasitol 24: 293-8.

Li AY, Davey RB, Miller RJ, Guerrero FD, George JE (2008) Genetics and mechanisms of permethrin resistance in the Santa Luiza strain of *Boophilus microplus* (Acari: Ixodidae). J Med Entomol 45: 427-38.

Lifschitz A, Entrocasso C, Alvarez L, Lloberas M, Ballent M, Manazza G, Virkel G, Borda B, Lanusse C (2010) Interference with P-glycoprotein improves ivermectin activity against adult resistant nematodes in sheep. Vet Parasitol 172: 291-8.

Martínez-Valladares M, Famularo MR, Fernández-Pato N, Cordero-Pérez C, Castañón-Ordóñez L, Rojo-Vázquez FA (2012). Characterization of a multidrug resistant *Teladorsagia circumcincta* isolate from Spain. Parasitol Res 110: 2083- 7.

Martins JR, Furlong J (2001) Avermectin resistance of the cattle tick *Boophilus microplus* in Brazil. Vet Rec 149: 64.

Miller RJ, Davey RB, George JE (2002) Modification of the food and agriculture organisation larval packet test to measure amitraz susceptibility against ixodidae. J Med Entomol 39: 645–651.

Mounsey KE, Pasay CJ, Arlian LG, Morgan MS, Holt DC, Currie BJ, Walton SF, McCarthy JS (2010) Increased transcription of Glutathione S-transferases in acaricide exposed scabies mites. Parasit Vectors 18: 3- 43.

Nolan J, Wilson JT, Green PE, Bird PE (1989) Synthetic pyrethroid resistance in field samples in the cattle tick (*Boophilus microplus*). Aust Vet J 66: 179-82.

Ortiz EM, Santamaría VM, Ortiz NA, Soberanes CN, Osorio MJ, Franco BR, Martínez IF, Quezada DR, Fragoso SH (1995) Caracterización de la resistencia de *Boophilus microplus* a ixodicidas em México. In: Memorias de IV Seminario Internacional de

Parasitología Animal, Resistencia y Control en Garrapatas y Moscas de Importancia Veterinaria, Acapulco, Guerrero, México, pp. 58–66.

Patarroyo JH, Costa JO (1980) Susceptibility of Brazilian samples of *Boophilus microplus* to organophosphorus acaricides. Trop Anim Health Prod 12: 6-10.

Perry T, Batterham P, Daborn PJ (2011) The biology of insecticidal activity and resistance. Insect Biochem Mol Biol 41: 411-422.

Pohl PC, Klafke GM, Carvalho DD, Martins JR, Daffre S, da Silva Vaz Jr I, Masuda A (2011) ABC transporter efflux pumps: A defense mechanism against ivermectin in *Rhipicephalus (Boophilus) microplus*. Int J Parasit 41: 1323-1333.

Porretta D, Gargani M, Bellini R, Medici A, Punelli F, Urbanelli S (2008) Defence mechanisms against insecticides temephos and diflubenzuron in the mosquito *Aedes caspius*: the P-glycoprotein efflux pumps. Med. Vet Entomol 22: 48-54.

Rosario-Cruz R, Almazan C, Miller RJ, Dominguez-Garcia DI, Hernandez-Ortiz R, de la Fuente J (2009a). Genetic basis and impact of tick acaricide resistance. Front Biosci 14: 2657-65.

Rosario-Cruz R, Guerrero FD, Miller RJ, Rodriguez-Vivas RI, Tijerina M, Dominguez-Garcia DI, Hernandez-Ortiz R, Cornel AJ, McAbee RD, Alonso-Diaz MA (2009b) Molecular survey of pyrethroid resistance mechanisms in Mexican field populations of *Rhipicephalus (Boophilus) microplus*. Parasitol Res 105: 1145-53.

Soberanes CN, Santamaría VM, Fragoso SH, García VZ (2002) Primer caso de resistencia al amitraz en la garrapata del ganado *Boophilus microplus* en México. Tec Pec Mex 40: 81–92.

Tompkins JB, Stitt LE, Morrissette AM, Ardelli BF (2011). The role of *Brugia malayi* ATP-binding cassette (ABC) transporters in potentiating drug sensitivity. Parasitol Res 109: 1311- 22.

Xu M, Molento M, Blackhall W, Ribeiro P, Beech R, Prichard R (1998) Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homolog. Mol Biochem Parasitol 91: 327-35.

Yoon KS, Strycharz JP, Baek JH, Sun W, Kim JH, Kang JS, Pittendrigh BR, Lee SH, Clark JM (2011) Brief exposures of human body lice to sublethal amounts of ivermectin over-transcribes detoxification genes involved in tolerance. Insect Mol Biol 20: 687-99.

3.2. PARTICIPAÇÃO DOS TRANSPORTADORES ABC NA RESISTÊNCIA À IVERMECTINA EM CÉLULAS EM CULTIVO

ARTIGO III

An ivermectin-resistant *Rhipicephalus (Boophilus) microplus* cell line and the contribution of ABC transporters in resistance

Paula C. Pohl[a], Danielle D. Carvalho[b], Sirlei Daffre[c], Itabajara da Silva Vaz Jr[a,d,#], Aoi Masuda[a,e]

[a]Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; [b]Departamento de Análises Especiais, SD&W Modelagem e Soluções Estratégicas Ltda., São Paulo, Brazil; [c]Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brazil; [d]Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; [e]Departamento de Biologia Molecular e Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

Manuscrito em preparação.

Abstract

Widespread resistance to acaricide is one of the greatest challenges to the control of the cattle tick *Rhipicephalus (Boophilus) microplus*. Understanding the development of acaricide resistance mechanism in the cattle tick is critical to prolong the efficacy of current acaricides, to identify markers for monitoring drug resistance, and to design new drugs. This study describes the establishment of an ivermectin (IVM)-resistant *R. microplus* embryonic cell line, called BME26-IVM, through step-wise exposure to increasing doses of IVM, starting with a dose of 0.5 µg/ mL up to 6 µg/ mL, for a 46-week treatment period. BME26-IVM cell line was 4.5 times more resistant to IVM than the parental BME26 cell line (LC_{50} 15.1 ± 1.6 and LC_{50} 3.35 ± 0.09, respectively). Resistance to IVM was significantly reduced by co-incubation with ATP-binding cassette (ABC) transporters inhibitor, and increased expression levels of ABC transporter genes were found in IVM exposed cells, suggesting the involvement of these proteins in IVM detoxification. These results strengthen previous report that showed the involvement of ABC transporters in tick IVM-resistance and suggest that similar resistance mechanisms may be induced both *in vitro* and *in vivo*, supporting this *in vitro* approach as a valuable alternative method to study drug-detoxification mechanism.

Keywords: *Rhipicephalus (Boophilus) microplus*; tick cell line; ivermectin resistance; ATP-binding cassette transporter.

1. Introduction

Rhipicephalus (Boophilus) microplus is one of the most important cattle parasites worldwide. It causes economic losses, significantly reducing cattle live-weight gain, milk production and transmitting tick-borne diseases (Graf et al., 2004, Jonsson et al., 2006).

The development of chemicals with acaricidal properties quickly led to the adoption of these substances as the main method of cattle tick control (Guerrero et al., 2012). However, *R. microplus* have become resistant to most acaricides commercially available, reducing the chances of effective tick control (Castro-Janer et al., 2010; Klafke et al., 2006; Martins and Furlong, 2001; Soberanes et al., 2002).

Macrocyclic lactones, such as ivermectin (IVM), are increasingly being used to control endo- and ectoparasites, including *R. microplus* (Fox, 2006; Guerrero et al., 2012). IVM activates glutamate-gated (GluCl) chloride ion channels in invertebrate nerve and muscle cells, paralyzing peripheral motor function and killing the parasite (Fox, 2006). However, recent reports about cattle tick populations resistant to IVM in Brazil (Klafke et al., 2006; Martins and Furlong, 2001), Mexico (Perez-Cogollo et al., 2010) and Uruguay (Castro-Janer et al., 2011) emphasize the need for a more in-depth understanding of the mechanisms behind acaricide resistance.

Numerous studies have associated ATP-binding cassette (ABC) transporters with drug resistance in nematodes, arthropods and cancer cells (James and Davey, 2009; Mounsey et al., 2010; Pohl et al., 2011). ABC transporters are membrane-integrated proteins expressed in all organisms and essential to several physiological processes (Holland and Blight, 1999). Some ABC transporters are involved in the cellular detoxification of xenobiotics by pumping drugs across membranes before these reach their target site (Szakács et al., 2008). These ABC transporters are able to transport a variety of

structurally and functionally different drugs, conferring the cells a phenotype termed multidrug resistance (MDR) (revised by Higgins, 2007 and Lage, 2003).

Recently, we showed the association of ABC transporters with macrocyclic lactones resistance (ivermectin, abamectin and moxidectin) and organophosphate resistance (Pohl et al., 2011; Pohl et al., data unpublished) in *R. microplus,* suggesting the implication of ABC transporters in drug resistance as a multidrug detoxification mechanism.

Currently, the detection of acaricide resistance is based on bioassay techniques, such as the larval packet test and the adult immersion test; however, bioassays are time-consuming and laborious (Drummond et al., 1973; Klafke et al., 2006). Development of more sensitive diagnosis methods, based on molecular biology methodologies, may be an alternative, but they rely on the understanding of the mechanisms involved in resistance. On the other hand, the identification of the mechanisms of drug resistance in tick populations is complex, since such knowledge depends on finding where acaricide resistance is already established in the field.

In this scenario, the use of cell cultures may be an attractive alternative, since experimental parameters can be rigorously controlled, reducing the variability between experiments. Additionally, cell cultures are a cost-effective approach where the drug resistance mechanism can be studied, even before resistance has established in the field. Moreover, the technique allows the rapid screening of new drugs under development. For example, selection of cancer cell lines for resistance to cytotoxic drugs has been a key element in searching for genetic alterations that are responsible for drug resistance (Edwards et al., 2008; Jovelet et al., 2012).

Several tick cell lines have been established and used in studies on host–vector–pathogen relationship, tick biology and genetic manipulation (Bell-Sakyi et al., 2007).

Here, we report the *in vitro* selection of an IVM-resistant *R. microplus* cell line and the use of this cell line to study the mechanisms of drug resistance showing the involvement of ABC transporters as a mechanism of IVM detoxification in ticks.

2. Materials and Methods

2.1. Cell lines and maintenance

Ivermectin (IVM)-resistant cell line (BME26-IVM) was derived from drug-sensitive BME26 cells, originally isolated from *R. microplus* embryos (Esteves et al., 2008). Cell lines were grown as adherent monolayers in L-15B300 medium supplemented with 5% heat-inactivated FBS (Gibco), 10% TPB (Difco), penicillin (100 units/ mL), streptomycin (100 mg/ mL) (Gibco) and 0.1% bovine lipoprotein concentrate (ICN), pH 7.2 (Esteves et al., 2008). BME26-IVM was maintained in the same medium supplemented with IVM (6 µg/ mL) (Sigma). Cultures were grown at 34 °C in 25 cm^2 plastic flasks (Falcon) in 5 mL of the medium, which was replaced once a week.

2.2. IVM toxicity on cell proliferation

IVM toxicity was primarily analyzed based on cell proliferation. BME26 cells (5 x 10^5 cells) were seeded in 25 cm^2 plastic flasks and cultured in 5 mL of medium for 4 hours; to cells adhere to the flasks. After this period, the medium was replaced with fresh medium alone or medium supplemented with IVM 0.5; 2.5; 12.5 or 62.5 µg/ mL, diluted from a stock solution of 2 mg/ mL IVM in 50% methanol. Every 7 days, cells were harvested with a cell scraper (Nunc), stained with trypan blue and counted in triplicate with a Neubauer hemocytometer; to generate a growth curve over a 28-day culture period.The experiment was performed in triplicates.

The effect of methanol alone (IVM solvent) was also determined. BME26 cells (1 x 10^6 cells) were seeded in 25 cm^2 plastic flasks and incubated in 5 mL of medium for 4 hours. The medium was replaced with fresh medium without or with increasing concentrations of methanol (0.025; 0.0625; 0.156; 0.39, 0.975%). After a 9-day incubation period, cell survival was evaluated by counting viable cells stained with trypan blue, using a Neubauer hemocytometer. The percentage of surviving cells in treated groups was calculated against the untreated control group (medium without methanol), considered as 100%.

2.3. Establishment of IVM-resistant cell line

BME26-IVM cells were derived from parental BME26 cells (23^{rd} passage) by continuous exposure of these cells to increasing concentrations of IVM in a gradual step-wise manner. Every 4 weeks, cells were harvested with a cell scraper and transferred onto a new plastic flask containing the same concentration of IVM or onto a flask containing a higher concentration of drug. Starting from an initial non-toxic concentration of 0.5 µg/ mL IVM, cells were able to grow at doses of 6 µg/ mL IVM after 46 weeks of selection.

2.4. Growth curves of BME26 and BME26-IVM

BME26 (40^{th} passage) and BME26-IVM (38^{th} passage) cells were seeded (5 x 10^5 cells) in 25 cm^2 plastic flasks and cultured in 5 mL of medium. After 4-hours incubation period, the medium was replaced with fresh medium alone or medium supplemented with 6 µg/ mL IVM. Every 7 days, cells were harvested with a cell scraper, stained with trypan blue and counted in triplicate with a Neubauer hemocytometer; to generate a growth curve over a 28-day culture period.The experiment was performed in triplicates.

2.5. Doubling time determination

The cells doubling times (dt) were calculated from exponential growth curve (7^{th} to 28^{th} days) using the equation dt = ln(2)/ n and n= [ln(final number of cells) - ln(initial number of cells)]/(final time) – (initial time).

2.6. Lethal concentration 50% and resistance ratio determination

BME26 (41^{st} passage) and BME26-IVM (39^{th} passage) cells were seeded ($1x10^6$ cells) in 25 cm^2 plastic flasks and incubated in 5 mL of the medium for 4 hours.; to cells adhere to the flasks. After this period the medium was replaced with fresh medium without or with 1, 3, 6 or 9 µg/ mL IVM. The medium was supplemented with 5 µM cyclosporin A (CsA) (predetermined non-toxic dose) to assess inhibitor effect. After a 9-day incubation period, cells were harvested, stained with trypan blue and counted in triplicate with a hemocytometer. The percentage of survival cells in treated groups was calculated against the untreated control group, considered as 100%. The experiment was performed in triplicates. Concomitantly, a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was performed to confirm the results.

Lethal concentration 50 (LC_{50}), defined as the concentration necessary to reduce cell survival by 50%, was calculated from dose-response curves in presence or absence of CsA. The resistance ratio (RR) was estimated by dividing the LC_{50} for the BME26-IVM cells by that of parental BME26 cells. Fold-reversal factor was estimated by dividing the LC_{50} between IVM and IVM plus CsA treatments.

2.7. Light microscopy

BME26 and BME26-IVM cells incubated in medium alone or medium supplemented with 6 µg/ mL IVM for 9 days were collected by scraping and centrifuged

onto microscope slides at 1,000 × g for 5 min, using a cytocentrifuge (Fanem). Cells were air-dried, stained with Panoptico (Newprov) stain and observed under oil immersion in an Axiofhot microscope (Zeiss).

2.8. Quantitative PCR (qPCR)

To evaluate the mRNA expression of ABC transporters, BME26 and BME26-IVM cells incubated for 9 days in medium alone or medium with 6 or 9 µg/ mL IVM, were collected by scraping and centrifuged at 5,000 x g for 5 mim. Total RNA was extracted from cells using TRIzol® reagent (Invitrogen) following the manufacturer's recommendations. RNA quantity was estimated spectrophotometrically at 260 nm in NanoDrop 1000 instrument (Thermo Fisher Scientific). One microgram of total RNA was treated with DNase I (Invitrogen, USA) and reverse-transcribed using the High-capacity cDNA Reverse Transcription kit with random primers, according to the manufacturer's recommendations (Applied Biosystems). Quantitative PCR (qPCR) was carried out to quantify mRNA of ABC transporters using the Quantimix Easy Sybr Green amplification kit (Biotools) in Mastercycler® ep realplex real-time PCR instrument (Eppendorf). The specific primers designed for gene amplification are described in Table 1. Gene amplification of 40-S tick protein was used for normalization (Pohl et al., 2008; Fabres et al., 2010). Cycling parameters were 10 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s and extension at 72 °C for 20 s. To confirm primer specificity to produce a single amplification product, the dissociation curve analysis was performed using the default parameters of the instrument and representative products from qPCR were electrophoresed. Primer efficiency was measured with 6-fold serially diluted cDNA in triplicate, while for the analyses 300 ng cDNA was added to each reaction. The relative amount of each ABC transporter mRNA produced per 40-S protein

was calculated for each sample. Each analysis was conducted in triplicate. The relative expression ratio of ABC transporter genes in each experiment was calculated according to the mathematical model described by Pfaffl (2001) in Relative Expression Software Tool (REST-MCS©, version 2) (Pfaffl et al., 2002).

Table 1- Primers used in the relative quantification of *R. microplus* ABC transporter mRNAs by qPCR.

Gene	NCBI accession no.	Primer	Sequence 5'-3'	Tm	Amplicon size (bp)
40S ribosomal	EW679928	Sense	ACG ACC GAT GGC TAC CTC CTC CGC	59.1	106
		Antisense	TGA GGC GAA CCT GGT TGT GCT GAG CG	59.2	
RmABCC1	JN098447	Sense	GAC ACC ATT CAC CGA GAG TTC AGT AGC AC	61.9	120
		Antisense	GCC CTG CTC CAC TAT TTC GCC ACC	64.2	
RmABCC2	JN098448	Sense	CGC GGG ACC TTC TGA AGC	58.9	84
		Antisense	GGT AGC TCG GTA TAG GGC TAG ACG	59.8	
RmABCB7	JX170903	Sense	AGTGATGGCAGAGTGTGTGAACGG	59.9	109
		Antisense	CATTCTCCTGGTGGGCTTGATGC	61.4	
RmABCB10	JN098446	Sense	GCC GCA GTT GTC ACT TGT TGG TTT G	61.3	95
		Antisense	ACG TCC GCT GCC ACT TGC CTC	64.9	

2.7. Statistical analysis

Statistical significance of cytotoxicity and doubling time results were analyzed with two-way ANOVA and Bonferroni tests. Statistical significance of qPCR results were analyzed by one-way ANOVA and Tukey's tests. Results are expressed as mean ± S.D. *P* values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Selection of ivermectin-resistant cell line

To select IVM-resistant cells, a preliminary analysis of IVM toxicity was performed on the parental BME26 cells (Fig. 1). Growth curves for BME26 in increasing IVM concentrations were compared. When treated with 0.5 µg/ mL of IVM, BME26 cells were able to survive without significant effect on doubling time, when compared with cells not exposed to IVM (control). They had doubling times of 11 and 13 days, respectively. When treated with 2.5 µg/ mL of IVM, BME26 cells were able to survive, though with a significant longer doubling time (30 days). When treated with higher IVM doses (12.5 and 62.5 µg/ mL) the cells did not survive (Fig. 1). Cell viability was not affected by methanol (IVM solvent) (data not shown).

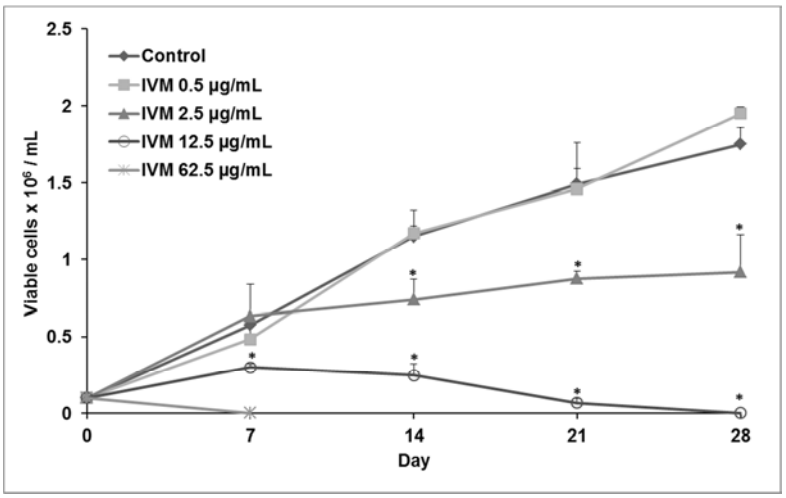


Fig. 1- Growth curves for BME26 in increasing IVM concentrations. Initially, $5x10^5$ cells were seeded into 25 cm^2 plastic flasks and cultured in medium alone or medium supplemented with IVM 0.5; 2.5; 12.5 or 62.5 µg/ mL. Cells were harvested and counted in triplicate with a hemocytometer every 7 days over a 28-day culture period. All measurements were expressed as mean ± S.D., *n*= 3. Asterisks (*) denote significant differences from control ($p \leq 0.05$).

Parental BME26 cells were maintained on medium containing initially 0.5 µg/ mL of IVM. Weekly, medium was replaced by fresh medium and each four weeks the IVM concentration was increased. Fig. 2 shows the time course of IVM resistance selection. After 46 weeks, the new IVM-resistant cell line was established and cells were able to grow at concentration of 6 µg/ mL IVM.

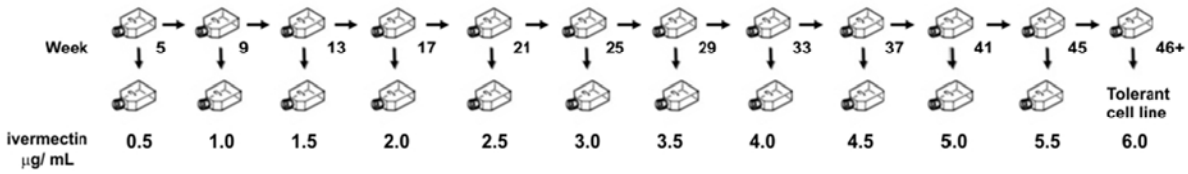


Fig. 2- Schematic summary of the protocol used to generate IVM-resistant cell line (BME26-IVM) from sensible parental cell line (BME26). At week 1, BME26 cells were incubated with 0.5 µg/ ml IVM. Every 4 weeks, cells were harvested and cultured in increased IVM concentration, as indicated. The process was stopped upon reaching 6 µg/ ml IVM.

3.2. Growth curves of BME26 and BME26-IVM

Growth curves of BME26 and BME26-IVM in control medium or medium with 6 µg/ mL IVM were compared and the doubling time in each treatment was measured. The parental drug-sensitive BME26 cells and the IVM-resistant BME26-IVM cells had the same doubling time of 16 days, when growing in medium without IVM (Fig. 3, control groups). BME26-IVM incubated with IVM had a doubling time of 12 days, not significantly different from control cells. BME26 had a much longer doubling time of 34 days (Fig. 3, IVM groups), evidencing the cytotoxic effect of IVM on drug-sensitive cells.

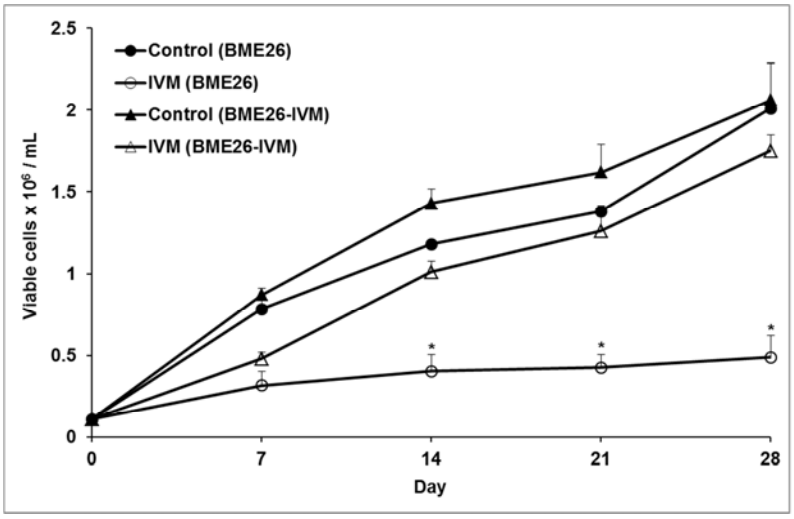


Fig. 3- Growth curves for BME26 and BME26-IVM cells in control medium or in medium with 6 µg/ mL IVM. Initially, $5x10^5$ cells were seeded into 25 cm^2 plastic flasks and cultured. Cells were harvested and counted in triplicate with a hemocytometer every 7 days over a 28-day culture period. All measurements were expressed as mean ± S.D., *n*= 3. Asterisks (*) denote significant differences from both controls ($p \leq 0.05$).

3.3. Effect of IVM and ABC transporters inhibitor on cell viability

Survival profiles of BME26 and BME26-IVM cell lines to IVM were determined incubating the cells in increasing IVM concentrations. Survival of BME26 was significantly reduced after IVM treatment, compared to BME26-IVM cells (Fig. 4A). Percent cell viability values obtained by counting viable trypan-blue staining cells in hemocytometer were confirmed by a MTT assay (data not shown).

As an effect of IVM cytotoxicity, BME26 cells grew larger and vacuolated, while BME26-IVM cells remained round and rich in cytoplasmic vesicles, similarly to what was observed in cells not exposed to IVM (Fig. 4B).

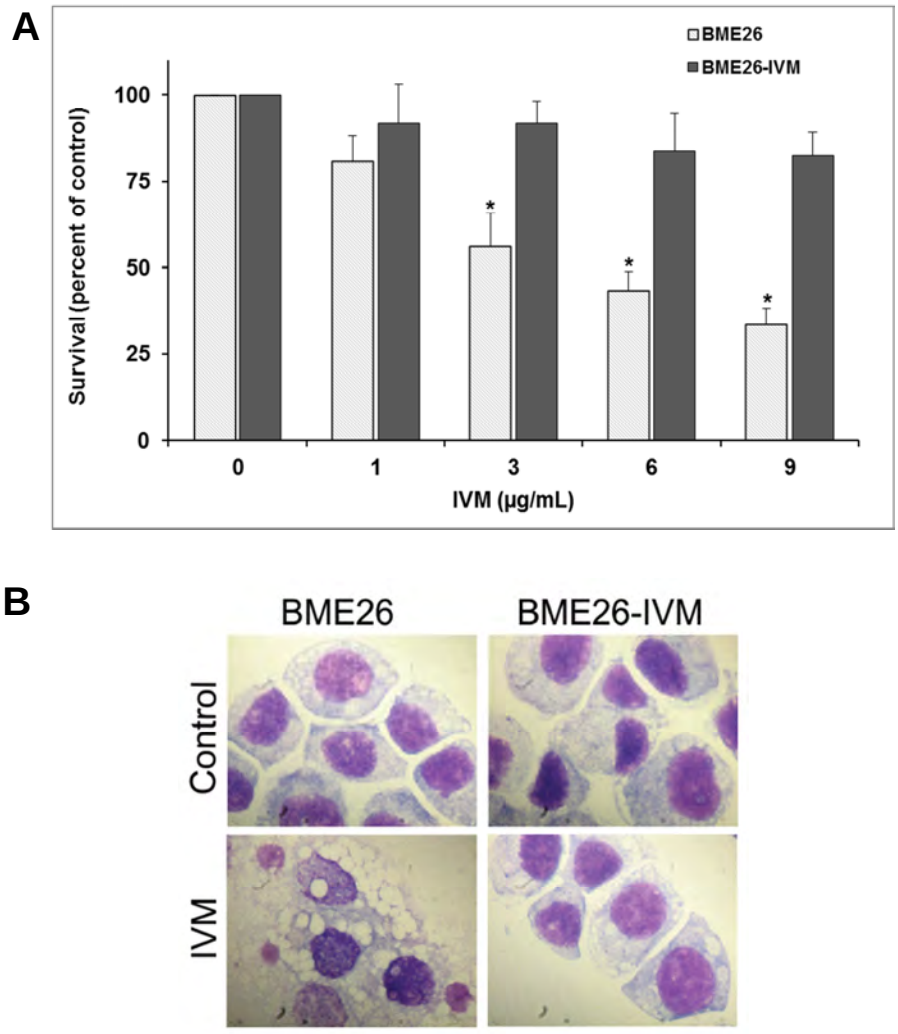


Fig. 4- Viability of BME26 and BME26-IVM cells after IVM treatment. **A**- Cell viability was measured by counting viable cells stained with trypan blue using a hemocytometer following 9 days incubation in increasing IVM concentration at 34 °C. Results are expressed as percentage of survival cells from control (100% cell viability). All measurements were expressed as mean ± S.D., *n*= 3. Asterisks (*) denote significant differences from BME26-IVM ($p \leq 0.05$). **B-** Panoptico-stained BME26 and BME26-IVM cells, after 9 days incubation in control medium or in medium with 6 µg/ mL IVM. Observed by light microscopy (100x).

The LC_{50} of BME26-IVM cells (15.1 ± 1.6) was significantly increased, compared to that observed in parental BME26 cells (3.35 ± 0.09), showing that BME26-IVM was 4.5 times more resistant to IVM than BME26 (Table 2).

The LC_{50} of IVM in BME26-IVM cells co-cultured with CsA decreased significantly, from 15.1 ± 1.6 to 8.42 ± 0.83 (Table 2), indicating a 1.79-fold reversal of resistance to IVM. In contrast, CsA had no effect on the sensitivity of BME26 to IVM.

Table 2- Effect of cyclosporin A (CsA) on reversing ABC-transporter mediated IVM-resistance.

Cell line	Treatment	LC_{50} ± SE (µg/ mL)	Fold-reversal factor
BME26	IVM	3.35 ± 0.09^{a}	
	IVM + CsA 5µM	3.24 ± 0.16^{a}	1.03
BME26-IVM	IVM	15.10 ± 1.60^{b}	
	IVM + CsA 5µM	8.42 ± 0.83^{c}	1.79

Different letters refer to statistically different values ($p \leq 0.05$).

3.3. *Expression of ABC transporters in IVM-resistant cells*

ABC transporters expression profiles (*RmABCB7*, *RmABCB10*, *RmABCC1* and *RmABCC2)* were determined by qPCR in BME26 and BME26-IVM cells, in presence or absence of IVM. As shown in Figure 5, *RmABCB10* gene expression was significantly increased in BME26-IVM cells exposed to IVM, compared with control cells (not exposed to IVM). We also observed a significant increase in *RmABCC1* expression in both BME26 and BME26-IVM cells in response to 9 µg/ mL IVM, compared to BME26 control. Whilst,

RmABCB7 was upregulated only in BME26 cells exposed to IVM. The expression level of *RmABCC2* was not significantly different. These results show that ABC transporters are required in cells exposed to IVM and that *RmABCB10* was selected in IVM-resistant cells.

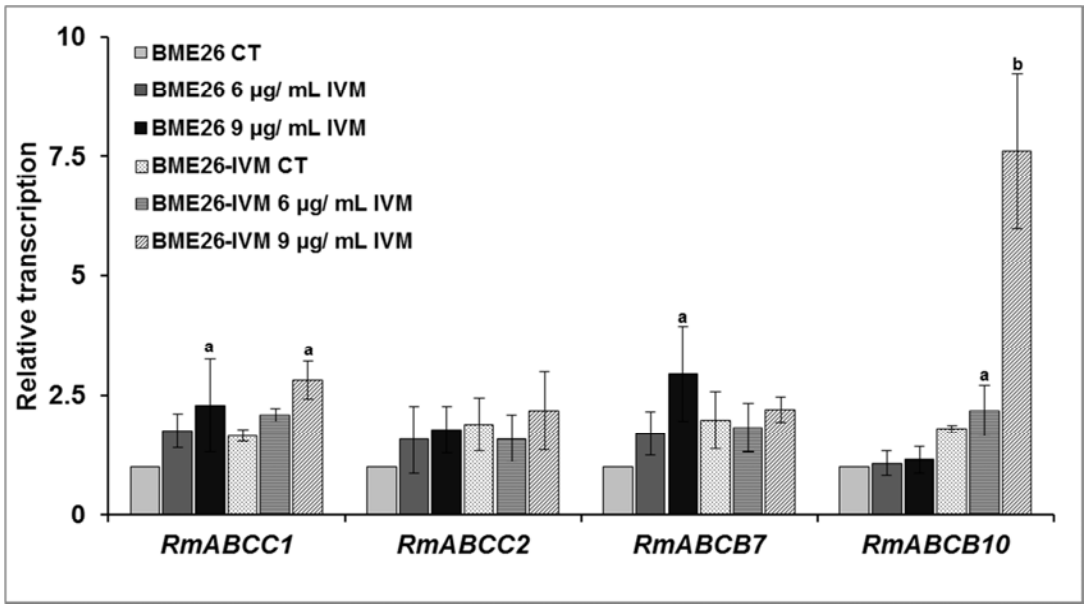


Fig. 5- Relative transcription levels of ABC transporter genes in BME26 and BME26-IVM cells cultured in control medium, 6 µg/ mL IVM medium and 9 µg/ mL IVM medium. After 9 days RNA was extracted from cells and ABC transporters genes were amplified from cDNA using gene-specific primers. Data represent mean ± SD of three independent experiments, normalized relative to 40-S transcript levels. The letter **a** denotes significant differences ($p \leq 0.05$) from BME26 CT and **b** denote significant differences ($p \leq 0.05$) from all treatments.

4. Discussion

Acaricide resistance represents a serious problem for *R. microplus* control (revised by Guerrero et al., 2012). Drug treatment remains the major method of tick control; however, it is necessary to understand the mechanism of resistance to the drugs, since it is essential to improving the detection and prevention of acaricide resistant tick populations (Perry et al., 2011; Rosario-Cruz et al., 2009)

The *in vitro* selection of drug-resistant cell lines has provided useful tools to understand of the mechanisms of cellular drug resistance. So, to characterize drug resistance mechanisms involved in IVM resistance at cellular level, we have developed a new cell line resistant to IVM. This line was obtained exposing an embryonic cell line BME26 to non-lethal doses of IVM in a step-wise approach. The resistance ratio observed was similar to that observed in field tick populations resistant to IVM (Pohl et al., 2011). Besides the importance of *in vitro* models based on human cancer-derived cell lines in the search for genetic alterations that are responsible for drug resistance (Gillet et al., 2007; Wind and Holen, 2011), some studies have also showed that insect and tick-derived cell lines are useful to evaluate the molecular mechanism of drug resistance, since similar phenotypes are observed *in vivo* and *in vitro*. Joussen and coworkers (2008) showed that cells of a *Nicotiana tabacum,* that metabolize the insecticides DDT and imidacloprid, overexpress the gene *Cyp6g1*, a cytochrome P450 monooxigenase. The same gene is overexpressed in *Drosophila melanogaster* strains resistant to a wide range of insecticides, including DDT and imidacloprid (Daborn et al., 2001). It is similar to that observed in *R. microplus,* since organophosphate-resistant cell lines showed increased levels of esterase activity, similar that was observed in organophasphate-resistant tick populations (Cossio-Bayugar et al., 2002).

Over the past 30 years, *in vitro* studies have led to the enumeration of close to 400 genes whose expression affects the response to chemotherapy in human cancer cells (Gillet et al., 2010). Among those genes, ABC transporters, a superfamily of 48 highly homologous members classified in seven subfamilies, have an important role in exporting chemotherapeutic agents from cells (Gillet et al., 2007, Szakács et al., 2006). Overexpression of human ABC transporter homologues has also been associated with drug resistance in arthropods and nematodes. Overexpression of ABC transporters was associated with ivermectin resistance in *Haemonchus contortus* (Xu et al., 1998), *Caenorhabditis elegans* (James and Davey, 2009), *Pediculus humanus humanus* (Yoon et al., 2011) and *Sarcoptes scabiei* (Mounsey et al., 2010).

Corroborating this hypothesis, *RmABCC1*, *RmABCB7* and *RmABCB10* were overexpressed in cells treated with IVM, showing a possible involvement in IVM detoxification. However, *RmABCB10* is probably more important, since it was overexpressed in BME26-IVM cells even before IVM treatment. These genes belong to the same subfamilies of genes identified previously in mammalian cells to be associated with drug resistance (Leslie et al., 2005). Furthermore, incubation of BME26-IVM with known ABC-transporter inhibitor, CsA, partly reverses the resistance to IVM, suggesting that ABC transporter proteins play a significant role in the development of resistance to IVM in this cell line. Nevertheless, the partial reversion of resistance with ABC transporter inhibitor, suggests that IVM resistance in *R. microplus* is multifactorial. More than one ABC transporter may be important in IVM resistance, as shown by the expression profile of the ABC transporters in the cell lines. Moreover, the importance of detoxification enzymes, such as esterases, cytochrome P450 monooxigenases and glutathione-S-transferases, should also be considered, showing the need for additional studies on this subject.

In a previous study, we reported that the inhibitor CsA increased IVM toxicity in IVM-resistant tick larvae and adults. The same ABC transporter gene, that was shown to be upregulated in the present study (*Rmabcb10*), had the same expression profile in ticks from an IVM-resistant population (Pohl et al., 2011). These findings suggest that similar resistance mechanisms may be induced both *in vitro* and *in vivo*, validating the use of acaricide resistant cell lines to study resistance development in the tick. Moreover, *in vitro* assays allow experimental parameters to be rigorously controlled, reducing the variability between experiments, which often is difficult to achieve in a bioassay.

In conclusion, selection of acaricide resistant cell lines plays a consistent role in the study of drug resistance mechanisms, not only concerning IVM resistance but also other drugs, whose resistance mechanisms remain to be characterized.

Acknowledgements

We thank CNPq - Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular, CAPES, FAPESP, FINEP and FAPERGS for supporting our work.

References

Bell-Sakyi, L., Zweygarth, E., Blouin, E. F., Gould, E. A., Jongejan, F., 2007. Tick cell lines: tools for tick and tick-borne disease research. Trends Parasitol. 23, 450- 457.

Castro-Janer, E., Martins, J. R., Mendes, M.C., Namindome, A., Klafke, G. M., Schumaker, T. T., 2010. Diagnoses of fipronil resistance in Brazilian cattle ticks (*Rhipicephalus (Boophilus) microplus*) using in vitro larval bioassays. Vet. Parasitol. 173, 300- 306.

Castro-Janer, E., Rifran, L., González, P., Niell, C., Piaggio, J., Gil, A., Schumaker, T.T.S. 2011. Determination of the susceptibility of *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) to ivermectin and fipronil by Larval Immersion Test (LIT) in Uruguay. Vet. Parasitol. 178, 148- 155.

Cossio-Bayugar, R., Wagner, G. G., Holman, P. J., 2002. In vitro generation of organophosphate resistant *Boophilus microplus* (Acari: Ixodidae) cell lines. J. Med. Entomol. 39, 278- 284.

Daborn, P., Boundy, S., Yen, J., Pittendrigh, B., ffrench-Constant, R., 2001. DDT resistance in *Drosophila* correlates with Cyp6g1 over-expression and confers cross-resistance to the neonicotinoid imidacloprid. Mol. Genet. Genomics. 266, 556- 563.

Drummond, R. O., Ernst, S. E., Trevino, J. L., Gladney, W. J., Graham, O. H., 1973. *Boophilus annulatus* and *B. microplus*: laboratory tests of insecticides. J. Econ. Entomol. 66, 130- 133.

Edwards, S. L., Brough, R., Lord, C. J., Natrajan, R., Vatcheva, R., Levine, D. A., Boyd, J., Reis-Filho, J. S., Ashworth, A., 2008. Resistance to therapy caused by intragenic deletion in BRCA2. Nature. 451, 1111- 1115.

Esteves, E., Lara, F. A., Lorenzini, D. M., Costa, G.H., Fukuzawa, A. H., Pressinotti, L. N., Silva, J. R., Ferro, J. A., Kurtti, T. J., Munderloh, U. G., Daffre, S., 2008. Cellular and molecular characterization of an embryonic cell line (BME26) from the tick *Rhipicephalus (Boophilus) microplus.* Insect Biochem. Mol. Biol. 38, 568- 580.

Fabres, A., De Andrade, C.P., Guizzo, M., Sorgine, M.H., Paiva-Silva, G., Masuda, A., da Silva Vaz Junior, I., Logullo, C., 2010. Effect of GSK-3 activity, enzymatic inhibition and gene silencing by RNAi on tick oviposition and egg hatching. Parasitology. 137, 1537- 1546.

Fox, L.M., 2006. Ivermectin: uses and impact 20 years on. Curr Opin Infect Dis. 19, 588-93.

Gillet, J. P., Efferth, T., Remacle, J., 2007. Chemotherapy-induced resistance by ATP-binding cassette transporter genes. Biochim Biophys Acta. 1775, 237- 262.

Gillet, J. P., Gottesman, M. M., 2010. Mechanisms of multidrug resistance in cancer. Multi-Drug Resistance in Cancer, ed Zhou J (Humana, Totowa, NJ), pp 47- 76.

Graf, J. F., Gogolewski, R., Leach-Bing, N., Sabatini, G. A., Molento, M. B., Bordin, E. L., Arantes, G. J. Tick control: an industry point of view. Parasitology. 129, S427- 42.

Guerrero, F. D., Lovis, L., Martins, J. R., 2012. Acaricide resistance mechanisms in *Rhipicephalus (Boophilus) microplus.* Rev Bras Parasitol Vet. 21, 1- 6.

Higgins, C.F., 2007. Multiple molecular mechanisms for multidrug resistance transporters. Nature. 446, 749- 757.

Holland, I.B., Blight, M.A., 1999. ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. J. Mol. Biol. 293, 381- 399.

James, C.E., Davey, M.W., 2009. Increased expression of ABC transport proteins is associated with ivermectin resistance in the model nematode *Caenorhabditis elegans*. Int. J. Parasitol. 39, 213- 220.

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.

Joussen, N., Heckel, D. G., Haas, M., Schuphan, I., Schmidt, B., 2008. Metabolism of imidacloprid and DDT by P450 CYP6G1 expressed in cell cultures of *Nicotiana tabacum* suggests detoxification of these insecticides in Cyp6g1-overexpressing strains of *Drosophila melanogaster*, leading to resistance. Pest Manag Sci. 64, 65- 73.

Jovelet, C., Bénard, J., Forestier, F., Farinotti, R., Bidart, J. M, Gil, S., 2012. Inhibition of P-glycoprotein functionality by vandetanib may reverse cancer cell resistance to doxorubicin. Eur J Pharm Sci. 46, 484- 491.

Klafke, G.M., Sabatini, G.A., de Albuquerque, T.A., Martins, J.R., Kemp, D.H., Miller, R.J., Schumaker, T.T., 2006. Larval immersion tests with ivermectin in populations of the cattle tick *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) from State of São Paulo, Brazil. Vet. Parasitol. 142, 386- 390.

Lage, H., 2003. ABC-transporters: implications on drug resistance from microorganisms to human cancers. Int. J. Antimicrob. Agents. 22, 188- 199.

Leslie, E.M., Deeley, R.G., Cole, S.P., 2005. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicol. Appl. Pharmacol. 204, 216- 237.

Martins, J.R., Furlong, J., 2001. Avermectin resistance of the cattle tick *Boophilus microplus* in Brazil. Vet. Rec. 149, 64.

Mounsey, K. E., Pasay, C. J., Arlian, L. G., Morgan, M. S., Holt, D. C., Currie, B. J., Walton, S. F., McCarthy, J. S., 2010. Increased transcription of Glutathione S-transferases in acaricide exposed scabies mites. Parasit Vectors. 18, 3- 43.

Perez-Cogollo, L.C., Rodriguez-Vivas, R.I., Ramirez-Cruz, G.T., Miller, R.J., 2010. First report of the cattle tick *Rhipicephalus microplus* resistant to ivermectin in Mexico. Vet. Parasitol. 168, 165- 169.

Perry, T., Batterham, P., Daborn, P. J., 2011. The biology of insecticidal activity and resistance. Insect Biochemistry and Molecular Biology 41, 411- 422.

Pfaffl , M. W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, 2002- 2007.

Pfaffl, M. W., Horgan, G. W., Dempfle, L., 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30, 01- 10.

Pohl, P. C., Sorgine, M. H., Leal, A. T., Logullo, C., Oliveira, P. L., Vaz, I., Masuda, A., 2008. An extraovarian aspartic protease accumulated in tick oocytes with vitellin-degradation activity. Comp Biochem Physiol B Biochem Mol Biol.151, 392- 399.

Pohl, P. C., Klafke, G. M., Carvalho, D. D., Martins, J. R., Daffre, S., da Silva Vaz Jr., I., Masuda, A., 2011. ABC transporter efflux pumps: A defense mechanism against ivermectin in *Rhipicephalus (Boophilus) microplus*. Int. J. Parasitol. 41, 1323- 1333.

Rosario-Cruz, R., Almazan, C., Miller, R.J., Dominguez-Garcia, D.I., Hernandez-Ortiz, R., de la Fuente, J., 2009. Genetic basis and impact of tick acaricide resistance. Front. Biosci. 14, 2657- 2665.

Soberanes, C. N., Santamaría, V. M., Fragoso, S.H., García, V. Z., 2002. Primer caso de resistencia al amitraz en la garrapata del ganado *Boophilus microplus* en México. Tec. Pec. Mex. 40, 81- 92.

Szakács, G., Paterson, J. K., Ludwig, J. A., Booth-Genthe, C., Gottesman, M.M., 2006. Targeting multidrug resistance in cancer. Nat. Rev. Drug Discov. 5, 219- 234.

Szakács, G., Váradi, A., Ozvegy-Laczka, C., Sarkadi, B., 2008. The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME-Tox). Drug Discov. Today.13, 379- 393.

Wind, N. S., Holen, I., 2011. Multidrug resistance in breast cancer: from *in vitro* models to clinical studies. Int J Breast Cancer. 2011, 967419.

Xu, M., Molento, M., Blackhall, W., Ribeiro, P., Beech, R., Prichard, R., 1998. Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homolog. Mol Biochem Parasitol. 91, 327- 335.

Yoon, K. S., Strycharz, J. P., Baek, J. H., Sun, W., Kim, J. H., Kang, J. S., Pittendrigh, B. R., Lee, S. H., Clark, J. M., 2011. Brief exposures of human body lice to sublethal amounts of ivermectin over-transcribes detoxification genes involved in tolerance. Insect Mol Biol. 20, 687- 699.

3.3. DESTOXIFICAÇÃO DE HEME E ACARICIDA NO INTESTINO DO CARRAPATO É MEDIADO POR TRANSPORTADOR ABC

ARTIGO IV

An ATP binding cassette transporter mediates both heme and pesticide detoxification in tick midgut cells

Running head: ABC-Mediated Heme and Pesticide Detoxification in Ticks

Flavio Alves Lara[1], Paula C. Pohl[2], Maria Clara Nascimento-Silva[3], Marcos H. F. Sorgine[3,4], Igor C. Almeida[5], John Furlong[6], Itabajara da Silva Vaz Jr.[2,4], Pedro L. Oliveira[3,4]

Participação no trabalho: experimentos de clonagem e caracterização da sequencia completa do transportador *RmABCB10*, experimentos de silenciamento gênico, alimentação artificial, análises de expressão gênica do transportador *RmABCB10* e redação do artigo.

[1]Laboratório de Microbiologia Celular, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil; [2]Centro de Biotecnologia e Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; [3]Programa de Biologia Molecular e Biotecnologia, Instituto de Bioquímica Médica, UFRJ, Rio de Janeiro, Brazil; [4]Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular –INCTEM; [5]The Border Biomedical Research Center, University of Texas at El Paso, El Paso, Texas; [6]Centro Nacional de Pesquisa de Gado de Leite, Empresa Brasileira de Pesquisa Agropecuária, EMBRAPA, Minas Gerais, Brazil.

Submetido para publicação na revista PLoS ONE

Author Summary

Blood-feeding animals must cope with high heme concentrations; therefore, they provide useful models for the study of the cell biology of heme. Heme is a ubiquitous molecule that participates in respiration, photosynthesis and a vast array of reactions involving redox chemistry that are essential to life. However, the same molecule can be toxic, as it can form reactive oxygen species. The study of how eukaryotic cells transport heme between intracellular compartments has made important progress in the last few years, and several novel transporters have recently been described. Here, we show that heme can also be a substrate for ABC transporters, a class of proteins that has been shown to transport heme in prokaryotes but not in eukaryotic cells. ABC transporters also perform an essential function in the detoxification of toxic compounds, and we show that the same ABC transporter that is used to transport heme in the tick midgut – leading to its accumulation in an intracellular compartment called the hemosome – serves to detoxify a pesticide. Taken together, our findings indicate that ABC transporters play a role in intracellular heme compartmentalization and that this same detoxification pathway is used to sequester a pesticide in an intracellular compartment, revealing a novel mechanism for insecticide resistance.

Blurb

The present work reveals a heme detoxification pathway in the midgut cells of the cattle tick that involves the ABC transporter-mediated uptake of heme from the cytosol into an organelle called the hemosome by a mechanism that is also used to detoxify pesticides.

Abstract

In ticks, the digestion of blood occurs intracellularly, and the heme moiety of hemoglobin that is released upon proteolytic digestion of the globin polypeptide chain is accumulated inside specialized organelles called hemosomes. In the present work, we studied the uptake of fluorescent metalopophyrins, used as heme analogs, and amitraz, one of the most regularly used acaricides to control cattle tick infestations, by *Rhipicephalus (Boophilus) microplus* midgut cells. Both compounds were taken up by midgut cells in vitro and accumulated inside the hemosomes. Transport of both molecules was sensitive to cyclosporine A (CsA), a well-known inhibitor of ATP binding cassette (ABC) transporters. Rhodamine 123, a fluorescent probe that is also a recognized ABC substrate, was similarly directed to the hemosome in a CsA-sensitive manner. Using antibodies against conserved domains of PgP-1- and MRP-1-type ABC transporters, we were able to immunolocalize PgP-1 in hemosome membranes and MRP-1 in basophilic cells. Comparison between two *R. microplus* strains that were resistant and susceptible to amitraz revealed that the resistant strain detoxified both amitraz and Sn-Pp IX more efficiently than the susceptible strain, a process that was also sensitive to CsA. A transcript containing an ABC transporter signature exhibited 2.5-fold increased expression in the amitraz-resistant strain when compared with the susceptible strain. RNAi-induced down-regulation of this ABC transporter led to the accumulation of Zn-Pp-IX in the digestive vacuole, interrupting heme traffic to the hemosome. This evidence further confirms that this transcript codes for a heme transporter. This is the first report of heme transport mediated by an ABC transporter in eukaryotic cells. While the primary physiological function of the hemosome is to detoxify heme and attenuate its toxicity, we suggest that the use of this acaricide detoxification pathway by ticks may represent a new molecular mechanism of resistance to pesticides.

Introduction

Ticks and tick-borne diseases began to be considered economic and public health concerns at the end of the nineteenth century, when the number of cattle increased in an attempt to feed a growing human population (DAVEY et al. 2004). Tick-borne diseases were some of the first arthropod-borne diseases described (SMITH and KILBORNE 1893). *R. microplus* is the major vector of *Babesia* spp. and *Anaplasma* sp., which cause severe economic losses in the largest cattle farms in tropical and subtropical countries (BRAM et al. 2002). In the midgut of ticks, hemoglobin from the blood of the vertebrate host is endocytosed by the so-called "digest cells," and its degradation is accomplished by hydrolytic lisosomal-type enzymes inside acidic digestive vacuoles (AGBEDE and KEMP 1985; LARA et al. 2005a; MENDIOLA et al. 1996; WALKER and FLETCHER 1987). The free heme that is produced by this process is transferred from these vacuoles to the cytosol and subsequently moved to a very specialized organelle called the hemosome, wherein heme aggregates accumulate (LARA et al. 2005b). The physiological relevance of this process is considered to be responsible for alleviating digest cells – and the tick as a whole – from the potentially deleterious effects of heme. In hematophagous insects, a wide array of adaptations have been reported that provide protection against heme toxicity and contribute to the adaption of the animal to a diet based on vertebrate blood (GRACA-SOUZA et al. 2006). In the case of the digest cell of the tick midgut, however, an intracellular pathway dedicated to heme transport from digestive vesicles to hemosomes has been implicated as a key aspect of heme detoxification, although the molecular nature of the mechanisms involved in transport across cellular membranes or through the cytosol remain poorly understood.

Intracellular pathways involved in heme transport inside cells have been studied in several organisms. In some species of pathogenic bacteria, ABC transporters have been

shown to transport heme obtained from the host (BURKHARD and WILKS 2008; THOMPSON et al. 1999). In contrast, the participation of ABC transporters in heme movement across membranes in eukaryotic cells has not yet been demonstrated. However, in the last few years, important advances have been made, including the discovery of three new types of non-ABC heme transporters: the feline leukemic virus receptor (FLVCR), (KEEL et al. 2008; QUIGLEY et al. 2004), intestinal heme receptors (SHAYEGHI et al. 2005) and heme regulated genes 1-4 (HRG) (RAJAGOPAL et al. 2008).

Drug detoxification in eukaryotic cells is generally described as a process that involves three steps: the chemical modification of xenobiotics, followed by conjugation to anionic groups such as glutathione, glucuronate or sulfate, and finally, excretion by ABC transporters (HOMOLYA et al. 2003). Resistance to insecticides has been shown to involve enzymes from the first two classes, including esterases, P-450 cytochromes and glutathione-S-transferases (KOSTAROPOULOS et al. 2001; SWAIN et al. 2009; WONDJI et al. 2009), but the participation of ABC transporters in the development of resistance still has not been clearly defined (BUSS 2008). Here, we show that heme transport to the hemosome is mediated by an ABC transporter. We also present evidence that a common acaricide, amitraz, is accumulated into the hemosomes through a pathway that uses the same ABC transporter; therefore, this pathway may represent a novel type of acaricide resistance.

Results

The transport of heme by members of the ABC family has been reported in organisms such as pathogenic bacteria and protozoa but not in higher eukaryotes (BURKHARD and WILKS 2008; GOLDMAN et al. 1996; KRISHNAMURTHY et al. 2006; LARA et al. 2007; WANDERSMAN and STOJILJKOVIC 2000). Digest cells of the

tick midgut degrade hemoglobin and direct heme into an intracellular pathway that involves digestive vacuoles and ultimately leads to heme accumulation in hemosomes. To test whether this heme intracellular transport is dependent on an ABC transporter, digest cells isolated from the midgut of *R. microplus* were incubated with Rhodamine 123, a classical ABC transporter substrate (POURTIER-MANZANEDO et al. 1992). The fluorescent dye was found to be associated with both intracellular vacuoles and hemosomes after incubation. Furthermore, uptake was markedly inhibited by CsA, a commonly used ABC inhibitor (STAPF et al. 1994), which suggests the participation of a transporter of this type (Figure 1).

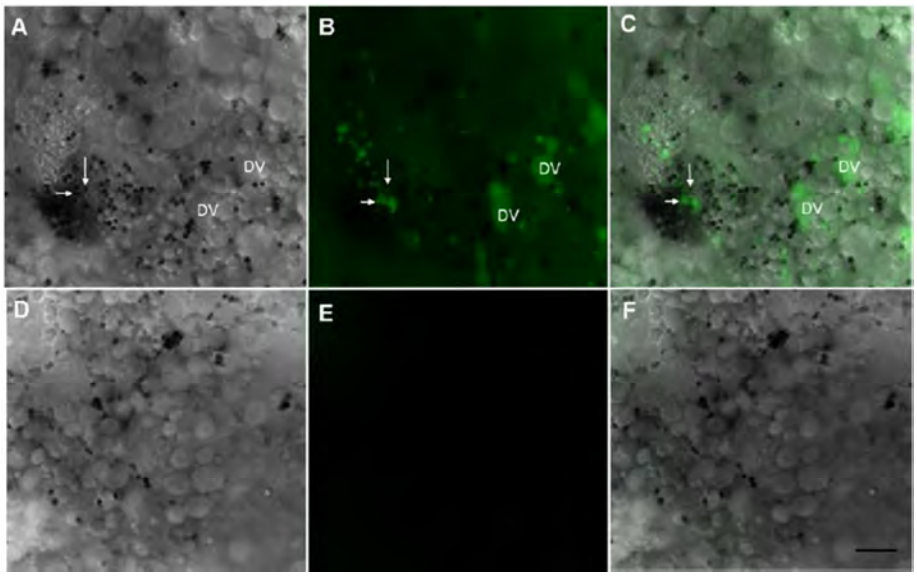


Figure 1 – Uptake of Rhodamine 123 by midgut digest cells: Digest cells from fully engorged adult females were obtained as described in the Materials and Methods. Digest cells were incubated in the presence of 0.5 µM Rhodamine 123 for 2 h. (A-C) Control; (D-E) cells pre-incubated with 10 µM CsA. (A and D). Panels are differential interference contrast (DIC) (A and D), fluorescence images (B and E) or fluorescence merged with DIC (C and F) showing stained hemosomes (white arrows) and digestive vesicles (DV). The scale bar is 10 µm.

This conclusion was also supported using immunocytochemistry with polyclonal antibodies that recognize conserved motifs of PgP-1 and MRP-1, representatives of two distinct subfamilies of ABC transporters. While the anti-MRP-1 antibody identified epitopes that were expressed only in basophilic epithelial cells (Figure 2B), the anti-PgP-1 antibody reacted markedly with hemosomes and digest cell membranes (Figure 2A and 2C).

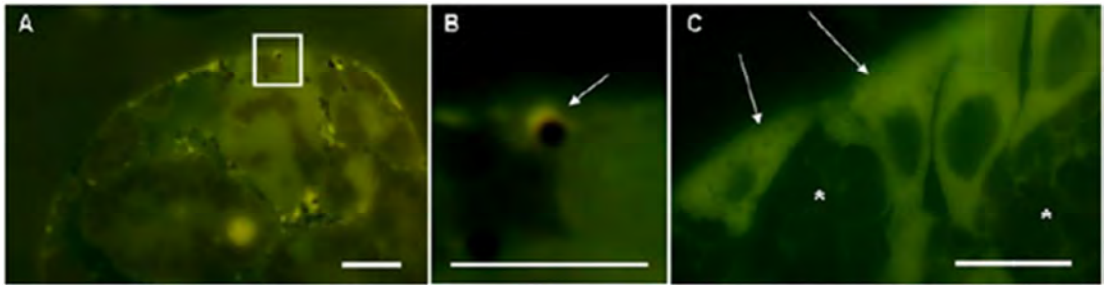


Figure 2 – Immunolocalization of ABC transporters in the tick midgut: Fully engorged females were dissected, and cryosections were stained using polyclonal antibodies against the domains of PgP-1 (A and B) or MRP-1 (C), followed by FITC-labeled secondary antibodies. (B) Higher magnification of the area indicated in the box in panel A. The arrow indicates hemosomes labeled with anti-PgP-1 antibodies. (C) An epithelial basophilic cell labeled by anti-MRP-1 antibodies is indicated by the arrow. The scale bars is 50 µm in A and C and 20 µm in B.

Further demonstration of the presence of an ABC in the hemosome was obtained by the histochemical localization of its ATPase activity in the hemosome. We used a cerium-based method to visualize ATPase activity at hemosome membranes. Phosphate is released by ATPase activity, and cerium is a heavy metal atom whose phosphate salt is highly insoluble (HULSTAERT et al. 1983). In figure 3A-B, we show that hemosomes

(asterisks) and digestive vesicles (DV) exhibit high ATPase activity associated with their membranes (black and white arrows, respectively) and that this activity can be inhibited by CsA (Figure 3C-D).

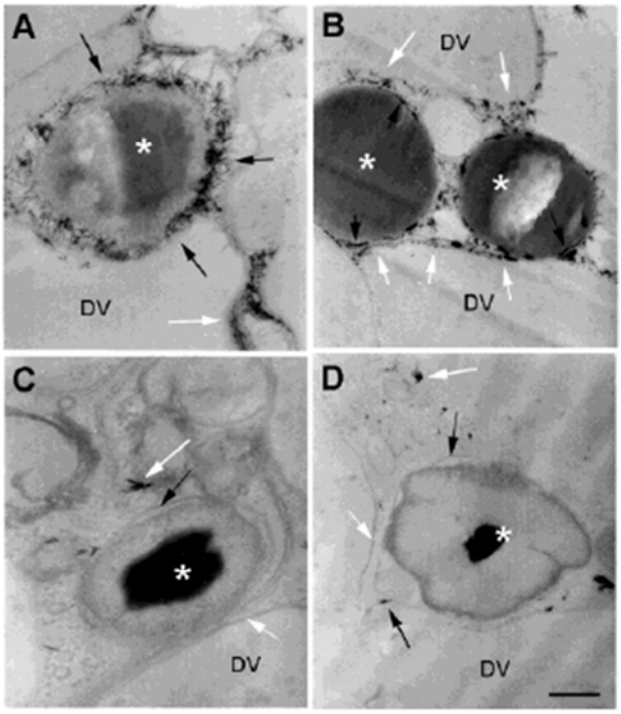


Figure 3 – Identification of a CsA-sensitive ATPase activity in the hemosome membrane: (A-B) Digest cells from fully engorged tick females 2 days ABM, showing hemosome (asterisk) and digestive vesicle (DV) membranes exhibiting strong ATPase activity, as revealed by the precipitation of cerium phosphate (arrows); (C-D) section from a different midgut diverticulum from the same tick, incubated with 10 µM CsA for 30 min before the ATPase assay. The scale bar is 200 nm (B) or 100 nm (A, C and D).

In a previous report, it was determined that an ABC transporter was involved in the detoxification of ivermectin (Pohl et al. 2011). We therefore hypothesized that the presence of ABC transporters in the heme transport pathway that ends in the hemosome could also turn this organelle into a site for the disposal of other toxic compounds such as

xenobiotics, in addition to its primary role as a site for the disposal of excess heme. As we had one *R. microplus* isolate that was amitraz-sensitive (hereafter referred to as the Porto Alegre strain – POA) and a naturally amitraz-resistant population (the Ibirapuã strain), we compared the uptake of Sn-Pp IX by digest cells from both strains to observe the relationship between amitraz resistance and heme detoxification by ABC transporters. After 2 h of incubation in medium with Sn-Pp IX, digest cells of the amitraz-resistant strain presented higher metalloporphyrin uptake than cells from the susceptible strain (Figure 4B and D, white arrows). Sn-Pp IX uptake was markedly inhibited by CsA, a classical ABC transporter inhibitor (LOOR et al. 2002), which reinforces the conclusion that ABC transporters are involved in this process (Figure 4F and H). When cells were incubated with SnPp IX and the amount of SnPp IX in isolated hemosomes was evaluated by HPLC, organelles from the resistant strain also exhibited a higher level of accumulation (Figure 5A). CsA, indometacin and verapamil, all known ABC inhibitors, were able to reduce the relative amount of SnPp IX that reached the hemosomes (Figure 5B), confirming that members of this class of transporter are responsible for the sequestration of metalloporphyrin into the hemosome. When added to the cell culture medium, amitraz was also sequestered into hemosomes, and uptake of this pesticide was higher in the amitraz-resistant Ibirapuã strain (Figure 5 C). In both strains, SnPp IX transport was sensitive to CsA (Figure 5 C). In the experiments shown in all three panels of Figure 5, low temperature incubations (4 °C) were performed as a control to confirm that uptake was dependent on active metabolism, and low levels of accumulation of SnPp IX were observed under these conditions.

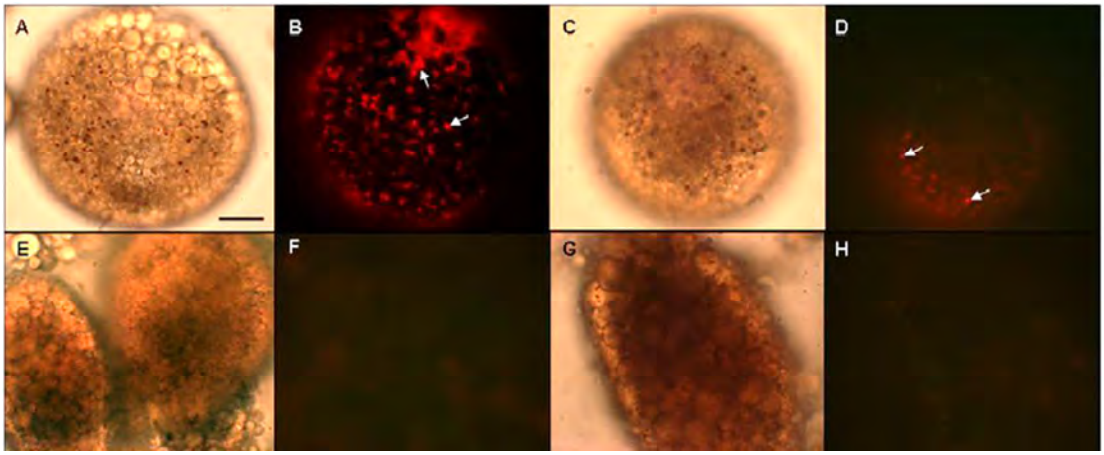


Figure 4 - CsA-sensitive uptake of Sn-Protoporphyrin IX (Sn-Pp IX) is higher in amitraz-resistant ticks: Digest cells from tick strains sensitive or resistant to amitraz were incubated in the presence of 100 µM Sn-Pp IX for 2 h. (A,B) Amitraz-resistant strain; (C,D) amitraz-sensitive strain; (E,F) amitraz-resistant strain pre-incubated with 10 µM CsA; (G,H) amitraz-sensitive strain pre-incubated with 10 µM CsA. A, C, E and G are DIC images. B, D, F and H are fluorescence images of the metalloporphyrin. Arrows indicate Sn-Pp IX fluorescence associated with hemosomes. The scale bar is 60 µm.

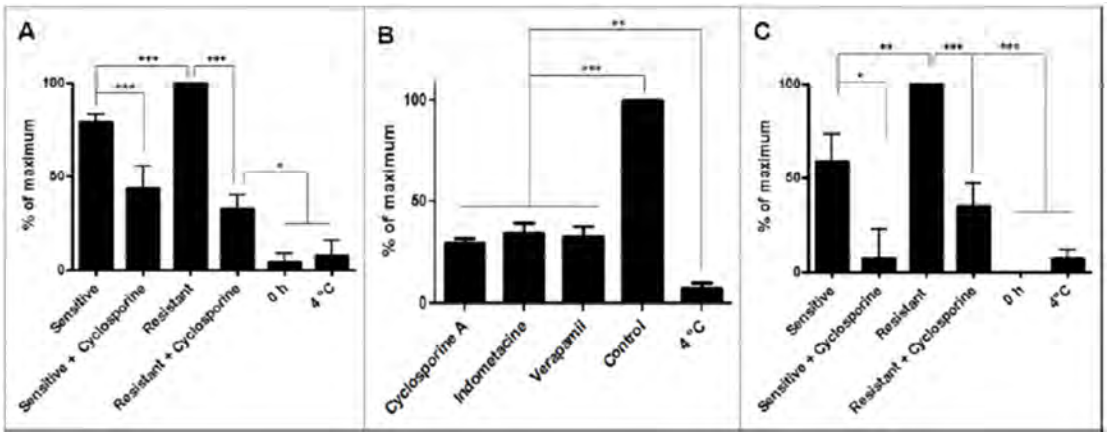


Figure 5 - Sn-Protoporphyrin IX and amitraz accumulation into hemosomes is due to ABC transporters: Digest cells were incubated either with 10 µM Sn-Pp IX (A, B) or 10 µM amitraz (C), and hemosomes were isolated as described in the Materials and Methods. Hemosomes were fractionated by reverse phase HPLC chromatography, and the Sn-Pp IX or amitraz content was calculated in relation to the area of the heme peak, with the highest value set at 100%. (A) Cells were collected from either the resistant strain or the sensitive strain and were pre-incubated or not with 10 µM CsA. The Sn-Pp IX content was evaluated. (B) Cells were collected from the amitraz-resistant strain, and all inhibitors were used at 10 µM. The Sn-Pp IX content was evaluated. (C) Cells were collected from either the resistant strain or the sensitive strain and were pre-incubated or not with 10 µM CsA, and the amitraz content was evaluated. Incubations of the cells from the resistant strain at 4 °C were included in all panels. (*) represents $p < 0.001$; (**) represents $p < 0.05$; (***) represents $p < 0.01$. Data are the mean ± SEM (n=3).

Because the RmABCB10 transporter has been implicated in ivermectin resistance (POHL et al. 2011), we analyzed its involvement in amitraz resistance and heme trafficking in digest cells. Based on the partial sequence available, the full-length sequence of RmABCB10 was obtained with rapid amplification of cDNA ends (RACE)-PCR. A phylogenetic analysis comparing RmABCB10 with transporters from different ABC subfamilies places this transcript in the half-transporter group of the B subfamily (Figure 6A). An analysis of the deduced amino acid sequence found a C-terminal nucleotide-binding domain (NBD) with typical Walker A, Walker B and signature motifs and a N-terminal transmembrane domain (TMD) (Figure 6B and Supporting Figures 1) with six membrane-spanning α-helices (Figure 6C). An analysis of the transcription levels in digest cells by real-time PCR showed significantly higher expression of RmABCB10 in the amitraz-resistant strain Ibirapuã than in the sensitive strain POA (Figure 6D).

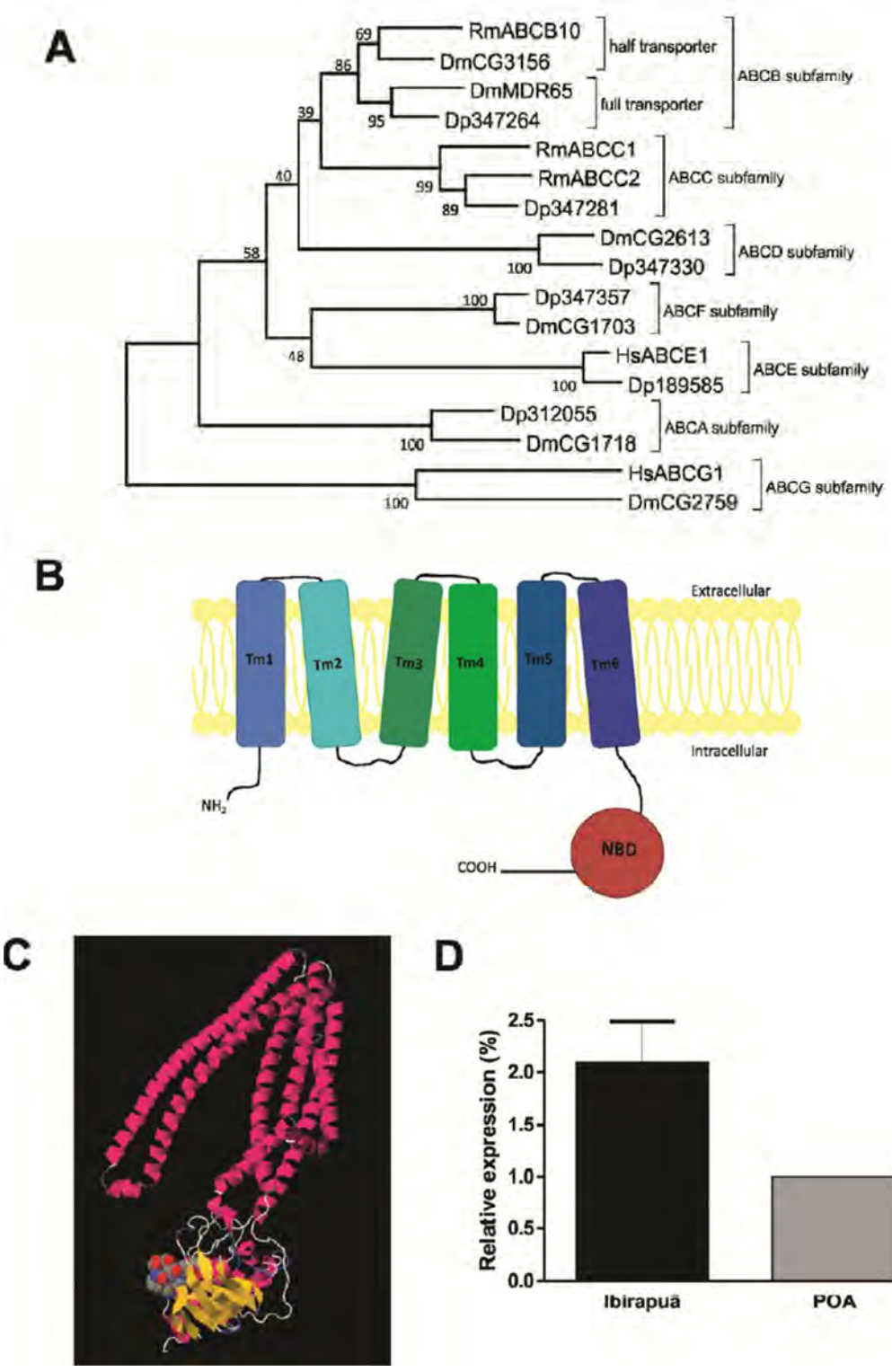
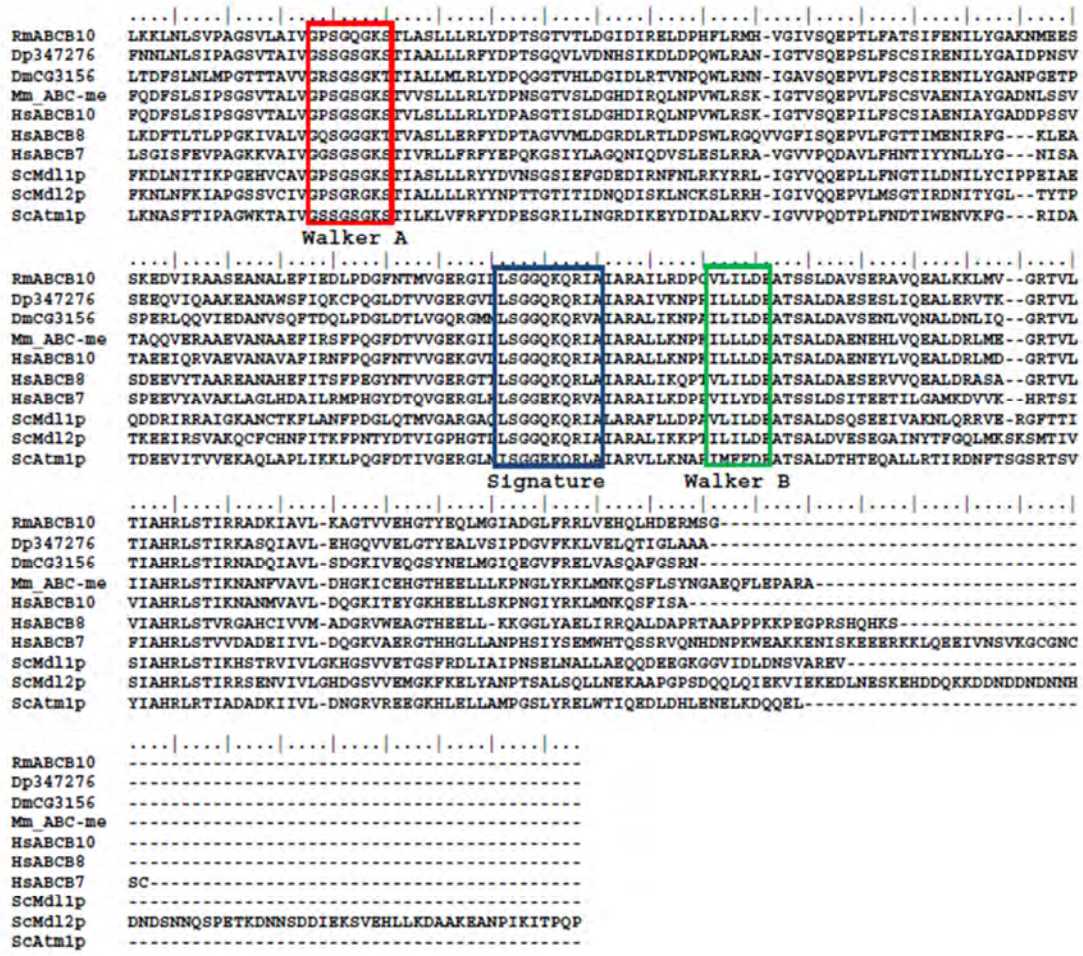

A
RmABCB10
DmCG3156
DmMDR65
Dp347264
half transporter
full transporter
ABCB subfamily
RmABCC1
RmABCC2
Dp347281
ABCC subfamily
DmCG2613
Dp347330
ABCD subfamily
Dp347357
DmCG1703
ABCF subfamily
HsABCE1
Dp189585
ABCE subfamily
Dp312055
DmCG1718
ABCA subfamily
HsABCG1
DmCG2759
ABCG subfamily
69
86
95
39
40
99
89
58
100
100
48
100
100
B
Extracellular
Intracellular
Tm1
Tm2
Tm3
Tm4
Tm5
Tm6
NH2
COOH
NBD
C
D
Relative expression (%)
2.5
2.0
1.5
1.0
0.5
0.0
Ibirapuã
POA

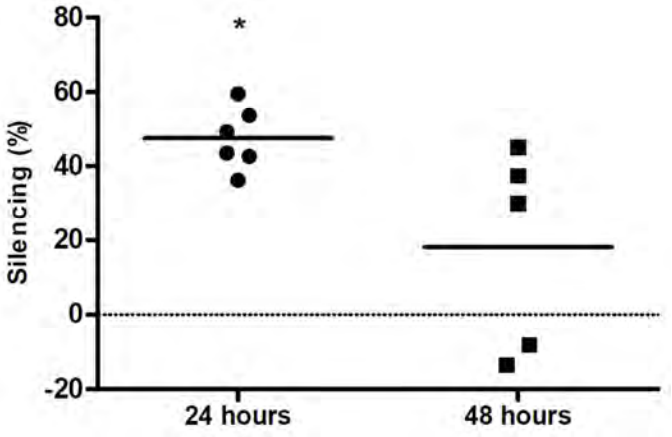
Figure 6 - Identification and expression of ABC transporters in midgut digest cells: (A) Phylogenetic analysis based on the alignment of RmABCB10 (bold) with ABC transporters from other organisms. Dm: *Drosophila melanogaster*, Dp: *Daphnia pulex*, Hs: *Homo sapiens*, Rm: *Rhipicephalus (Boophilus) microplus*. Accession numbers of the sequences are described in the Materials and Methods. (B) Proposed scheme showing the secondary structure of the RmABCB10 protein consisting of six transmembrane domains (Tm1 to Tm6) and one cytosolic nucleotide-binding domain (NBD). (C) Predicted tertiary structure based on the amino acid sequence of the RmABCB10 protein based on the known structure of human ATP-binding cassette sub-family B member 10 (2YL4). (D) The relative expression on *RmABCB10* was evaluated by qPCR in digest cells from both sensitive and resistant strains dissected 3 days ABM. The mean ± SEM are shown (n=3); (**) represents $p < 0.05$.


RmABCB10
Dp347276
DmCG3156
Mm_ABC-me
HsABCB10
HsABCB8
HsABCB7
ScMdl1p
ScMdl2p
ScAtm1p
Tm1
Tm2
Tm3
Tm4
Tm5
Tm6



Supporting Figure 1 - Multiple sequence alignment of the predicted amino acid sequence of the *R. microplus* and other species: ABC transporter RmABCB10 (AEI91123.2) and amino acid sequences of *H. sapiens* ABCB10 (NP036221.2), ABCB8 (NP009119.2) and ABCB7 (NP004290.2); *M. musculus* ABC-me (NP062425.1); *D. melanogaster* G3156 (NP569844.2); *Daphnia pulex* 347276 (EFX65703.1); and *S. cerevisiae* Mdl1p (NP13289.1), Mdl2p (NP015053.2) and Atm1p (NP014030.1). Conserved motifs of the nucleotide-binding domain (NBD) are indicated by boxes, and transmembrane regions are highlighted. Alignments were performed using the Muscle algorithm with the default settings in Mega 5 software.

To further test the involvement of RmABCB10 in heme trafficking, partially engorged females were injected with dsRNA and artificially fed (GONSIOROSKI et al. 2012), which resulted in efficient down-regulation of *RmABCB10* mRNA in the midgut (Supporting Figure 2). When Zn-Pp IX was added to the blood meal in *RmABCB10*-silenced femeals, a reduction in the labeling of hemosomes was observed in parallel with an increase in the labeling of digestive vacuoles (Figure 7). These data, together with the higher capacity of the Ibirapuã-strain hemosomes to sequester Sn-Pp IX and amitraz, strongly support the involvement of the same ABC transporter system in the detoxification of both heme and pesticides.



Supporting Figure 2 - Analysis of RNAi-mediated *RmABCB10* silencing in the midgut of ticks after repletion. The relative expression of *RmABCB10* was determined by quantitative PCR of the total RNA extracted from the midguts of female ticks collected 24 hours and 48 hours after repletion and injected with *RmABCB10* dsRNA (dsABC) or control dsRNA (dsCont). The points represent the percent of *RmABCB10* silencing of six females from each group, and the means are indicated with a bar. Asterisks (*) denote a significant difference as determined by a one-way ANOVA followed by Tukey's test ($p \leq 0.05$).

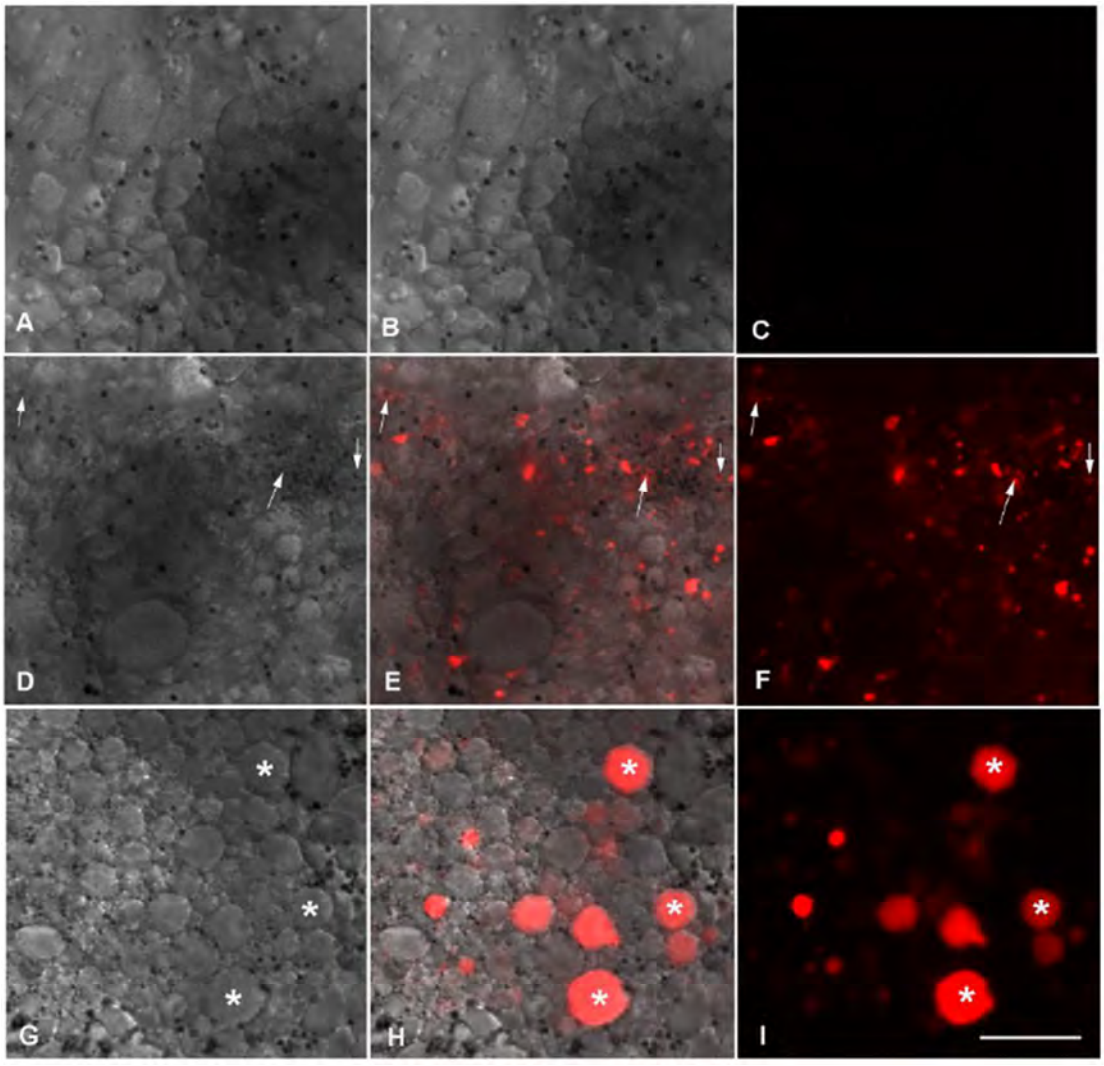


Figure 7 - ABC transporter silencing impairs Zn-PP IX traffic in digest cells. Partially engorged females were collected from cattle and were artificially fed with blood supplemented with dsABC (A-C), with Zn-PP plus dsCont (D-F) or with Zn-PP IX plus dsABC (G-H). In all cases, the blood meal contained 0.5% DMSO. After 48 h ABM, digest cells were detached from the tissue, and differential interference contrast (DIC) (A, D and G) and Zn-PP fluorescence (C, F and I) images were acquired. Merged images are shown in B, E and H. The arrows indicate early hemosomes exhibiting a Zn-PP signal; asterisks show digestive vesicles within the Zn-PP signal. The scale bars are 20 µm in all images.

Discussion

Despite the importance of heme as the prosthetic group of several fundamental enzymes and its presence in almost all organisms, the intracellular trafficking of heme is still poorly understood. ABC transporters are a family of large membrane proteins that are involved in the ATP-powered transport of a wide array of biologically relevant molecules, frequently against a concentration gradient (BORST and ELFERINK 2002). ABC transporters have been shown to perform transmembrane heme transport in bacteria (GOLDMAN et al. 1996) and trypanosomatids (LARA et al. 2007). In higher eukaryotes, however, other classes of transporters (non-ABC) have recently been implicated in heme transport across cellular membranes (QUIGLEY et al. 2004; RAJAGOPAL et al. 2008; SHAYEGHI et al. 2005). The participation of an ABC transporter in heme metabolism in typical eukaryotic cells has been shown only in mitochondria, where an ABC transporter was implicated in the uptake of a heme precursor, coproporphyrinogen III, but not in heme transport directly (KRISHNAMURTHY et al. 2006). In the midgut of tick digest cells, we previously identified specialized organelles called hemosomes that are dedicated to the accumulation of large amounts of heme released during the digestion of a blood meal (LARA et al. 2003). Hemosome formation is accomplished by a pathway that starts with the removal of heme from the hemoglobin polypeptide chain inside an acidic digestive vacuole, the subsequent transfer of heme to the cytosol, and finally, the uptake of heme by hemosomes. Heme can account for up to 90% of the composition of hemosomes. Here, we showed that an ABC transporter is responsible for heme sequestration into hemosomes. The presence of an ABC transporter in the hemosome and in the digestive vesicle was demonstrated by immunoreactivity with specific antibodies against conserved domains of a PgP-type ABC ATPase, together with the identification of ATPase enzymatic activity by immunohistochemistry and the accumulation of Rhodamine 123, both of which were

inhibited by CsA (Figures 1-3). Direct demonstration of ABC-dependent SnPp-IX transport provides evidence that this mechanism is involved in the accumulation of heme (Figures 4 and 5). There are a number of reports relating ABC transporters to heme metabolism, showing that cells that lack BCRP/ABCG2 or ABCB6 accumulate porphyrin (KRISHNAMURTHY et al. 2004; KRISHNAMURTHY et al. 2006). ABCB6 is located on the mitochondrial outer membrane (ZUTZ et al. 2009), whereas the BCRP protein is localized to inner mitochondrial cristae (SOLAZZO et al. 2009); both are thought to participate in the uptake of heme precursors into the mitochondria, fueling the final steps of the heme biosynthesis pathway. A role in heme transport has also been hypothesized (KHAN and QUIGLEY 2011). The cattle tick has been shown to lack heme biosynthesis (BRAZ et al. 1999), which excludes any connection of the heme cellular trafficking route studied here to this metabolic pathway. Therefore, to our knowledge, this is the first report that demonstrates heme transportation by an ABC enzyme in a higher eukaryote. These results also indicate that this is an ancestral mechanism capable of mediating heme transport across membranes, similar to its role in bacteria (BRAUN and HANTKE 2011) and trypanosomatids (CUPELLO et al. 2011; LARA et al. 2007). While the hemosome itself is clearly an adaptation developed during the evolution of ticks because of their blood-based diet, it is possible that heme transport across membranes through ABC proteins may occur in other eukaryotes as well, thus adding a new player in the rapidly growing model of the intracellular trafficking of heme.

ABC transporters have been primarily related to the export of toxic molecules but have also been shown to play a role in the import of nutrients and the transport of many other physiological substrates. As a consequence, a relatively large number of genes from this family are found in most eukaryotic organisms, such as in humans, where 48 genes have been identified (DEAN et al., 2001), or in the mosquito genome, which contains 64

ABC genes (NENE et al. 2007). However, as the variety of molecules that are ABC substrates is very large, these transporters frequently have a broad specificity; as a result, there are several different molecules that can be translocated by the same protein (DEAN et al. 2001). The ABC-mediated accumulation of the acaricide amitraz inside the hemosome reported here may be due to this non-stringent specificity. Alternatively, these results could be explained by the presence of more than one ABC transporter in the hemosome membrane, but the presence of increased heme transport and RmABCB10 expression in a tick strain resistant to acaricide suggests that the same transporter is used to detoxify both compounds.

As mentioned above, the most intensively studied role of ABC transporters is the detoxification of xenobiotics, as well as the capacity of ABCs to detoxify pesticides, has already been described in mammalian cells (BRAYDEN and GRIFFIN 2008; NOBMANN et al. 2001; SHABBIR et al. 2005). With respect to the detoxification of pesticides by insects and ticks, however, the involvement of ABCs has been generally overlooked. One exception was a report that the susceptibility of *Culex pipiens* to insecticides was increased by verapamil, thereby suggesting the participation of ABC transporters in the resistance to this insecticide (BUSS et al. 2002). Here, we clearly show ABC-dependent accumulation of amitraz in the hemosome, an intracellular organelle primarily dedicated to sequestration of heme, which is also a toxic molecule. The validity of this trafficking pathway as a potentially new mechanism of resistance stems from the fact that a tick strain resistant to this acaricide demonstrated increased transport of both amitraz and heme to the hemosome. The increased expression of the mRNA of at least one ABC in digest cells provides additional support for this hypothesis. This hypothesis is also supported by the observation of increased expression of ABC genes in resistant strains of mosquitoes (Ranson H., personal communication). The relationship between ABC transporters and the pesticide

resistance of arthropods has already been described (BUSS et al. 2002; BUSS 2008). The ABC activity observed here was associated with the membrane of the hemosome, and this unique location may be used to create new tick control strategies, as this ABC transporter pumps toxic compounds to the inner side of an organelle, the hemosome, and not into the external medium, such as most other ABC transporters described thus far. Based on this data, control strategies that can disrupt the accumulation of heme into the hemosome (CITELLI et al. 2007) could also increase the sensitivity of these animals to commonly used pesticides.

Taken together, our data show that, similar to bacteria and trypanosomatid protozoa, complex eukaryotes also employ ABC transporters in the intracellular trafficking of heme and that the same mechanism can also be used in the detoxification of xenobiotics such as pesticides, which has potential implications in both the understanding of the drug detoxification metabolism in arthropod pests and the basic knowledge of heme trafficking within cells in eukaryotic organisms.

Material and Methods

Animals

Rhipicephalus (Boophilus) microplus of the Porto Alegre strain, free of *Babesia* spp. and *Anaplasma* spp., were reared on calves obtained from a tick-free area and maintained at the Faculdade de Veterinária of Universidade Federal do Rio Grande do Sul (UFRGS), Brazil. Fully engorged adult females were kept in Petri dishes at 28°C and 80% relative humidity until use. The animals were handled in compliance with the UFRGS and EMBRAPA review committee for animal care. The amitraz-resistant strain of *R. microplus* was collected from a farm in the Ibirapuã district, Bahia state, Brazil, and maintained on calves reared in a tick-free area at Empresa Brasileira de Agropecuária (EMBRAPA, Gado

de Leite) from Juiz de Fora (MG), Brazil. Amitraz resistance was evaluated using the adult immersion test (AIT), a bioassay applied to fully engorged female ticks, performed as described by Drummond et al. (1973). Briefly, groups of 10 females were immersed for 5 min in the acaricide solutions, usually using the acaricide concentrations recommended by the manufacturer. The control group was immersed in distilled water. The viability was estimated using the POA strain as a susceptible standard, which showed 0% survival to all types of acaricides tested (Klafke et al., 2006). The effect of pesticides on the Ibirapuã strain, in contrast, ranged from a lack of resistance to some organophosphates, moderate resistance to pyrethroids and 100% survival to 85 µM of amitraz.

Digest cell culture

The digest cell culture was performed as described by Lara et al. (2005a). Briefly, fully engorged females on the second day after a blood meal were rinsed in 70% ethanol for 1 minute and dissected in sterile phosphate buffered saline (PBS) containing 200 U ml^{-1} streptomycin and penicillin. Midguts were isolated in sterile Petri dishes, and digest cells were detached from the gut wall with sterile tweezers. The cells were carefully collected using a Pasteur pipette, washed three times in the same buffer and then placed in a 12-well culture plate with L-15 Leibowitz's medium supplemented with 150 mM NaCl plus streptomycin and penicillin, both at 100 U ml^{-1}. The cells were kept at 28°C in the dark until use.

Uptake of metalloporphyrins and Rhodamine 123 by digest cells

In a previous report (Lara et al., 2005), we used a fluorescent metalloporphyrin, palladium mesoporphyrin, as a fluorescent heme analog to characterize heme intracellular pathways in the digest cells of *R. microplus*. Here, we used two other metalloporphyrins as

heme analogs, tin-protoporphyrin IX (Sn-Pp-IX) and zinc-protoprophyrin (Zn-Pp-IX) (RAO et al. 2005), as the fluorescence of these compounds exhibits a higher quantum yield than the palladium complex (data not shown). A 20 mM stock solution of Sn-Pp IX was prepared in DMSO and further diluted 1:1 with 0.1 N NaOH immediately before its addition to cells. Solutions (100 µM) were prepared by diluting the stock solution directly into culture medium. Fluorescence spectra were collected using an Eclipse 100 spectrofluorimeter (Varian, Palo Alto, USA) and showed two excitation peaks at 410 nm and 550 nm. The emission spectrum of both porphyrins showed a strong red fluorescence, with peaks at 582 nm and 630 nm. Zn-Pp IX was used in the artificial feeding of partially engorged ticks for RNA interference experiments, as described below.

The fluorescent images of Sn-Pp IX and Zn-Pp IX uptake by digest cells were obtained using a 100 W mercury lamp as the excitation light source with a Zeiss-15 filter set (BP 546-12 nm / FT 580 nm / LP 590 nm) and an Axioplan 2 microscope (Zeiss, Gottingen, Germany). In experiments to observe Sn-Pp IX uptake, the cells were pre-incubated or not in culture medium containing 10 µM of the ABC inhibitor indicated. After 2 h, 100 µM of Sn-Pp IX was added to the medium. Images were acquired after 4 h of incubation. To study the uptake of Rhodamine 123, a canonical ABC transporter superfamily substrate, we performed a pulse-and-chase experiment, where 0.5 µM of rhodamine was added to the digest cell culture. After 8 h, the media was replaced by media without the fluorescent probe, followed by a chase period of 12 h. Images were acquired using a Zeiss-09 (BP 450–490 nm/FT 510 nm/LP 515 nm) filter set.

HPLC analysis of the accumulation of Sn-Pp IX and amitraz in the hemosome

Digest cells from resistant or sensitive strains were placed in culture media and pre-treated for 30 minutes with ABC transporter superfamily modulators such as 10 µM

CsA, 300 µM indometacine, 50 µM verapamil, or 50 µM of trifluorperazine. After this pre-incubation, 50 µM of Sn-Pp IX or 35 µM of amitraz was added to the media. A control was performed by incubating the cells at 4 °C to decrease metabolic activity. After 30 min of incubation with amitraz or Sn-Pp IX, the cells were disrupted by repeatedly pipetting with a 100 µl automatic pipette. Hemosomes were purified through centrifugation at 100 × g for 2 min in a 30% sucrose cushion. The pellets formed below the cushions were dissolved by the addition of 0.025% ammonium hydroxide and 5% acetonitrile and vortexing. The suspensions were centrifuged at 14000 × g to remove insoluble particles, and the supernatant was applied to a Poros reverse-phase HPLC column (C8-C18; Applied Biosystems, California, USA) using 0.025% hydroxide ammonium and a gradient of 5 to 100% acetonitrile as the mobile phase. HPLC was performed using a diode array detector (SPD-M10A, Shimadzu, Tokyo, Japan) with an HPLC system LC-10AT (Shimadzu, Tokyo, Japan). Spectra from the heme, Sn-Pp IX and amitraz peaks were recorded during chromatography. As heme was determined to account for 90% of the hemosomes dry weight (LARA et al. 2003), the relative amount of Sn-Pp IX and amitraz found in hemosomes from digest cells incubated with these compounds was calculated using the heme content of hemosomes as an internal reference. To normalize the data, the amount of Sn-Pp IX or amitraz found in digest cells from the acaricide-resistant strain after 2 h exposure was defined as 100%. Absorbance values at 400 nm for heme and Sn-Pp IX and at 360 nm for amitraz and the area under the corresponding peaks were used to calculate the relative amount of amitraz and Sn-Pp IX that had accumulated inside the hemosomes.

Immunolocalization

Fully engorged females were dissected in PBS, and midguts were fixed in PBS with 4% paraformaldehyde for 12 h at 4 °C. The tissues were further incubated in PBS

with 20% sucrose for 12 h at 4 °C, embedded in O.C.T. (TissueTek, Minnesota, USA) and frozen under liquid nitrogen. Transversal 10 µm-thick sections were cut using a CM1900 cryostat (Leica, Wetzlar, Germany). Sections were collected on a glass slide covered with poly-L-lysine, blocked with 5% BSA and 0.05% Triton X-100 in PBS for 1 h, and then incubated for 4 h at 4 °C with polyclonal antibodies against the conserved regions of human glycoprotein (PgP-1) or multidrug resistance protein-1 (MRP-1) transporters (DAKO, California, USA; diluted 1:200 in PBS with 5% BSA), two distinct subtypes of the ABC superfamily. The sections were then washed and stained with Alexa-488 conjugated anti-rabbit secondary antibody (Sigma, Missouri, USA) diluted 1:500 in PBS containing 5% BSA for 4 h and then observed in a fluorescence microscope Axioplan 2 (Zeiss, Gottingen, German).

Histochemical staining of ATPase activity

Midgut sections of engorged females dissected on the third day ABM were fixed in 1% glutaraldehyde and 0.0005% Triton X-100, 50 mM HEPES, pH 7.2, for 10 minutes at 4 °C. Subsequently, the tissues were incubated with a reaction mixture composed of 5 mM ATP, 2.5 mM $MgCl_2$, 5 mM $CeCl_3$, 50 mM KCl, and 50 mM HEPES, pH 7.2, for 2 hours at 28 °C, as described by Hulstaert et al. (1983). The samples were further fixed in 2.5% glutaraldehyde, 4% paraformaldehyde, and 50 mM HEPES, pH 7.2, for 72 hours at 4 °C. After gradual dehydration in acetone, the tissues were embedded in Epon resin. Ultra-thin sections (60 nm) were observed under a Morgagni 268 transmission electron microscope (Fei, Oregon, USA) operating at 80 Kv.

RNA extraction and cDNA synthesis

Midgut or digest cells were homogenized in TRIzol® reagent (Invitrogen). After extraction following the manufacturer's recommendations, RNA was treated with DNase I (Invitrogen, USA), and the concentration and quality of the RNA was estimated using a NanoDrop 1000 (Thermo Fisher Scientific, USA). For cloning experiments, the total RNA was reverse-transcribed using an oligo-dT primer and SuperScript II (Invitrogen, USA), and for quantitative PCR, total RNA was reverse-transcribed using the High-capacity cDNA Reverse Transcription kit with random primers, according to the manufacturer's recommendations (Applied Biosystems, USA). cDNA was stored at -80 °C until use.

Cloning and sequence analysis of RmABCB10 transcript

To obtain the full-length sequence of *RmABCB10*, 5'RACE was performed using the 5'RACE System for Rapid Amplification of cDNA Ends kit (Invitrogen) according to the manufacturer's directions. Total RNA prepared from gut digest cells was reverse transcribed using a gene-specific primer (ABCBm-GSP1- CGAATTTATAGAAG-ACCTTC) designed from the partial *RmABCB10* sequence available (Genbank accession number JN098446.1). cDNA was purified, a homopolymeric tail (dCTP) was added to the 3' end of the cDNA using a terminal deoxynucleotidyl transferase and PCR was performed with a nested gene-specific primer (ABCBm-GSP2-GAGAACATTCTTTATGGTGC-AAAGAATATGGAGGAAAGCTC) and the Abridged Anchor Primer (Invitrogen). cDNA amplification products were cloned into the pGEM-T vector (Promega), and the positive clones were sequenced using vector-specific primers (T7 and SP6) and two internal primers: 5'-AGCTTCTGACTGGACAGTAG-3' (sense) and 5'-CCCACCGTAATAGAGGAC-3' (antisense). The full-length sequence was deposited in NCBI with the accession number JN098446.2.

The predicted amino acid sequence of full-length RmABCB10 was searched for the presence of the ABC signature, and the Walker A and Walker B conserved motifs using the Conserved Domain search program (MARCHLER-BAUER et al. 2011) available on the NCBI website. Multiple sequence alignments and phylogenetic analyses were performed using the MUSCLE algorithm (EDGAR 2004) using the default settings in MEGA software version 5 (TAMURA et al. 2011). SwissModel First Approach Mode was used for tertiary structure modeling. Membrane topology was predicted using SOSUI and TMHMM (GUEX and PEITSCH 1997; HIROKAWA et al. 1998; KALL et al. 2004). The sequences used in sequence alignments and phylogenetic analyses were *R. microplus* RmABCB10 (AEI91123.2), RmABCC1 (AEI91124.1) and RmABCC2 (AEI91125.1); *D. melanogaster* CG3156 (NP569844.2), MDR65 (NP476831.1), CG2613 (AAF59366.1), CG1703 (NP572736.1), CG1718 (NP608445.1) and CG2759 (NP476787.1); *D.pulex* 347264 (EFX85237.1), 347281 (EPX72783.1), 347357 (EFX73813.1), 347330 (EFX8324.11), 189585 (EFX66734.1), 312055 (EPX87570.1), and 347276 (EFX65703.1); *H. sapiens* ABCE1 (NP001035809.1), ABCG1 (NP058198.2), ABCB10 (NP036221.2), ABCB8 (NP009119.2) and ABCB7 (NP004290.2); *M. musculus* ABC-me(NP062425.1), and *S. cerevisiae* Mdl1p (NP13289.1), Mdl2p (NP015053.2) and Atm1p (NP014030.1).

Real-time PCR

For quantitative analysis of mRNA expression levels using real-time PCR, two specific primers were designed to amplify a 95 bp fragment (5'- GCCGCAGTTGTCACT-TGTTGGTTTG-3' and 5'-ACGTCCGCTGCCACTTGCCTC-3') of RmABCB10. A fragment of the β-actin gene (DA SILVA VAZ et al. 2005) that produces a 205 bp amplicon was used as a reference (5'GAGGAAGTACTCCGTCTGGATCGGCG3' and 5'CCGTAGGGTGGCGTTGCCGG3'). Cycling parameters were 10 min at 95 °C followed

by 40 cycles of denaturation at 95 ℃ for 15 s, annealing at 60 ℃ for 15 s and extension at 72 ℃ for 20 s. After amplification was complete, a melting curve analysis was performed using the default parameters of the instrument. Primer efficiency was measured with 6-fold serially diluted cDNA in triplicate, and for the analyses, 300 ng cDNA was added to each reaction. All samples were analyzed in triplicate. The relative expression ratio of *RmABCB10* gene in each experiment was calculated according to the mathematical model described by Pfaffl (2001) and used in the Relative Expression Software Tool (REST-MCS©, version 2) (Pfaffl et al. 2002).

RNA interference

The full-length *RmABCB10* sequence (1964bp) was amplified from gut cells cDNA using the primers 5'-ATGAACCCTACAGTTGAGTCCCAC-3' (sense) and 5'-TCACCCAGACATTCTCTCATCATGTAAC-3' (antisense) and cloned into the pGEM-T vector (Promega). A 578-bp fragment of *RmABCB10* was amplified by PCR from the recombinant plasmid with gene specific primers containing the T7 promoter recognition sites (sense: 5'-GGATCCTAATACGACTCACTATAGGGGCTCTCAGTTGTTTCGTC-3') (antisense: 5'- GGATCCTAATACGACTCACTATAGGGACATCCCAACCAGCG-3'). The PCR product was purified using a Gene Jet PCR Purification Kit (Fermentas) and used to synthesize dsRNA with a T7 Ribo Max Express RNA Kit (Promega) according to the manufacturer's protocol. The dsRNA synthesis was evaluated by 1.5% agarose gel electrophoresis, and the concentration was determined spectrophotometrically at 260 nm in a NanoDrop 1000 instrument (Thermo Fisher Scientific, USA).

The dsRNA of a tick-unrelated gene, *MSP1* from Plasmodium falciparum (accession number AF061132), was used as a negative control (dsCont). This gene was obtained from a plasmid kindly provided by Dr. Gerhard Wunderlich (Departamento de

Parasitologia, USP, Brazil). The 1110 bp sequence was recovered after digestion of the plasmids with Fast Digest SpEI (Fermentas), and the dsRNA of *MSP1* was synthesized as described above. Partially engorged tick females (weighing between 25 and 70 mg) of the POA strain were manually removed from experimentally infested bovines. Groups of 15 tick females were immobilized on a glass plate covered with double-sided adhesive tape, and dsRNA solutions were injected into the hemocoel (1 µL, 5 µg/tick). These ticks were artificially fed using microhematocrit capillary tubes filled with blood from non-infested bovines collected in the presence of sodium citrate (GONSIOROSKI et al. 2012; POHL et al. 2011). Initially, females were fed with 50 µl of blood supplemented with either the fluorescent heme analog Zn-PP IX diluted in DMSO (1:200, 100 µM) or DMSO alone (1:200), as a control for autofluorescence. After this initial meal, the females were fed until repletion with blood alone, without metaloporphyrin or DMSO. The females were allowed to feed for approximately 28 h, and then kept in separate vials at 27–28 °C and 80–90% relative humidity. After 24 h or 48 h feeding, the females were dissected, and the midguts were collected for RNA extraction and microscopy.

Statistical analysis

Comparisons between groups were performed by a one-way ANOVA analysis of variance and an a posteriori Tukey's test for pair-wise comparisons, using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA).

Acknowledgements

We would like to thank Prof. Rosalia Mendez Otero, from the Instituto de Biofísica Carlos Chagas Filho (UFRJ), for the use of the cryosection apparatus and Prof. Ulysses Lins, from the Instituto de Microbiologia (UFRJ), for the use of the differential

interference contrast microscope and the transmission electron microscope. We also would like to thank S. R. Cássia for technical assistance and Dr. Carlos Frederico Leite Fontes and Dra. Vivian M Rumjanek, from the Instituto de Bioquimica Médica (UFRJ), Dr. Alberto Félix da Nóbrega and Dr. Antônio Ferreira Pereira, from the Instituto de Microbiologia Prof. Paulo de Góes (UFRJ), and Dra. Cristina Vidal Pessolani, from Pavilhão Hanseníase (FioCruz Foundation) for scientific discussions.

Financial Disclosure

This work was supported by CNPq, FAPERJ, CAPES, FAPERGS, PADCT, PRONEX and HHMI. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

AGBEDE, R. I., and D. H. KEMP, 1985 Digestion in the cattle-tick *Boophilus microplus*: light microscope study of the gut cells in nymphs and females. Int J Parasitol 15: 147-157.

BORST, P., and R. O. ELFERINK, 2002 Mammalian ABC transporters in health and disease. Annu Rev Biochem 71: 537-592.

BRAM, R. A., J. E. GEORGE, R. E. REICHAR and W. J. TABACIINIC, 2002 Threat of foreign arthropod-borne pathogens to livestock in the United States. J Med Entomol 39: 405-416.

BRAUN, V., and K. HANTKE, 2011 Recent insights into iron import by bacteria. Current Opinion in Chemical Biology 15: 328-334.

BRAYDEN, D. J., and J. GRIFFIN, 2008 Avermectin transepithelial transport in MDR1- and MRP-transfected canine kidney monolayers. Vet Res Commun 32: 93-106.

BRAZ, G. R., H. S. COELHO, H. MASUDA and P. L. OLIVEIRA, 1999 A missing metabolic pathway in the cattle tick *Boophilus microplus*. Curr Biol 9: 703-706.

BURKHARD, K. A., and A. WILKS, 2008 Functional characterization of the *Shigella dysenteriae* heme ABC transporter. Biochemistry 47: 7977-7979.

BUSS, D. S., A. R. MCCAFFERY and A. CALLAGHAN, 2002 Evidence for p-glycoprotein modification of insecticide toxicity in mosquitoes of the *Culex pipiens* complex. Med Vet Entomol 16: 218-222.

BUSS, D. S. C., A., 2008 Interaction of pesticides with p-glycoprotein and other ABC proteins: A survey of the possible importance to insecticide, herbicide and fungicide resistance. . Pesticide biochemistry and physiology 90: 141–153.

CITELLI, M., F. A. LARA, I. DA SILVA VAZ, JR. and P. L. OLIVEIRA, 2007 Oxidative stress impairs heme detoxification in the midgut of the cattle tick, *Rhipicephalus (Boophilus) microplus*. Mol Biochem Parasitol 151: 81-88.

CUPELLO, M. P., C. F. SOUZA, C. BUCHENSKY, J. B. SOARES, G. A. LARANJA et al., 2011 The heme uptake process in *Trypanosoma cruzi* epimastigotes is inhibited by heme analogues and by inhibitors of ABC transporters. Acta Trop 120: 211-218.

DA SILVA VAZ, I., JR., S. IMAMURA, C. NAKAJIMA, F. C. DE CARDOSO, C. A. FERREIRA et al., 2005 Molecular cloning and sequence analysis of cDNAs encoding for *Boophilus microplus*, *Haemaphysalis longicornis* and *Rhipicephalus appendiculatus* actins. Vet Parasitol 127: 147-155.

DAVEY, R. B., J. E. GEORGE and R. J. MILLER, 2004 Control of an organophosphate-resistant strain of *Boophilus microplus* (Acari: Ixodidae) infested on cattle after a series of dips in coumaphos applied at different treatment intervals. J Med Entomol 41: 524-528.

DEAN, M., Y. HAMON and G. CHIMINI, 2001 The human ATP-binding cassette (ABC) transporter superfamily. J Lipid Res 42: 1007-1017.

EDGAR, R. C., 2004 MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32: 1792-1797.

GOLDMAN, B. S., K. K. GABBERT and R. G. KRANZ, 1996 Use of heme reporters for studies of cytochrome biosynthesis and heme transport. J Bacteriol 178: 6338-6347.

GONSIOROSKI, A. V., I. A. BEZERRA, K. U. UTIUMI, D. DRIEMEIER, S. E. FARIAS et al., 2012 Anti-tick monoclonal antibody applied by artificial capillary feeding in *Rhipicephalus (Boophilus) microplus* females. Exp Parasitol. 130: 359-63.

GRACA-SOUZA, A. V., C. MAYA-MONTEIRO, G. O. PAIVA-SILVA, G. R. BRAZ, M. C. PAES et al., 2006 Adaptations against heme toxicity in blood-feeding arthropods. Insect Biochem Mol Biol 36: 322-335.

GUEX, N., and M. C. PEITSCH, 1997 SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling. Electrophoresis 18: 2714-2723.

HIROKAWA, T., S. BOON-CHIENG and S. MITAKU, 1998 SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics 14: 378-379.

HOMOLYA, L., A. VARADI and B. SARKADI, 2003 Multidrug resistance-associated proteins: Export pumps for conjugates with glutathione, glucuronate or sulfate. Biofactors 17: 103-114.

HULSTAERT, C. E., D. KALICHARAN and M. J. HARDONK, 1983 Cytochemical demonstration of phosphatases in the rat liver by a cerium-based method in combination with osmium tetroxide and potassium ferrocyanide postfixation. Histochemistry 78: 71-79.

KALL, L., A. KROGH and E. L. L. SONNHAMMER, 2004 A combined transmembrane topology and signal peptide prediction method. Journal of Molecular Biology 338: 1027-1036.

KEEL, S. B., R. T. DOTY, Z. YANG, J. G. QUIGLEY, J. CHEN et al., 2008 A heme export protein is required for red blood cell differentiation and iron homeostasis. Science 319: 825-828.

KHAN, A. A., and J. G. QUIGLEY, 2011 Control of intracellular heme levels: heme transporters and heme oxygenases. Biochim Biophys Acta 1813: 668-682.

KOSTAROPOULOS, I., A. I. PAPADOPOULOS, A. METAXAKIS, E. BOUKOUVALA and E. PAPADOPOULOU-MOURKIDOU, 2001 Glutathione S-transferase in the defence against pyrethroids in insects. Insect Biochem Mol Biol 31: 313-319.

KRISHNAMURTHY, P., D. D. ROSS, T. NAKANISHI, K. BAILEY-DELL, S. ZHOU et al., 2004 The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. J Biol Chem 279: 24218-24225.

KRISHNAMURTHY, P. C., G. DU, Y. FUKUDA, D. SUN, J. SAMPATH et al., 2006 Identification of a mammalian mitochondrial porphyrin transporter. Nature 443: 586-589.

LARA, F. A., U. LINS, G. H. BECHARA and P. L. OLIVEIRA, 2005a Tracing heme in a living cell: hemoglobin degradation and heme traffic in digest cells of the cattle tick *Boophilus microplus*. Journal of Experimental Biology 208: 3093-3101.

LARA, F. A., U. LINS, G. H. BECHARA and P. L. OLIVEIRA, 2005b Tracing heme in a living cell: hemoglobin degradation and heme traffic in digest cells of the cattle tick *Boophilus microplus*. J Exp Biol 208: 3093-3101.

LARA, F. A., U. LINS, G. PAIVA-SILVA, I. C. ALMEIDA, C. M. BRAGA et al., 2003 A new intracellular pathway of haem detoxification in the midgut of the cattle tick *Boophilus microplus*: aggregation inside a specialized organelle, the hemosome. J Exp Biol 206: 1707-1715.

LARA, F. A., C. SANT'ANNA, D. LEMOS, G. A. LARANJA, M. G. COELHO et al., 2007 Heme requirement and intracellular trafficking in *Trypanosoma cruzi* epimastigotes. Biochem Biophys Res Commun 355: 16-22.

LOOR, F., F. TIBERGHIEN, T. WENANDY, A. DIDIER and R. TRABER, 2002 Cyclosporins: structure-activity relationships for the inhibition of the human FPR1 formylpeptide receptor. J Med Chem 45: 4613-4628.

MARCHLER-BAUER, A., S. LU, J. B. ANDERSON, F. CHITSAZ, M. K. DERBYSHIRE et al., 2011 CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Res 39: D225-229.

MENDIOLA, J., M. ALONSO, M. C. MARQUETTI and C. FINLAY, 1996 *Boophilus microplus*: multiple proteolytic activities in the midgut. Exp Parasitol 82: 27-33.

NENE, V., J. R. WORTMAN, D. LAWSON, B. HAAS, C. KODIRA et al., 2007 Genome sequence of Aedes aegypti, a major arbovirus vector. Science 316: 1718-1723.

NOBMANN, S., B. BAUER and G. FRICKER, 2001 Ivermectin excretion by isolated functionally intact brain endothelial capillaries. Br J Pharmacol 132: 722-728.

PFAFFL, M. W., 2001 A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45.

PFAFFL, M. W., G. W. HORGAN and L. DEMPFLE, 2002 Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30: e36.

POHL, P. C., G. M. KLAFKE, D. D. CARVALHO, J. R. MARTINS, S. DAFFRE et al., 2011 ABC transporter efflux pumps: a defense mechanism against ivermectin in *Rhipicephalus (Boophilus) microplus*. Int J Parasitol 41: 1323-1333.

POURTIER-MANZANEDO, A., A. D. DIDIER, C. D. MULLER and F. LOOR, 1992 SDZ PSC 833 and SDZ 280-446 are the most active of various resistance-modifying agents in restoring rhodamine-123 retention within multidrug resistant P388 cells. Anticancer Drugs 3: 419-425.

QUIGLEY, J. G., Z. YANG, M. T. WORTHINGTON, J. D. PHILLIPS, K. M. SABO et al., 2004 Identification of a human heme exporter that is essential for erythropoiesis. Cell 118: 757-766.

RAJAGOPAL, A., A. U. RAO, J. AMIGO, M. TIAN, S. K. UPADHYAY et al., 2008 Haem homeostasis is regulated by the conserved and concerted functions of HRG-1 proteins. Nature 453: 1127-1131.

RAO, A. U., L. K. CARTA, E. LESUISSE and I. HAMZA, 2005 Lack of heme synthesis in a free-living eukaryote. Proc Natl Acad Sci U S A 102: 4270-4275.

SHABBIR, A., S. DISTASIO, J. ZHAO, C. P. CARDOZO, M. S. WOLFF et al., 2005 Differential effects of the organochlorine pesticide DDT and its metabolite p,p'-DDE on p-glycoprotein activity and expression. Toxicol Appl Pharmacol 203: 91-98.

SHAYEGHI, M., G. O. LATUNDE-DADA, J. S. OAKHILL, A. H. LAFTAH, K. TAKEUCHI et al., 2005 Identification of an intestinal heme transporter. Cell 122: 789-801.

SMITH, T., and F. L. KILBORNE, 1893 Investigations into the nature, causation, and prevention of Texas or southern cattle fever. Washington: USDA, 1893. 301 p. (USDA. Bulletin, 1).

SOLAZZO, M., O. FANTAPPIE, M. D'AMICO, C. SASSOLI, A. TANI et al., 2009 Mitochondrial expression and functional activity of breast cancer resistance protein in different multiple drug-resistant cell lines. Cancer Res 69: 7235-7242.

STAPF, V., T. THALHAMMER, R. HUBER-HUBER, F. FELBERBAUER, L. GAJDZIK et al., 1994 Inhibition of rhodamine 123 secretion by cyclosporin A as a model of P-glycoprotein mediated transport in liver. Anticancer Res 14: 581-585.

SWAIN, V., R. K. SETH, K. RAGHAVENDRA and S. S. MOHANTY, 2009 Characterization of biochemical based insecticide resistance mechanism by thermal bioassay and the variation of esterase activity in *Culex quinquefasciatus*. Parasitol Res 104: 1307-1313.

TAMURA, K., D. PETERSON, N. PETERSON, G. STECHER, M. NEI et al., 2011 MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 28: 2731-2739.

THOMPSON, J. M., H. A. JONES and R. D. PERRY, 1999 Molecular characterization of the hemin uptake locus (hmu) from Yersinia pestis and analysis of hmu mutants for hemin and hemoprotein utilization. Infect Immun 67: 3879-3892.

WALKER, A. R., and J. D. FLETCHER, 1987 Histology of digestion in nymphs of *Rhipicephalus appendiculatus* fed on rabbits and cattle naive and resistant to the ticks. Int J Parasitol 17: 1393-1411.

WANDERSMAN, C., and I. STOJILJKOVIC, 2000 Bacterial heme sources: the role of heme, hemoprotein receptors and hemophores. Curr Opin Microbiol 3: 215-220.

WONDJI, C. S., H. IRVING, J. MORGAN, N. F. LOBO, F. H. COLLINS et al., 2009 Two duplicated P450 genes are associated with pyrethroid resistance in Anopheles funestus, a major malaria vector. Genome Res 19: 452-459.

ZUTZ, A., S. GOMPF, H. SCHAGGER and R. TAMPE, 2009 Mitochondrial ABC proteins in health and disease. Biochim Biophys Acta 1787: 681-690.

4. DISCUSSÃO GERAL

O controle de parasitas se tornou um sério problema nas últimas décadas. Fundamentalmente, devido ao aparecimento de populações resistentes após o uso indiscriminado de antiparasitários (CASIDA & QUISTAD, 1998; FAO, 2004). Este quadro não é diferente ao observado para o *R. microplus*, em que a resistência aos acaricidas é identificada em, praticamente, todos os países produtores de gado (GUERREIRO *et al.*, 2012a). Uma vez que, o desenvolvimento de novos acaricidas tem declinado (CASIDA & QUISTAD, 1998; FAO, 2004), o controle químico depende das drogas existentes e de um manejo adequado destas para retardar o aparecimento da resistência.

O objetivo desta tese foi contribuir ao conhecimento dos mecanismos moleculares envolvidos no desenvolvimento de resistência no carrapato *R. microplus* e identificar alvos para estratégias de diagnóstico da resistência e de controle desse parasita.

Os transportadores ABC são um grupo de proteínas que tem atraído interesse especial com relação aos estudos de resistência às drogas. Conforme apresentado na introdução, a importância destes transportadores é reconhecida em um grande número de organismos, onde são considerados a primeira linha de defesa contra a entrada de drogas na célula. Sendo assim, responsáveis pela resistência a um grande número de compostos diferentes.

No presente trabalho, apresentamos a participação de transportadores ABC na resistência à ivermectina no carrapato *R. microplus* (**artigo I**). O tratamento com inibidores de transportadores ABC causou um aumento significativo dos efeitos tóxicos da ivermectina em larvas e fêmeas adultas provenientes de populações resistentes a esta

droga, mostrando a participação destes transportadores na destoxificação da ivermectina. Em larvas, os efeitos tóxicos da ivermectina foram observados pelo aumento da mortalidade, enquanto que, em fêmeas, o efeito mais considerável foi a redução da postura e a viabilidade dos ovos.

Sabe-se que a ivermectina promove um melhor controle do carrapato quando o tratamento atinge larvas, sendo menos efetiva contra carrapatos no estágio final de ingurgitamento (DAVEY *et al.*, 2005; DAVEY *et al.*, 2010). DAVEY e colaboradores (2010) mostraram que o tratamento com ivermectina em bovinos, promoveu um melhor controle do carrapato somente quando a infestação ocorreu até o 14° dia de tratamento, onde os níveis de ivermectina no soro dos animais se encontravam em torno de 30ng/ mL. Após este período, quando os níveis de concentração no sangue já eram menores, o principal efeito tóxico da ivermectina foi a redução no índice de fertilidade. Semelhantemente, observamos que a alimentação artificial de fêmeas com sangue acrescido de 20ng/ mL de ivermectina, reduziu significativamente o índice de fertilidade e a viabilidade dos ovos na população sensível ao acaricida. Na população resistente ao acaricida, efeito semelhante foi atingido somente quando o inibidor de transportadores ABC, ciclosporina (CsA), foi adicionado ao tratamento, observando-se, também, um significativo aumento de mortalidade das fêmeas.

Inibidores de transportadores ABC promovem o aumento da concentração das drogas nas células pelo bloqueio ou modulação da destoxificação destas pelos transportadores ABC. Por este motivo, tem sido amplamente estudados com a finalidade de melhorar os tratamentos de câncer e o controle de patógenos, principalmente, quando a resistência mediada por estes transportadores reduz o número de drogas disponíveis (LESPINE *et al.*, 2008, YANG *et al.*, 2010). Esta abordagem se mostrou eficiente na melhoria do controle de nematódeos com a ivermectina. A eficiência da ivermectina contra

H. contortus, em ovelhas, foi aumentada de 0%, quando administrada sozinha; para 72,5% quando coadministrada com loperamida (inibidor de transportador ABC), que aumentou também a disponibilidade da ivermectina no plasma dos animais (LIFSCHITZ *et al*., 2010b).

Estes resultados suportam a ideia que, aumentar a disposição da ivermectina nos carrapatos, através da coadministração de inibidores de transportador ABC, pode ser uma alternativa eficiente para aumentar a suscetibilidade do parasita e reduzir a dose e a frequência de aplicação do acaricida, fator relevante para retardar o aparecimento da resistência e reduzir os resíduos presentes na carne e no leite. Entretanto, tal estratégia requer disponibilidade de inibidores mais específicos para evitar efeitos colaterais da inibição de transportadores ABC no hospedeiro. E a primeira etapa para a identificação destes inibidores é caracterizar genes codificadores de transportadores ABC e determinar a importância do produto codificado por cada gene na destoxificação de drogas.

O aumento da transcrição de genes codificadores de transportadores ABC é associado ao aparecimento da resistência em diversas espécies de parasitas. Corroborando com a literatura, fêmeas resistentes à ivermectina apresentaram transcrição induzida do gene *RmABCB10* no intestino, porta de entrada da ivermectina que é ingerida durante o repasto sanguíneo. A importância desse transportador foi validada pelo silenciamento do gene no intestino, que levou ao aumento da toxicidade da ivermectina no carrapato, observada pela diminuição na viabilidade dos ovos das fêmeas (**anexo I**). Resultado semelhante ao que foi obtido com o uso da CsA, inibidor de transportadores ABC da mesma subfamília cuja RmABCB10 pertence (**artigo I**).

Por sua capacidade de transportar diversos tipos de drogas, os transportadores ABC são responsabilizados pelo fenótipo de resistência a múltiplas drogas em diversos organismos. Uma vez que, populações de carrapato resistentes a múltiplas drogas são

comumente relatadas (BENAVIDES *et al*., 2000; FERNÁNDEZ-SALAS *et al*., 2012; ORTIZ *et al*., 1995), analisamos a participação destas proteínas na resistência a outros acaricidas em uma população de carrapato resistente a quatro classes de acaricidas (lactonas macrocíclicas, organofosforados, piretróides e amitraz) (**artigo II**). Interessantemente, inibidores de transportador ABC também aumentaram significativamente a toxidade de duas outras lactonas macrocíclicas, moxidectina e abametina, e do organofosforado, clorpirifós, em larvas dessa população. Da mesma forma, a toxicidade do amitraz foi significativamente aumentada quando as larvas foram expostas ao inibidor CsA, embora a razão de resistência não tenha sido significativamente aumentada. Este resultado mostra que a destoxificação de drogas pelos transportadores ABC não é um mecanismo exclusivo da ivermectina, mas sim, um mecanismo comum para diferentes classes de acaricidas e que deve ter importância fundamental na defesa desse parasita.

O mecanismo molecular que permite que um transportador ABC seja capaz de transportar substratos estruturalmente distintos é tópico de constantes debates. A comparação de 44 compostos, conhecidamente transportados pela P-gp (ABCB1), mostrou algumas propriedades comuns entre eles. Por exemplo, possuir pelo menos uma estrutura cíclica, uma massa molecular de mais de 300 Da e uma lipofilicidade moderada (BAIN *et al*., 1997). Estas características são comuns a muitos inseticidas e acaricidas de diferentes classes que já mostraram ser substrato de transportadores ABC. Em modelos de roedores, a P-gp (ABCB1) conferiu proteção à ivermectina e ao organofosforado clorpirifós (LANNING *et al*., 1996; SCHINKEL *et al*., 1994). E em modelos celulares de mamíferos, ciclodienos, organofosforados, piretróides e avermectinas foram transportadas ou interagiram com transportadores ABC (BAIN & LEBLAN, 1996; BAIN *et al*., 1997). Inseticidas e acaricidas também são substratos de transportadores ABC em artrópodes. Em

Chironomus riparius, a toxicidade da ivermectina foi significativamente aumentada quando larvas deste mosquito foram tratadas com doses subletais dos inibidores de transportadores ABC, verapamil e CsA (PODSIADLOWSKI *et al*., 1998). No mosquito *Culex pipiens*, a toxicidade a três classes de inseticidas (cipermetrina, endosulfan e ivermectina) foi aumentada após o tratamento com verapamil (BUSS *et al*., 2002), e em larvas de *Aedes caspius*, o tratamento com o mesmo inibidor aumentou a toxicidade de temefós e diflubenzuron (PORRETTA *et al*., 2008).

Estudar os mecanismos de resistência no carrapato, assim como em outros organismos, depende da caracterização de populações de carrapato em que a resistência está estabelecida. Em geral, estas populações são, ou foram expostas a vários acaricidas diferentes, não se observando o efeito da seleção pela exposição de uma droga isolada. Por isso, culturas de células são uma alternativa interessante para obter melhores controles em experimentos básicos de mecanismos de resistência. Linhagens derivadas de células cancerígenas são bastante usadas como modelos para estudar o transporte de drogas pelos transportadores ABC, bem como identificar possíveis inibidores com vista à aplicação em tratamentos clínicos (GILLET *et al*., 2007; JOVELET *et al*., 2012). Nesta tese, mostramos o estabelecimento de uma linhagem de células resistentes à ivermectina a partir da linhagem parental de células embrionárias BME26 (**artigo III**). Estas células foram 4,5 vezes mais resistentes à ivermectina que as células parentais. Uma taxa de resistência semelhante ao que foi observado em populações de campo (**artigo I**). A toxicidade da ivermectina foi aumentada quando as células foram tratadas com o inibidor CsA, indicando a participação de transportadores ABC na destoxificação da droga. Além disso, o mesmo gene induzido em fêmeas (*RmABCB10*) (**artigo I**) foi significativamente induzido nas células resistentes quando estas foram expostas ao acaricida, o que indica que,

provavelmente, o mesmo mecanismo induzido *in vivo* foi selecionado *in vitro*, validando os resultados obtidos.

Os transportadores ABC são importantes também na destoxificação de compostos endógenos, como a molécula heme, que, quando livre, tem grande potencial de formar espécies reativas de oxigênio. Devido a sua dieta de sangue, artrópodes hematófagos apresentam adaptações que os protegem do potencial tóxico do heme liberado pela digestão da hemoglobina (GRAÇA-SOUZA *et al*., 2006; LARA *et al*., 2005). Conforme discutido no **artigo IV**, em contraste ao que é observado na maioria dos artrópodes hematófagos, em que a digestão dos componentes do sangue ocorre no lúmen do intestino, no carrapato *R. microplus*, a digestão ocorre no interior das células intestinais, levando a liberação de heme dentro delas (LIYOU *et al*., 1996; LARA *et al*., 2005). Para analisar a rota intracelular do heme dentro das células digestivas do carrapato *R. microplus*, utilizou-se paládio-mesoporfirina IX, um análogo fluorescente de heme (LARA *et al*., 2005). Este estudo mostrou que nas células digestivas, a hemoglobina é hidrolisada em vacúolos, liberando uma imensa quantidade de heme nessas células. O heme livre é, então, transportado pelo citosol, por uma proteína ligadora-de-heme até os hemossomos, organelas especializadas na sua destoxificação. E o seu transporte para o interior destas é dependente de uma proteína transportadora de membrana (LARA *et al*., 2005).

Na presente tese, identificamos que o transporte de um análogo fluorescente do heme para o interior dos hemossomos, é inibido quando as células são incubadas com diferentes inibidores de transportador ABC. Estas organelas são, ainda, capazes de acumular rodamina 123, um substrato clássico de transportadores ABC, sendo este acúmulo bloqueado pelo inibidor CsA (**artigo IV**). Além disso, ensaios de imunolocalização e de atividade ATPásica indicaram a presença de transportadores ABC

na membrana dos hemossomos. Estes resultados evidenciam que transportadores ABC são responsáveis pelo transporte de heme nestas organelas.

Vista a participação dos transportadores ABC na destoxificação de acaricidas no *R. microplus* e a presença destes transportadores na membrana nos hemossomos, avaliamos a possibilidade destas organelas funcionarem também, como sítio de destoxificação de compostos exógenos, como os acaricidas. Esta hipótese foi confirmada, uma vez que, hemossomos foram capazes de acumular o acaricida amitraz, sendo o transporte inibido por CsA. A transcrição de *RmABCB10* também foi induzida em uma população resistente a este acaricida, assim como observado para a população resistente à ivermectina (**artigo I**). Além disso, hemossomos provenientes de células digestivas da população resistente ao amitraz foram capazes de acumular maiores quantidades desse acaricida e de heme. O silenciamento gênico do *RmABCB10* causou significativa redução no acúmulo de heme nos hemossomos (**artigo IV**), assim como, aumentou a toxicidade da ivermectina nas fêmeas (**anexo I**). Em conjunto, todos estes resultados validam a participação do transportador RmABCB10 no transporte de heme e de acaricidas no carrapato *R. microplus*.

O gene codificador deste transportador foi também induzido na linhagem de células resistentes à ivermectina (**artigo III**). Embora hemossomos, propriamente ditos, não foram até o momento caracterizados nestas células, elas possuem vesículas capazes de sequestrar heme (ESTEVES *et al*., 2008), que podem também, ter participação na destoxificação de drogas. Porém, não podemos afirmar a localização exata deste transportador ao nível celular. No carrapato a transcrição do gene *RmABCB10* foi identificada no intestino, ovários e túbulos de Malpighi (**anexo I**).

De forma geral, transportadores ABC destoxificam as células, transportando a droga ou toxina para o meio externo. O sequestro de compostos tóxicos para o interior de

organelas ou vesículas, conforme visto aqui, para o interior dos hemossomos, não é um mecanismo comum, mas relatado em alguns tipos celulares como um importante mecanismo de destoxificação das células. Em células leucêmicas humanas, o aumento da expressão do transportador *ABCA3* está associado à resistência de um amplo espectro de drogas, entre elas, a imatinibe, droga muito utilizada no tratamento da leucemia mielóide aguda. Este transportador está localizado na membrana dos lisossomos e de outros corpos multivesiculares, e é responsável pelo sequestro, para o interior destas organelas, de um grande número de drogas quimioterápicas (CHAPUY *et al*., 2008 e 2009). Em células de câncer de mama, o transportador ABCG2 confere resistência à mitoxantrona através do sequestro desta para o interior de vesículas extracelulares. Altos níveis de resistência ao topodecano e amidazoacridinonas, nestas células, são também associados à capacidade de sequestrar estas drogas para o interior das vesículas extracelulares, indicando que o sequestro de diversas drogas é um mecanismo de proteção destas células (GOLER-BARON & ASSARAF, 2011).

Considerações finais

No presente trabalho, mostramos que a destoxificação de acaricidas por transportadores ABC, constitui-se em um importante mecanismo de resistência no carrapato *R. microplus*. Por ser uma das portas de entrada dos acaricidas, o intestino tem papel fundamental na proteção do carrapato. A destoxificação de acaricidas no intestino, pelo sequestro das drogas nos hemossomos, inibe ou reduz a consequente circulação desta por todo o organismo, principalmente, em carrapatos resistentes, em que o número de transportadores ABC é maior e a destoxificação é mais eficiente. Ainda que a droga escape por esta barreira, ou penetre no carrapato por outra via, como a cutícula, provavelmente,

transportadores ABC presentes em outros órgãos, destoxificam os acaricidas e protegem o carrapato dos efeitos tóxicos destas drogas.

Este mecanismo descrito no carrapato representa um novo alvo para o desenvolvimento de drogas que interfiram com transportadores ABC e/ou vacinas, já que é, também, importante para a destoxificação do heme. Além disso, uma vez que o transportador RmABCB10 mostrou ter um papel importante na resistência, o aumento de transcrição deste gene pode ser um indício precoce da seleção de resistência e funcionar como um método de diagnóstico.

5. REFERÊNCIAS BIBLIOGRAFICAS

ABDULLAH, S.; YADAV, C. L. & VATSYA, S. Esterase profile of *Rhipicephalus (Boophilus) microplus* populations collected from Northern India exhibiting varied susceptibility to deltamethrin. *Exp Appl Acarol.* (no prelo), 2012.

ABEL, I.; CORRÊA, F. N.; CASTRO, A. A.; CUNHA, N. C.; MADUREIRA, R. C. & FONSECA, A. H. Artificial feeding of *Amblyomma cajennense* (Acari: Ixodidae) fasting females through capillary tube technique. *Rev. Bras. Parasitol. Vet.* 17: 128-32 2008.

ALMAZÁN, C.; MORENO-CANTÚ, O.; MORENO-CID, J. A.; GALINDO, R. C.; CANALES, M.; VILLAR, M. & DE LA FUENTE, J. Control of tick infestations in cattle vaccinated with bacterial membranes containing surface-exposed tick protective antigens. *Vaccine.* 30: 265-72, 2012.

AMBUDKAR, S. V.; DEY, S.; HRYCYNA, C. A.; RAMACHANDRA, M.; PASTAN, I. & GOTTESMAN, M. M. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annu. Rev. Pharmacol. Toxicol.* 39: 361-98, 1999.

ANDREOTTI, R. Situação atual da resistência do carrapato-do-boi *Rhipicephalus (Boophilus) microplus* aos acaricidas no Brasil. Embrapa Gado de Corte, Mato Grosso do Sul, n. 180, 2010.[online] Disponível em: http://www.cnpgc.embrapa.br/publicacoes/doc/DOC180.pdf. Arquivo capturado em abril de 2012.

ANDREOTTI, R.; GUERRERO, F. D.; SOARES, M. A.; BARROS, J. C.; MILLER, R. J. & LÉON, A. P. Acaricide resistance of *Rhipicephalus (Boophilus) microplus* in State of Mato Grosso do Sul, Brazil. *Rev. Bras. Parasitol. Vet*. 20: 127-33, 2011.

ANGUS, B. M. The history of the cattle tick *Boophilus microplus* in Australia and achievements in its control. *Int. J. Parasitol.* 26: 1341-55, 1996.

ARGENTINE, J. A.; CLARK, J. M. & LIN, H. Genetics and biochemical mechanisms of abamectin resistance in two isogenic strains of Colorado potato beetle. *Pestic. Biochem. Physiol*. 44, 191- 207, 1992.

BAFFI, M. A.; DE SOUZA, G. R.; DE SOUSA, 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). *Mol. Biochem. Parasitol.* 160: 70-3, 2008.

BAIN, L. J. & LEBLANC, G. A. Interaction of structurally diverse pesticides with the human MDR1 gene product P-glycoprotein. *Toxicol. Appl. Pharmacol.* 141: 288-298, 1996.

BAIN, L. J.; MCLACHLAN, J. B. & LEBLANC, G. A. Structure-activity relationships for xenobiotic transport substrates and inhibitory ligands of P-glycoprotein. *Environ. Health Perspect.* 105: 812-8, 1997.

BARTLEY, D. J.; MCALLISTER, H.; BARTLEY, Y.; DUPUY, J.; MÉNEZ, C.; ALVINERIE, M.; JACKSON, F. & LESPINE, A. P-glycoprotein interfering agents

potentiate ivermectin susceptibility in ivermectin sensitive and resistant isolates of *Teladorsagia circumcincta* and *Haemonchus contortus*. *Parasitology*. 136, 1081-1088, 2009.

BASS, C. & FIELD, L. M. Gene amplification and insecticide resistance. *Pest Manag. Sci*. 67: 886-90, 2011.

BELL-SAKYI, L.; ZWEYGARTH, E.; BLOUIN, E. F.; GOULD, E. A. & JONGEJAN, F. Tick cell lines: tools for tick and tick-borne disease research. *Trends Parasitol*. 23, 450- 457, 2007.

BENAVIDES, E.; RODRÍGUEZ, J. L. & ROMERO, A. Isolation and partial characterization of the Montecitos strain of *Boophilus microplus* (Canestrini, 1877) multiresistant to different acaricides. *Ann. N.Y. Acad. Sci.* 916: 668- 671, 2000.

BENZ, G. W.; RONCALLI, R. A. & GROSS, S. J. Use of ivermectina in cattle, sheep, goats and swine. In: CAMPBELL, W. C., Ivermectin and Abamectin. Springer-Verlag, New York, v.1, cap. 15, p. 215- 229, 1989.

BORGES, L. M.; SOUSA, L. A. & BARBOSA, CDA. S. Perspectives for the use of plant extracts to control the cattle tick *Rhipicephalus (Boophilus) microplus*. *Rev. Bras. Parasitol. Vet.* 20: 89-96, 2011.

BORTHS, E. L.; LOCHER, K. P.; LEE, A. T. & REES, D. C. The structure of *Escherichia coli* BtuF and binding to its cognate ATP binding cassette transporter, *Proc. Natl. Acad. Sci. U.S.A.* 99: 16642–16647, 2002.

BOURGUINAT, C.; ARDELLI, B. F.; PION, S. D.; KAMGNO, J.; GARDON, J.; DUKE, B. O.; BOUSSINESQ, M. & PRICHARD, R. K. P-glycoprotein-like protein, a possible genetic marker for ivermectin resistance selection in *Onchocerca volvulus*. *Mol. Biochem. Parasitol*. 158: 101-11, 2008.

BURG, R. W.; MILLER, B. M.; BAKER, E. E.; BIRNBAUM, J.; CURRIE, S. A.; HARTMAN, R.; KONG, Y. L.; MONAGHAN, R. L.; OLSON, G.; PUTTER, I.; TUNAC, J. B.; WALLICK, H.; STAPLEY, E. O.; OIWA, R. & OMURA, S. Avermectins, new family of potent anthelmintic agents: producing organism and fermentation. *Antimicrob. Agents Chemother*. 15: 361-7, 1979.

BUSS, D. S.; A. R. MCCAFFERY & A. CALLAGHAN, Evidence for p-glycoprotein modification of insecticide toxicity in mosquitoes of the *Culex pipiens* complex. *Med. Vet. Entomol* 16: 218-222, 2002.

CAMILLO, G.; VOGELI, F. F.; SANGIONI, L. A.; CADORE, G. C., & FERRARI, R. Eficiência in vitro de acaricidas sobre carrapatos de bovinos no Estado do Rio Grande do Sul, Brasil. *Ciência Rural*, 39: 490-495, 2009.

CAMPOS, R. A.; BOLDO, J. T.; PIMENTEL, I. C.; DALFOVO, V.; ARAÚJO, W. L.; AZEVEDO, J. L.; VAINSTEIN, M. H. & BARROS, N. M. Endophytic and entomopathogenic strains of *Beauveria* sp to control the bovine tick *Rhipicephalus (Boophilus) microplus*. *Genet. Mol. Res.* 9: 1421-30, 2010.

CASIDA, J. E. & QUISTAD, G. B. Golden age of insecticide research: past, present, or future? *Annu. Rev. Entomol*. 43:1-16, 1998.

CASIDA, J. E. Pest toxicology: the primary mechanisms of pesticide action.Chem *Res. Toxicol*. 22: 609-19, 2009.

CASTRO-JANER, E.; MARTINS, J. R.; MENDES, M. C.; NAMINDOME, A.; KLAFKE, G. M. & SCHUMAKER, T. T. Diagnoses of fipronil resistance in Brazilian cattle ticks (*Rhipicephalus (Boophilus) microplus*) using in vitro larval bioassays. *Vet. Parasitol.* 173: 300-306, 2010a.

CASTRO-JANER, E.; RIFRAN, L.; GONZÁLEZ, P.; PIAGGIO, J.; GIL, A. & SCHUMAKER TT. *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) resistance to fipronil in Uruguay evaluated by in vitro bioassays. *Vet. Parasitol.* 169: 172-7, 2010b.

CASTRO-JANER, E.; RIFRAN, L.; GONZÁLEZ, P.; NIELL, C.; PIAGGIO, J.; GIL, A. & SCHUMAKER, T. T. Determination of the susceptibility of *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) to ivermectin and fipronil by Larval Immersion Test (LIT) in Uruguay. *Vet. Parasitol.* 178, 148- 155, 2011.

CHAPUY, B.; KOCH, R.; RADUNSKI, U.; CORSHAM, S.; CHEONG, N.; INAGAKI, N.; BAN, N.; WENZEL, D.; REINHARDT, D.; ZAPF, A.; SCHWEYER, S.; KOSARI, F.; KLAPPER, W.; TRUEMPER, L. & WULF, G. G. Intracellular ABC transporter A3 confers multidrug resistance in leukemia cells by lysosomal drug sequestration. *Leukemia.* 22: 1576-86, 2008.

CHAPUY, B.; PANSE, M.; RADUNSKI, U.; KOCH, R.; WENZEL, D.; INAGAKI, N.; HAASE, D.; TRUEMPER, L. & WULF, G. G. ABC transporter A3 facilitates lysosomal sequestration of imatinib and modulates susceptibility of chronic myeloid leukemia cell lines to this drug. *Haematologica*. 94: 1528-36, 2009.

CLAUDIANOS, C.; RUSSELL, R. J. & OAKESHOTT, J. G. The same amino acid substitution in orthologous esterases confers organophosphate resistance on the house fly and a blowfly. *Insect Biochem. Mol. Biol.* 29: 675-86, 1999.

CNPC: CONSELHO NACIONAL DA PECUÁRIA DE CORTE. Fórum nacional permanente da pecuária de corte: balanço da ecuária bovídea de corte 2001 a 2010. [online] Disponível em www.cnpc.org.br/arquivos/Balanco.xls. Arquivo capturado em junho de 2012.

COLES, G. C.; WATSON, C. L. & ANZIANI, O. S. Ivermectin-resistant *Cooperia* in cattle. *Vet. Rec*. 148, 283-4, 2001.

COLES, G. C.; RHODES, A. C. & WOLSTENHOLME, A. J. Rapid selection for ivermectin resistance in Haemonchus contortus.*Vet. Parasitol.* 129: 345-7, 2005.

CORDOVÉS. C.O. Carrapato: controle ou erradicação. Alegrete: Gralha, 1996.

CURRIE, B. J.; HARUMAL, P.; MCKINNON, M. & WALTON, S. F. First documentation of in vivo and in vitro ivermectin resistance in *Sarcoptes scabiei*. *Clin. Infect. Dis.* 39: 8-12, 2004.

DA SILVA VAZ JR, I.; LERMEN, T. T.; MICHELON, A.; FERREIRA, C.A.S.; DE FREITAS, D.R.J.; TERMIGNONI, C. & MASUDA, A. Effect of acaricides on the activity of a *Boophilus microplus* glutathione S-transferase. *Vet. Parasitol*. 119: 237-45, 2004.

DASSA, E. Phylogenetic and functional classification of ABC (Atp-binding cassette) systems. In: HOLLAND, I. B., COLE, S. P. C., KUCHLER, K. & HIGGINS, C. F.

ABC proteins: from bacteria toman. *Academic Press Elsevier Science*, London, v. 1, cap.1, p.3- 35, 2003.

DAVEY, R.B.; MILLER, J.A.; GEORGE, J.E. & MILLER, R.J. Therapeutic and persistent efficacy of a single injection treatment of ivermectin and moxidectin against *Boophilus microplus* (Acari: Ixodidae) on infested cattle. *Exp. Appl. Acarol.* 35: 117-29, 2005.

DAVEY, R. B.; POUND, J. M.; MILLER, J. A. & KLAVONS, J. A.Therapeutic and persistent efficacy of a long-acting (LA) formulation of ivermectin against *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) and sera concentration through time in treated cattle. *Vet. Parasitol.* 169: 149-56, 2010.

DE ABREU, L. A.; FABRES, A.; ESTEVES, E.; MASUDA, A.; DA SILVA VAZ JR, I.; DAFFRE, S. & LOGULLO, C. Exogenous insulin stimulates glycogen accumulation in *Rhipicephalus (Boophilus) microplus* embryo cell line BME26 via PI3K/AKT pathway. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 153: 185-90, 2009.

DE LA FUENTE, J.; ALMAZÁN, C.; CANALES, M.; LASTRA, J. M. P.; KOCAN, K. M. & WILLADSEN, P. A ten-year review of commercial vaccine performance for control of tick infestations on cattle. *Anim. Health Res. Rev.* 8: 23-8, 2007.

DEAN, M.; HAMON, Y. & CHIMINI, G. The human ATP-binding cassette (ABC) transporter superfamily. *J. Lipid. Res.* 42: 1007-1017, 2001.

DEELEY, R. G.; WESTLAKE, C. & COLE, S. P. Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. *Physiol Rev.* 86: 849-99, 2006.

DESPRÉS, L.; DAVID, J. P. & GALLET, C. The evolutionary ecology of insect resistance to plant chemicals. *Trends Ecol. Evol.* 22: 298-307, 2007.

DEVONSHIRE, A. L. Role of esterases in resistance of insects to insecticides. *Biochem. Soc. Trans.* 19: 755- 9, 1991.

DICKER, A. J.; NISBET, A. J. & SKUCE, P. J. Gene expression changes in a P-glycoprotein (Tci-pgp-9) putatively associated with ivermectin resistance in *Teladorsagia circumcincta*. *Int J Parasitol.* 41: 935-42, 2011.

DOMINGUES, L. N.; BRASIL, B. S. A. F.; BELLO, A. C. P. P; CUNHA, A. P.; BARROS, A. T. M.; LEITE, R. C.; SILAGHI, C.; PFISTER, K. & PASSOS, L. M. F. Survey of pyrethroid and organophosphate resistance in Brazilian field populations of *Rhipicephalus (Boophilus) microplus*: Detection of C190A mutation in domain II of the para-type sodium channel gene. *Vet. Parasitol*., (no prelo), 2012.

DOS SANTOS, T. R.; PAPPEN, F. G.; DA ROSA FARIAS, N. A. & DA SILVA VAZ JUNIOR, I. In vitro analysis of amitraz efficacy against *Rhipicephalus (Boophilus) microplus* (Canestrini, 1887) populations of southern region of Rio Grande do Sul state. *Rev. Bras. Parasitol. Vet.* 18: 54-7, 2009.

DRUMMOND, R. O.; ERNST, S. E.; TREVINO, J. L.; GLADNEY, W. J. & GRAHAM, O. H. *Boophilus annulatus* and *B. microplus*: laboratory tests of insecticides. *J. Econ. Entomol.* 66: 130- 133, 1973.

EDMONDS, M. D.; JOHNSON, E. G. & EDMONDS, J. D. Anthelmintic resistance of *Ostertagia ostertagi* and *Cooperia oncophora* to macrocyclic lactones in cattle from the western United States. *Vet Parasitol*. 170: 224- 9, 2010.

ENAYATI, A. A.; RANSON, H. & HEMINGWAY, J. Insect glutathione transferases and insecticide resistance. *Insect Mol. Biol*. 14: 3-8, 2005.

ESTEVES, E.; LARA, F. A.; LORENZINI, D. M.; COSTA, G.H.; FUKUZAWA, A. H.; PRESSINOTTI, L. N.; SILVA, J. R.; FERRO, J. A.; KURTTI, T. J.; MUNDERLOH, U. G. & DAFFRE, S. Cellular and molecular characterization of an embryonic cell line (BME26) from the tick *Rhipicephalus (Boophilus) microplus*. *Insect Biochem. Mol. Biol.* 38: 568- 580, 2008.

ESTEVES, E.; BASTOS, C. V.; ZIVKOVIC, Z.; DE LA FUENTE, J.; KOCAN, K.; BLOUIN, E.; RIBEIRO, M. F.; PASSOS, L. M. & DAFFRE, S. Propagation of a Brazilian isolate of *Anaplasma marginale* with appendage in a tick cell line (BME26) derived from *Rhipicephalus (Boophilus) microplus*. *Vet. Parasitol.* 161: 150-3, 2009.

ESTRADA-PEÑA, A.; BOUATTOUR, A.; CAMICAS, J. L.; GUGLIELMONE, A.; HORAK, I.; JONGEJAN, F.; LATIF, A.; PEGRAM, R. & WALKER, A. R. The known distribution and ecological preferences of the tick subgenus *Boophilus* (Acari: Ixodidae) in Africa and Latin America. *Exp. Appl. Acarol.* 38: 219-35, 2006.

FABRES, A.; DE ANDRADE, C.P.; GUIZZO, M.; SORGINE, M.H.; PAIVA-SILVA, G.; MASUDA, A.; DA SILVA VAZ JUNIOR, I. & LOGULLO, C. Effect of GSK-3 activity, enzymatic inhibition and gene silencing by RNAi on tick oviposition and egg hatching. *Parasitology*. 137: 1537- 1546, 2010.

FAO. Resistance Management and Integrated Parasite Control in Ruminants- guidelines, Executive Summary, Food and Agriculture Organization, Animal Production and Health Division, Rome, v.1, modulo 1, p. 25- 66, 2004.

FERNANDEZ-RUVALCABA, M.; PRECIADO-DE-LA TORRE, F.; CRUZ-VAZQUEZ, C. & GARCIA-VAZQUEZ, Z. Anti-tick effects of Melinis minutiflora and Andropogon gayanus grasses on plots experimentally infested with Boophilus microplus larvae. *Exp. Appl. Acarol.* 32: 293-9, 2004.

FERNÁNDEZ-SALAS, A.; RODRÍGUEZ-VIVAS, R. I. & ALONSO-DÍAZ, M. A. First report of a *Rhipicephalus microplus* tick population multi-resistant to acaricides and ivermectin in the Mexican tropics. *Vet. Parasitol.* 183: 338-42, 2012.

FFRENCH-CONSTANT, R. H.; DABORN, P. J. & LE GOFF, G. The genetics and genomics of insecticide resistance. *Trends Genet.* 20: 163-70, 2004.

FOURNIER, D.; KARCH, F.; BRIDE, J. M.; HALL, L. M.; BERGÉ, J. B. & SPIERER, P. *Drosophila melanogaster* acetylcholinesterase gene. Structure, evolution and mutations. *J. Mol. Biol.* 210: 15-22, 1989.

FOX, L. M. Ivermectin: uses and impact 20 years on. *Curr. Opin. Infect. Dis*. 19: 588- 93, 2006.

FREITAS, D.; POHL, P. C. & DA SILVA VAZ, I. JR. Caracterização da resistência para acaricidas no carrapato *Boophilus microplus*. *Acta Sci. Vet.*, 33: 109-117, 2005.

FURLONG, J. Diagnosis of the susceptibility of the cattle tick, *Boophilus microplus*, to acaracides in Minas Gerais state, Brazil. In: Seminário Internacional de Parasitologia Animal, IV, 1999, Puerto Vallarta, México. Proceedings. Puerto Vallarta: CONASAG, p. 41–46, 1999.

GEORGE, J. E. Present and future technologies for tick control. *Ann. N Y Acad. Sci*. 916: 583-8, 2000.

GIGLIOTI, R.; FORIM, M. R.; OLIVEIRA, H. N.; CHAGAS, A. C.; FERREZINI, J.; BRITO, L. G.; FALCOSKI, T. O.; ALBUQUERQUE, L. G. & OLIVEIRA, M. C. In vitro acaricidal activity of neem (Azadirachta indica) seed extracts with known azadirachtin concentrations against *Rhipicephalus microplus*. *Vet. Parasitol.* 181: 309-15, 2011.

GILLET, J. P.; EFFERTH, T. & REMACLE, J. Chemotherapy-induced resistance by ATP-binding cassette transporter genes. *Biochim. Biophys. Acta*. 1775: 237- 262, 2007.

GLENDINNING, S. K.; BUCKINGHAM, S. D.; SATTELLE, D. B.; WONNACOTT, S. & WOLSTENHOLME, A. J. Glutamate-gated chloride channels of *Haemonchus contortus* restore drug sensitivity to ivermectin resistant *Caenorhabditis elegans*. *PLoS One*. 6: e22390, 2011.

GOLER-BARON, V. & ASSARAF, Y. G. Structure and function of ABCG2-rich extracellular vesicles mediating multidrug resistance. *PLoS One*. 6: e16007, 2011.

GONSIOROSKI, A. V.; BEZERRA, I. A.; UTIUMI, K. U.; DRIEMEIER, D.; FARIAS, S. E.; DA SILVA VAZ JR, I. & MASUDA, A. Anti-tick monoclonal antibody applied by artificial capillary feeding in *Rhipicephalus (Boophilus) microplus* females. *Exp. Parasitol.* 130: 359-63, 2012.

GONZALES, J.C. O controle do carrapato do boi. 2ed. Porto Alegre: Edição do autor, 1995.

GRAÇA-SOUZA, A.V.; MAYA-MONTEIRO, C.; PAIVA-SILVA, G. O.; BRAZ, G. R., PAES, M.C.; SORGINE, M.H.; OLIVEIRA, M. F. & OLIVEIRA P. L. Adaptations against heme toxicity in blood-feeding arthropods. *Insect Biochem. Mol. Biol.* 36: 322-335, 2006.

GRAF, J. F.; GOGOLEWSKI, R.; LEACH-BING, N.; SABATINI, G. A.; MOLENTO, M. B.; BORDIN, E. L. & ARANTES, G. J. Tick control: an industry point of view. *Parasitology*. 129, S427- 42, 2004.

GRISI, L.; MASSARD, C. L.; MOYA-BORJA, G. E. & PEREIRA, J. B. Impacto econômico das principais ectoparasitoses em bovinos no Brasil. *A Hora Veterinária*, 21:8-10, 2002.

GROS, P.; CROOP, J. & HOUSMAN, D. Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. *Cell*. 47: 371-80, 1986.

GUERRERO, F. D.; DAVEY, R. B. & MILLER, R. J. Use of an allele-specific polymerase chain reaction assay to genotype pyrethroid resistant strains of *Boophilus microplus* (Acari: Ixodidae). *J. Med. Entomol.* 38: 44–50, 2001.

GUERRERO, F. D.; LOVIS, L. & MARTINS, J. R. Acaricide resistance mechanisms in *Rhipicephalus (Boophilus) microplus*. Rev Bras Parasitol Vet. 21: 1- 6, 2012a.

GUERRERO, F. D.; MILLER, R. J. & PÉREZ DE LEÓN, A. A. Cattle tick vaccines: Many candidate antigens, but will a commercially viable product emerge? *Int. J. Parasitol.* 42: 421-7, 2012b.

HANRAHAN, J. W. & WIOLAND, M. A. Revisiting cystic fibrosis transmembrane conductance regulator structure and function. *Proc Am Thorac Soc*. 1: 17-21, 2004.

HE, H.; CHEN, A. C.; DAVEY, R. B.; IVIE, G. W. & GEORGE, J. E. Identification of a point mutation in the para-type sodium channel gene from a pyrethroid-resistant cattle tick. *Biochem Biophys Res Commun*. 261: 558-61, 1999.

HGNC, HUGO Gene Nomenclature Committee. ATP binding-cassette transporters. [online] Disponível em http://www.genenames.org/genefamilies/ABC. Arquivo acessado em junho de 2012.

HIGGINS, C. F.; HILES, I. D.; SALMOND, G. P.; GILL, D. R.; DOWNIE, J. A.; EVANS, I. J.; HOLLAND, I. B.; GRAY, L.; BUCKEL, S. D.; BELL. A. W. & HERMODSON, M. A. A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria. *Nature*. 323: 448-50, 1986.

HIGGINS, C. F. ABC transporters: physiology, structure and mechanism-an overview. *Res. Microbiol*.152: 205-10, 2001.

HIGGINS, C. F. & LINTON, K. J. ABC transporters: an introduction and overview. In: HOLLAND, I. B., COLE, S. P. C., KUCHLER, K. & HIGGINS, C. F. ABC proteins: from bacteria toman. Academic Press Elsevier Science, London, v. 1, p.xvii- xxiii, 2003.

HIGGINS, C. F. Multiple molecular mechanisms for multidrug resistance transporters. *Nature*. 446: 749-57, 2007.

HOLLAND, I. B. & BLIGHT, M. A. ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. *J. Mol. Biol.* 293: 381- 399, 1999.

HOLMES, A. R.; LIN, Y. H.; NIIMI, K.; LAMPING, E.; KENIYA, M.; NIIMI, M.; TANABE, K.; MONK, B. C. & CANNON, R. D. ABC transporter Cdr1p contributes more than Cdr2p does to fluconazole efflux in fluconazole-resistant *Candida albicans* clinical isolates. *Antimicrob Agents Chemother*. 52: 3851- 62, 2008.

HONJO, Y.; HRYCYNA, C. A.,; YAN, Q. W.; MEDINA-PÉREZ, W. Y.; ROBEY, R. W.; VAN DE LAAR, A.; LITMAN, T.; DEAN, M. & BATES, S. E. Acquired mutations in the MXR/BCRP/ABCP gene alter substrate specificity in MXR/BCRP/ABCP-overexpressing cells. *Cancer Res*. 61: 6635- 9, 2001.

HUANG, Y. J. & PRICHARD, R. K. Identification and stage-specific expression of two putative P-glycoprotein coding genes in *Onchocerca volvulus*. *Mol. Biochem. Parasitol*. 102: 273-81, 1999.

HYDE, S. C.; EMSLEY, P.; HARTSHORN, M. J.; MIMMACK, M. M.; GILEADI, U.; PEARCE, S. R., GALLAGHER, M. P.; GILL, D. R.; HUBBARD, R. E. & HIGGINS, C. F. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature*. 346: 362- 5, 1990.

IBGE, Instituto Brasileiro de Geografia e Estatística. Produção Pecuária Municipal-2010, Rio de Janeiro, v. 38, p. 1-65, 2010.

ISHIKAWA, T. The ATP-dependent glutathione S-conjugate export pump. Trends Biochem. Sci. 17: 463-468, 1992.

JAMES, C. E. & DAVEY, M. W. Increased expression of ABC transport proteins is associated with ivermectin resistance in the model nematode *Caenorhabditis elegans*. *Int. J. Parasitol*. 39: 213- 220, 2009.

JONSSON, N. N. 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, 2006.

JONSSON, N. N.; BOCK, R. E. & JORGENSEN, W. K. Productivity and health effects of anaplasmosis and babesiosis on *Bos indicus* cattle and their crosses, and the effects of differing intensity of tick control in Australia. *Vet. Parasitol*. 155, 1- 9, 2008.

JONSSON, N. N.; CUTULLÈ, C.; CORLEY, S. W. & SEDDON, J. M. Identification of a mutation in the para-sodium channel gene of the cattle tick *Rhipicephalus microplus* associated with resistance to flumethrin but not to cypermethrin. *Int J Parasitol*. 40: 1659-64, 2010.

JOVELET, C.; BÉNARD, J.; FORESTIER, F.; FARINOTTI, R.; BIDART, J. M & GIL, S. Inhibition of P-glycoprotein functionality by vandetanib may reverse cancer cell resistance to doxorubicin. *Eur J Pharm Sci*. 46: 484- 491, 2012.

KAJIJI, S.; TALBOT, F.; GRIZZUTI, K.; VAN DYKE-PHILLIPS, V.; AGRESTI, M.; SAFA, A.R. & GROS, P. Functional analysis of P-glycoprotein mutants identifies predicted transmembrane domain 11 as a putative drug binding site. *Biochemistry* 32, 4185–4194, 1993.

KAPLAN, R.M. Drug resistance in nematodes of veterinary importance: a status report. *Trends Parasitol.* 20: 477-81, 2004.

KERBOEUF, D.; BLACKHALL, W.; KAMINSKY, R. & VON SAMSON-HIMMELSTJERNA, G. P-glycoprotein in helminths: function and perspectives for anthelmintic treatment and reversal of resistance. *Int. J. Antimicrob..Agents*. 22: 332-46, 2003.

KLAFKE, G. M.; SABATINI, G. A.; DE ALBUQUERQUE, T. A.; MARTINS, J. R.; KEMP, D. H.; MILLER, R. J. & SCHUMAKER, T. T. Larval immersion tests with ivermectin in populations of the cattle tick *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) from State of São Paulo, Brazil. *Vet. Parasitol*. 142: 386- 390, 2006.

KLAFKE, G. M. Resistência de *R. (B.) microplus* contra carrapaticidas. In. PEREIRA, M. C.; LABRUNA, M. B.; SZABÓ, M. P. J & KLAFKE, G. M. *Rhipicephalus (Boophilus) microplus:* Biologia, Controle e Resistência. MedVet, São Paulo, v.1, cap.6, 81- 105, 2008.

KOENDERINK, J. B.; KAVISHE, R. A.; RIJPMA, S. R. & RUSSEL, F. G. The ABCs of multidrug resistance in malaria. *Trends Parasitol*. 26: 440-6, 2010.

KREWULAK, K. D. & VOGEL, H. J. Structural biology of bacterial iron uptake. *Biochim. Biophys. Acta.* 1778: 1781-804, 2008.

KRISHNAMURTHY, P.; XIE, T. & SCHUETZ, J. D. The role of transporters in cellular heme and porphyrin homeostasis. *Pharmacol. Ther*. 114: 345- 58, 2007.

KRISHNAMURTHY, P. & SCHUETZ, J. D. The role of ABCG2 and ABCB6 in porphyrin metabolism and cell survival. *Curr. Pharm. Biotechnol*. 12: 647- 55, 2011.

KRÖBER, T. & GUERIN, P. M. In vitro feeding assays for hard ticks. *Trends Parasitol*. 23: 445-9, 2007a.

KRÖBER, T. & GUERIN, P. M. An in vitro feeding assay to test acaricides for control of hard ticks. *Pest. Manag. Sci*. 63: 17-22, 2007b.

KUNZ, S. E. & KEMP, D. H. Insecticides and acaricides: Resistance and environmental impact. *Rev. Sci. Tech*., 13: 1249–1286, 1994.

KWON, D. H.; YOON, K. S.; CLARK, J. M. & LEE, S. H. A point mutation in a glutamate-gated chloride channel confers abamectin resistance in the two-spotted spider mite, *Tetranychus urticae* Koch. *Insect. Mol. Biol*. 19: 583- 591, 2010.

LABRUNA, M. B. Combate contra *R. (B.) microplus*. In. PEREIRA, M. C.; LABRUNA, M. B.; SZABÓ, M. P. J & KLAFKE, G. M. *Rhipicephalus (Boophilus) microplus:* Biologia, Controle e Resistência. MedVet, São Paulo, v.1, cap.5, 65- 78, 2008.

LAGE, H. ABC-transporters: implications on drug resistance from microorganisms to human cancers. *Int. J. Antimicrob. Agents*. 22: 188-99, 2003.

LANNING, C.L.; FINE, R.L.; SACHS, C. W.; RAO, U.S; CORCORAN, J.J. & ABOU-DONIA, M.B. Chlorpyrifos oxon interacts with the mammalian multidrug resistance protein, P-glycoprotein. *J. Toxicol. Environ. Health*. 47: 395-407, 1996.

LARA, F. A.; LINS, U.; BECHARA, G. & OLIVEIRA, P.L. Tracing heme in a living cell: hemoglobin degradation and heme traffic in digest cells of the cattle tick *Boophilus microplus*. *J Exp. Biol*. 208: 3093-3101, 2005.

LEAL, A. T.; SEIXAS, A.; POHL, P. C.; FERREIRA, C. A., LOGULLO, C.; OLIVEIRA, P. L., FARIAS, S. E.; TERMIGNONI, C.; DA SILVA VAZ, I. JR & MASUDA, A. Vaccination of bovines with recombinant *Boophilus microplus* Yolk pro-Cathepsin. *Vet. Immunol. Immunopathol*. 114: 341-345, 2006.

LEEMON, D. M. & JONSSON, N. N. Comparison of bioassay responses to the potential fungal biopesticide Metarhizium anisopliae in *Rhipicephalus(Boophilus) microplus* and *Lucilia cuprina.Vet. Parasitol*. 185: 236- 47, 2012.

LESLIE, E. M.; DEELEY, R. G. & COLE, S. P. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicol. Appl. Pharmacol*. 204: 216- 237, 2005.

LESPINE, A.; DUPUY, J.; ORLOWSKI, S.; NAGY, T.; GLAVINAS, H.; KRAJCSI, P. & ALVINERIE, M. Interaction of ivermectin with multidrug resistance proteins (MRP1, 2 and 3). *Chem. Biol. Interact*. 159: 169-79, 2006.

LESPINE, A.; ALVINERIE, M.; VERCRUYSSE, J.; PRICHARD, R.K. & GELDHOF, P. ABC transporter modulation: a strategy to enhance the activity of macrocyclic lactone anthelmintics. *Trends Parasitol*. 24: 293-8, 2008.

LI, A. Y.; DAVEY, R. B; MILLER, R. J. & GEORGE, J. E. Resistance to coumaphos and diazinon in *Boophilus microplus* (Acari: Ixodidae) and evidence for the involvement of an oxidative detoxification mechanism. *J. Med. Entomol*. 40: 482- 90, 2003.

LI, A. Y.; DAVEY, R. B.; MILLER, R. J. & GEORGE, J. E. Detection and characterization of amitraz resistance in the southern cattle tick, *Boophilus microplus* (Acari: Ixodidae). *J. Med. Entomol.* 41: 193- 200, 2004.

LI, X.; SCHULER, M. A. & BERENBAUM, M. R. Molecular mechanisms of metabolic Resistance to synthetic and natural xenobiotics. Annu Rev Entomol 52: 231-53, 2007.

LIFSCHITZ, A.; ENTROCASSO, C.; ALVAREZ, L.; LLOBERAS, M.; BALLENT, M.; MANAZZA, G.; VIRKEL, G.; BORDA, B. & LANUSSE, C. Interference with P-glycoprotein improves ivermectin activity against adult resistant nematodes in sheep. *Vet. Parasitol.* 172: 291-8, 2010a.

LIFSCHITZ, A.; SUAREZ, V. H.; SALLOVITZ, J.; CRISTEL, S. L.; IMPERIALE. F.; AHOUSSOU, S.; SCHIAVI, C. & LANUSSE, C. Cattle nematodes resistant to macrocyclic lactones: comparative effects of P-glycoprotein modulation on the efficacy and disposition kinetics of ivermectin and moxidectin. *Exp Parasitol.* 125: 172-8, 2010b.

LINTON, K. J. & HIGGINS, C. F. The *Escherichia coli* ATP-binding cassette (ABC) proteins. *Mol. Microbiol*. 28: 5-13, 1998.

LINTON, K. J. Structure and function of ABC transporters. *Physiology (Bethesda).* 22: 122- 30, 2007.

LIYOU, N.; HAMILTON, S.; WATTERS, D. J.; TELLAM, R. & WILLADSEN, P. Endocytosis by digest cells of the cattle tick *Boophilus microplus*: regulation by protein Kinase C. *Insect Biochem. Mol. Biol*., 26: 147-154, 1996.

LYNCH, J.; FUKUDA, Y.; KRISHNAMURTHY, P.; DU, G. & SCHUETZ, J. D. Cell survival under stress is enhanced by a mitochondrial ATP-binding cassette transporter that regulates hemoproteins. *Cancer Res*. 69: 5560- 7, 2009.

MACALUSO, K. R.; SONENSHINE, D. E.; CERAUL, S. M. & AZAD, A. F. Infection and transovarial transmission of rickettsiae in *Dermacentor variabilis* ticks acquired by artificial feeding. *Vector Borne Zoonotic Dis*. 1: 45-53, 2001.

MARITZ-OLIVIER, C.; VAN ZYL, W. & STUTZER, C. A systematic, functional genomics, and reverse vaccinology approach to the identification of vaccine candidates in the cattle tick, *Rhipicephalus microplus. Ticks Tick Borne Dis*, (no prelo), 2012.

MARTIN, C.; HIGGINS, C. F. & CALLAGHAN, R. The vinblastine binding site adopts high- and low-affinity conformations during a transport cycle of P-glycoprotein. *Biochemistry*. 40: 15733- 42, 2001.

MARTINS, J.R. & FURLONG, J. Avermectin resistance of the cattle tick *Boophilus microplus* in Brazil. *Vet. Rec*. 149: 64, 2001.

MENDES, M.C.; LIMA, C.K.; NOGUEIRA, A.H.; YOSHIHARA, E.; CHIEBAO, D.P.; GABRIEL, F.H.; UENO, T.E.; NAMINDOME, A. & KLAFKE, G.M. Resistance to cypermethrin, deltamethrin and chlorpyriphos in populations of *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) from small farms of the State of São Paulo, Brazil. *Vet. Parasitol.* 178: 383-8, 2011.

MÉNEZ, C.; MSELLI-LAKHAL, L.; FOUCAUD-VIGNAULT, M.; BALAGUER, P.; ALVINERIE, M. & LESPINE A. Ivermectin induces P-glycoprotein expression and function through mRNA stabilization in murine hepatocyte cell line. *Biochem. Pharmacol.* 83: 269-78, 2012.

MILLER, R. J.; DAVEY, R. B. & GEORGE, J. E. Characterization of pyrethroid resistance and susceptibility to coumaphos in Mexican *Boophilus microplus* (Acari: Ixodidae). *J. Med. Entomol.* 36: 533-8, 1999.

MILLER, R. J.; LI, A. Y.; TIJERINA, M.; DAVEY, R. B & GEORGE, J. E. Differential response to diazinon and coumaphos in a strain of *Boophilus microplus* (Acari: Ixodidae) collected in Mexico. *J. Med. Entomol*. 45: 905-11, 2008.

MORGAN, J. A.; CORLEY, S. W.; JACKSON, L. A.; LEW-TABOR, A. E.; MOOLHUIJZEN, P. M. & JONSSON, N. N. Identification of a mutation in the para-sodium channel gene of the cattle tick *Rhipicephalus (Boophilus) microplus* associated with resistance to synthetic pyrethroid acaricides. *Int J Parasitol*. 39: 775-9, 2009.

MORITA, M.; SHIMOZAWA, N.; KASHIWAYAMA, Y.; SUZUKI, Y. & IMANAKA, T. ABC subfamily D proteins and very long chain fatty acid metabolism as novel targets in adrenoleukodystrophy. *Curr. Drug Targets*. 12: 694-706, 2011.

MOUNSEY, K. E.; PASAY, C. J.; ARLIAN, L. G.; MORGAN, M. S.; HOLT, D. C.; CURRIE, B. J.; WALTON, S. F. & MCCARTHY, J. S. Increased transcription of Glutathione S-transferases in acaricide exposed scabies mites. *Parasit Vectors*. 18: 3-43, 2010.

MURRELL, A. & BARKER, S. C. Synonymy of *Boophilus* Curtice, 1891 with *Rhipicephalus* Koch, 1844 (Acari: Ixodidae). *Syst. Parasitol*. 56: 169-72, 2003.

NARI, A. Strategies for control of one-host ticks and relationship with tick-borne diseases in South America. *Vet. Parasitol*. 57: 153-165, 1995.

NATARAJAN, K.; XIE, Y.; BAER, M. R. & ROSS, D. D. Role of breast cancer resistance protein (BCRP/ABCG2) in cancer drug resistance. *Biochem. Pharmacol*. 83: 1084-103, 2012.

NIIMI, M.; NIIMI, K.; TAKANO, Y.; HOLMES, A. R.; FISCHER, F. J.; UEHARA, Y. & CANNON, R. D. Regulated overexpression of CDR1 in *Candida albicans* confers multidrug resistance. *J. Antimicrob. Chemother*. 54: 999-1006, 2004.

NOLAN, J.; ROULSTON, W. J. & WHARTON, R. H. Resistance to synthetic pyrethroids in a DDT-resistant strain of Boophilus microplus. Pestic. Sci. 8: 484- 486, 1977.

OMURA, S. Ivermectin: 25 years and still going strong. *Int. J. Antimicrob. Agents*. 31: 91-8, 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 em México. In: Memorias de IV Seminario Internacional de Parasitología Animal, Resistencia y Control en Garrapatas y Moscas de Importancia Veterinaria, Acapulco, Guerrero, México, pp. 58–66, 1995.

OSWALD, C.; HOLLAND, I. B. & SCHMITT, L. The motor domains of ABC-transporters: What can structures tell us? *Naunyn-Schmiedebergs Arch Pharmacol*, 372: 385-399, 2006.

PARIZI, L.F.; UTIUMI, K.U.; IMAMURA, S.; ONUMA, M.; OHASHI, K.; MASUDA, A. & DA SILVA VAZ, I. JR. Cross immunity with *Haemaphysalis longicornis* glutathione S-transferase reduces an experimental *Rhipicephalus (Boophilus) microplus* infestation. *Exp. Parasitol*. 127, 113-8, 2011.

PECONICK, A. P.; SOSSAI, S.; GIRÃO, F. A.; RODRIGUES, M. Q.; SOUZA E SILVA, C. H.; GUZMAN, Q. F.; PATARROYO, V. A. M.; VARGAS, M. I. & PATARROYO, J. H. Synthetic vaccine (SBm7462) against the cattle tick *Rhipicephalus (Boophilus) microplus*: Preservation of immunogenic determinants in different strains from South America. *Exp. Parasitol.*, 119: 37-43, 2008.

PEREIRA, M. A.; JACINTO, M. A. C.; GOMES, A. & EVARISTO, L. G. S. Cadeia Produtiva do Couro Bovino: Oportunidades e Desafios. Embrapa Gado de Corte, Mato Grosso do Sul, n. 153, 2005. [online] Disponível em: http://www.cnpgc.embrapa.br/publicacoes/doc/doc_pdf/Doc153.pdf. Arquivo capturado em julho de 2012.

PEREIRA, M. C. & LABRUNA, M. B. *Rhipicephalus (Boophilus) microplus*. In. PEREIRA, M. C.; LABRUNA, M. B.; SZABÓ, M. P. J & KLAFKE, G. M. *Rhipicephalus (Boophilus) microplus:* Biologia, Controle e Resistência. MedVet, São Paulo, v.1, cap.3, 15- 53, 2008.

PEREZ-COGOLLO, L.C.; RODRIGUEZ-VIVAS, R.I.; RAMIREZ-CRUZ, G.T. & MILLER, R.J. First report of the cattle tick *Rhipicephalus microplus* resistant to ivermectin in Mexico. *Vet. Parasitol*. 168: 165- 169, 2010.

PERINOTTO, W. M.; ANGELO, I. C.; GOLO, P. S.; QUINELATO, S.; CAMARGO, M. G.; SÁ, F. A. & BITTENCOURT, V. R. Susceptibility of different populations of ticks to entomopathogenic fungi. *Exp. Parasitol*. 130: 257-60, 2012.

PERRY, T.; BATTERHAM, P. & DABORN, P. J. The biology of insecticidal activity and resistance. Insect Bioch. *Mol. Biol*. 41: 411- 422, 2011.

PIPER, E. K.; JACKSON, L. A.; BIELEFELDT-OHMANN, H.; GONDRO, C.; LEW-TABOR, A. E. & JONSSON, N. N. Tick-susceptible *Bos taurus* cattle display an increased cellular response at the site of larval *Rhipicephalus (Boophilus) microplus* attachment, compared with tick-resistant *Bos indicus* cattle. *Int. J. Parasitol*. 40: 431-41, 2010.

PODSIADLOWSKI, L.; MATHA, V. & VILCINSKAS, A. Detection of a P-glycoprotein related pump in *Chironomus* larvae and its inhibition by verapamil and cyclosporin A. *Comp Biochem Physiol B Biochem Mol Biol.* 121: 443-50, 1998.

POELARENDS, G. J.; MAZURKIEWICZ, P. & KONINGS, W. N. Multidrug transporters and antibiotic resistance in Lactococcus lactis. *Biochim Biophys Acta*. 1555: 1-7, 2002.

POHL, P. C.; KLAFKE, G. M.; CARVALHO, D. D.;, MARTINS, J. R.; DAFFRE, S.; DA SILVA VAZ JR., I. & MASUDA, A. ABC transporter efflux pumps: A defense

mechanism against ivermectin in *Rhipicephalus (Boophilus) microplus*. *Int. J. Parasitol*. 41: 1323- 1333, 2011.

PONTE-SUCRE, A. Availability and applications of ATP-binding cassette (ABC) transporter blockers. *Appl. Microbiol. Biotechnol*. 76: 279-86, 2007.

PORRETTA, D.; GARGANI, M.; BELLINI, R.; MEDICI, A.; PUNELLI, F. & URBANELLI, S. Defence mechanisms against insecticides temephos and diflubenzuron in the mosquito *Aedes caspius*: the P-glycoprotein efflux pumps. *Med. Vet. Entomol*., 22: 48-54, 2008.

PRUETT, J. H. Comparative inhibition kinetics for acetylcholinesterases extracted from organophosphate resistant and susceptible strains of *Boophilus microplus* (Acari: Ixodidae). *J. Econ. Entomol*. 95: 1239-44, 2002.

PRUETT, J. H. & POUND, J. M. Biochemical diagnosis of organophosphate-insensitivity with neural acetylcholinesterase extracted by sonication from the adult tick synganglion. *Vet. Parasitol.* 135: 355-63, 2006.

RANGEL, C. P.; DA CUNHA, N. C.; DE REZENDE, J.; DA SILVA, F. J.; CORRÊA, F. N.; TEIXEIRA, R. C.; DA SILVA, J. B.; BAÊTA, B. A. & DA FONSECA, A. H. Alimentaçao artificial por meio de tubos capilares de fêmeas parcialmente ingurgitadas do carrapato *Dermacentor (Anocentor) nitens*. *Ver. Bras. Parasitol. Vet.* 17: 35- 9, 2008.

REGITANO, L. C.; IBELLI, A. M.; GASPARIN, G.; MIYATA, M.; AZEVEDO, A. L.; COUTINHO, L. L.; TEODORO, R. L.; MACHADO, M. A.; SILVA, M. V.; NAKATA, L. C.; ZAROS, L. G.; SONSTEGARD, T. S.; SILVA, A. M.; ALENCAR, M. M. & OLIVEIRA, M. C. On the search for markers of tick resistance in bovines. *Dev. Biol. (Basel)*. 132: 225-30, 2008.

RIBEIRO, V. L.; DOS SANTOS, J. C.; MARTINS, J. R.; SCHRIPSEMA, J.; SIQUEIRA, I. R.; VON POSER, G. L. & APEL, M. A. Acaricidal properties of the essential oil and precocene II obtained from Calea serrata (Asteraceae) on the cattle tick *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae). *Vet. Parasitol*. 179: 195-8, 2011.

ROBERTS, J.A. Resistance of cattle to the tick *Boophilus microplus* (Canestrini). II. Stages of the cycle of the parasite against which resistance is manifest. *J. Parasitol*. 54: 667-673, 1968.

RODRÍGUEZ, M.; PENICHET, M. L.; MOURIS, A. E.; LABARTA, V.; LUACES, L. L.; RUBIERA, R.; CORDOVÉS, 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. *Vet. Parasitol*. 57: 339- 49, 1995.

RODRIGUEZ-VIVAS, R. I.; TREES, A. J.; ROSADO-AGUILAR, J. A.; VILLEGAS-PEREZ, S. L. & HODGKINSON, J. E. Evolution of acaricide resistance: phenotypic and genotypic changes in field populations of *Rhipicephalus (Boophilus) microplus* in response to pyrethroid selection pressure. *Int. J. Parasitol*. 41: 895- 903, 2011.

ROSARIO-CRUZ, R.; ALMAZAN, C.; MILLER, R. J.; DOMINGUEZ-GARCIA, D. I.; HERNANDEZ-ORTIZ, R. & DE LA FUENTE, J. Genetic basis and impact of tick acaricide resistance. *Front. Biosci*. 14: 2657-65, 2009a.

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*. *Parasitol Res.* 105: 1145-53, 2009b.

ROUSH, R. T. & MCKENZIE, J. A. Ecological genetics of insecticide and acaricide resistance. *Annu. Rev. Entomol.* 32: 361-380, 1987.

SABATINI, G. A.; KEMP, D. H.; HUGHES, S.; NARI, A. & HANSEN, J. Tests to determine LC50 and discriminating doses for macrocyclic lactones against the cattle tick, *Boophilus microplus*. *Vet. Parasitol*. 95: 53- 62, 2001.

SANGLARD, D.; KUCHLER, K.; ISCHER, F.; PAGANI, J. L.; MONOD, M. & BILLE, J. Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. *Antimicrob. Agents Chemother*. 39: 2378- 86, 1995.

SCHINKEL, A. H.; SMIT, J. J.; VAN TELLINGEN, O.; BEIJNEN, J. H.; WAGENAAR, E.; VAN DEEMTER, L.; MOL, C. A.; VAN DER VALK, M. A.; ROBANUS-MAANDAG, E. C.; TE RIELE, H. P. J.; BERNS, A. J. M. & BORST, P. Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell*. 77: 491-502, 1994.

SCHMITT, L. & TAMPÉ, R. Structure and mechanism of ABC transporters. *Curr Opin Struct Biol*. 12: 754-60, 2002.

SCHNEIDER, E. & HUNKE, S. ATP-binding-cassette (ABC) transport systems: functional and structural aspects of the ATP-hydrolyzing subunits/domains. *FEMS Microbiol. Rev*. 22, 1-20, 1998.

SCOTT, J. G. & WEN, Z. Cytochromes P450 of insects: the tip of the iceberg. *Pest Manag. Sci.* 57: 958-67, 2001.

SEELIG, A. A general pattern for substrate recognition by P-glycoprotein. *Eur J Biochem*. 251: 252- 61, 1998.

SEELIG, A. & LANDWOJTOWICZ, E. Structure–activity relationship of P- glycoprotein substrates and modifiers. *Eur. J. Pharm. Sci*. 12: 31–40, 2000.

SHAW, R. D. Culture of na organophosphorus resistant strain of Boophilus microplus (Canestrini) and assessment of its resistance spectrum. *Bull. Entomol. Res.* 56: 398-405, 1966

SOBERANES, C. N.; SANTAMARÍA, V. M.; FRAGOSO, S. H. & GARCÍA, V. Z. Primer caso de resistencia al amitraz en la garrapata del ganado *Boophilus microplus* en México. *Tec. Pec. Mex.* 40: 81- 92, 2002.

SOLL, M. D.; D'ASSONVILLE, J. A. & SMITH, C. J. Efficacy of topically applied ivermectin against sarcoptic mange (*Sarcoptes scabiei* var. *bovis*) of cattle. *Parasitol. Res*. 78: 120-2, 1992.

STONE, B. F. & HAYDOCK, K. P. A method for measuring the acaricide susceptibility of the cattle tick *Boophilus microplus* (Can.). *Bull. Entomol. Res.* 53: 563- 578, 1962.

SUTHERST, R.W.; MAYWALD, G. F.; KERR, J. D. & SIEGEMAN, D. A. The effect of the cattle tick (Boophilus microplus) on the growth of *Boss indicus* x *Bos taurus* steers. *Aust. J. Agr. Res.* 34: 317-327, 1983.

SZAKÁCS, G.; VÁRADI, A.; OZVEGY-LACZKA, C. & SARKADI, B. The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME-Tox). *Drug Discov. Today*. 13: 379- 393, 2008.

TAKIGUCHI, Y.; MISHIMA, H.; OKUDA, M.; TERAO, M.; AOKI, A. & FUKUDA, R. Milbemycins, a new family of macrolide antibiotics: fermentation, isolation and physico-chemical properties. *J. Antibiot. (Tokyo).* 33: 1120- 7, 1980.

TEMEYER, K. B.; OLAFSON, P. U. & MILLER, R. J. Genotyping mutations in BmAChE3: A survey of organophosphate-resistant and -susceptible strains of *Rhipicephalus (Boophilus) microplus*. *J. Med. Entomol.* 46: 1355- 60, 2009.

TEMEYER, K. B.; OLAFSON, P. U. & PRUETT, J. H. Sequence polymorphism in acetylcholinesterase transcripts and genotyping survey of BmAchE1 in laboratory and Mexican strains of *Rhipicephalus (Boophilus) microplus*. *J. Med. Entomol.* 49: 555-62, 2012.

TOWNSON, S.; TAGBOTO, S. K.; CASTRO, J.; LUJAN, A.; AWADZI, K. & TITANJI, V. P. Comparison of the sensitivity of different geographical races of *Onchocerca volvulus* microfilariae to ivermectin: studies *in vitro*. *Trans. R. Soc. Trop. Med. Hyg.* 88: 101-6, 1994.

TRIBBLE, N. D.; BURKA, J. F. & KIBENGE, F. S. Evidence for changes in the transcription levels of two putative P-glycoprotein genes in sea lice (*Lepeophtheirus salmonis*) in response to emamectin benzoate exposure. *Mol Biochem Parasitol*. 153: 59-65, 2007.

VALLE, M. R.; MÈNDEZ, L.; VALDEZ, M.; REDONDO, M.; ESPINOSA, C. M.; VARGAS, M.; CRUZ, R. 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. *Exp Appl Acarol.* 34: 375-82, 2004.

VARMA, M. G.; PUDNEY, M. & LEAKE, C. J. The establishment of three cell lines from the tick Rhipicephalus appendiculatus (Acari: Ixodidae) and their infection with some arboviruses. *J. Med. Entomol.* 11: 698–706, 1975.

VILLARINO, M. A.; WAGHELA, S. D. & WAGNER, G. G. Biochemical detection of esterases in the adult female integument of organophosphate-resistant *Boophilus microplus* (Acari: Ixodidae). *J. Med. Entomol.* 40: 52-7, 2003.

WALADDE, S. M.; YOUNG, A. S.; OCHIENG, S. A.; MWAURA, S. N. & MWAKIMA, F. N. Transmission of *Theileria parva* to cattle by *Rhipicephalus appendiculatus* adults fed as nymphae in vitro on infected blood through an artificial membrane. *Parasitology*. 107: 249-56, 1993.

WANDERSMAN, C. & DELEPELAIRE, P. Bacterial iron sources: from siderophores to hemophores. *Annu. Rev. Microbiol*., V. 58: 611–647, 2004.

WEILL, M.; FORT, P.; BERTHOMIEU, A.; DUBOIS, M. P.; PASTEUR, N. & RAYMOND, M. A novel acetylcholinesterase gene in mosquitoes codes for the insecticide target and is non-homologous to the ace gene in *Drosophila*. *Proc Biol Sci*. 269: 2007-16, 2002.

WILLADSEN, P.; RIDING, G. A.; MCKENNA, R. V.; KEMP, D. H.; TELLAM, R. L.; NIELSEN, J. N.; LAHNSTEIN, J.; COBON, G. S. & GOUGH, J. M. Immunologic control of a parasitic arthropod: Identification of a protective antigen from *Boophilus microplus*. *J. Immunol.*, 143:1346-1351, 1989.

WILLADSEN, P.; BIRD, P.; COBON, G. S. & HUNGERFORD J. Commercialisation of a recombinant vaccine against *Boophilus microplus*. *Parasitology*. 110: S43-50, 1995.

WILLADSEN, P.; COBON, G. & MCKENNA, R. V. Comparative vaccination of cattle against Boophilus microplus with recombinant antigen Bm86 or in combination with recombinant Bm91. *Parasite Immunol*., 18: 214-246, 1996.

WILLADSEN, P. Anti-tick vaccines. *Parasitology*, 129: S367-S387, 2004.

WILLADSEN, P. Tick control: thoughts on a research agenda. *Vet. Parasitol*., 138: 161-168, 2006.

WILSON, T. G. Resistance of *Drosophila* to toxins. *Annu. Rev. Entomol*. 46: 545-71, 2001.

WRIGHT, F. C. & AHRENS, E. H. Cholinesterase insensitivity: a mechanism of resistance in Mexican strains of *Boophilus microplus* (Acari: Ixodidae) against coumaphos. *J. Med. Entomol.* 25: 234-9, 1988.

WU, C. P.; HSIEH, C. H. . & WU, Y. S. The emergence of drug transporter-mediated multidrug resistance to cancer chemotherapy. *Mol. Pharm*. 8: 1996-2011, 2011.

XU, M.; MOLENTO, M.; BLACKHALL, W.; RIBEIRO, P.; BEECH, R. & PRICHARD, R. Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homolog. *Mol. Biochem. Parasitol*. 91: 327- 335, 1998.

YAN, S.; CUI, F. & QIAO, C. Structure, function and applications of carboxylesterases from insects for insecticide resistance. *Protein Pept. Lett.* 16: 1181-8, 2009.

YANG, A. K.; ZHOU, Z. W.; WEI, M. Q.; LIU, J. P. & ZHOU, S. F. Modulators of multidrug resistance associated proteins in the management of anticancer and antimicrobial drug resistance and the treatment of inflammatory diseases. *Curr. Top. Med. Chem*. 10: 1732-56, 2010.

YAO, N.; TRAKHANOV, S. & QUIOCHO, F. A. Refined 1.89-A structure of the histidine-binding protein complexed with histidine and its relationship with many other active transport/chemosensory proteins. *Biochemistry* 33: 4769–4779, 1994.

YOON, K. S.; STRYCHARZ, J. P.; BAEK, J. H.; SUN, W.; KIM, J. H.; KANG, J. S.; PITTENDRIGH, B. R.; LEE, S. H. & CLARK, J. M. Brief exposures of human body lice to sublethal amounts of ivermectin over-transcribes detoxification genes involved in tolerance. *Insect Mol. Biol*. 20, 687- 699, 2011.

YOUNG, A. S. & MORZARIA, S. P. Biology of Babesia. *Parasit. Today*, 2: 211-219, 1986.

ANEXO I: resultados suplementares

Transcrição do transportador RmABCB10

RNA total foi extraído de intestino, ovário, túbulos de Malpighi, glândula salivar e corpo gorduroso de teleóginas da população POA. Este foi reversamente transcrito e analisado por PCR com primers específicos para o gene *RmABCB10* descritos no artigo I. Os produtos da reação foram analisados por eletroforese confirmando a transcrição do gene *RmABCB10* em intestino, ovário e túbulos de Malpighi (Figura 1).

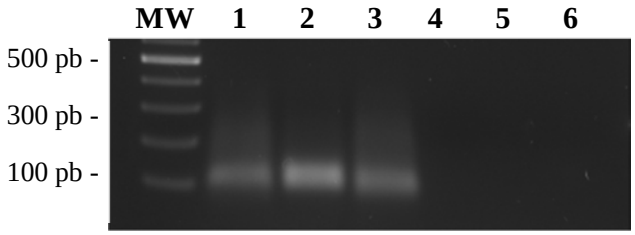


Fig 1- Análise dos produtos de PCR do gene *RmABCB10* por eletroforese em gel de agarose 1,5% . MW- marcador de massa molecular, 1- intestino, 2- ovário, 3- túbulos de Malpighi, 4- glândula salivar, 5- corpo gorduroso, 6- controle negativo (ausência de cDNA na reação).

Silenciamento gênico do transportador *RmABCB10*

Dado o aumento nos níveis de transcrição do gene *RmABCB10* na população JUA resistente a ivermectina (artigo I), foi realizado o silenciamento gênico deste, a fim de validar sua participação na destoxificação da ivermectina nesta população.

Para isto, dsRNA do gene *RmABCB10* e do gene controle (*MSP1*) foram sintetizado. Fêmeas parcialmente ingurgitadas da população JUA foram coletadas de bovinos e divididas em grupos homogêneos de 25 fêmeas (30 a 80 mg cada) que foram injetadas na hemocele com dsRNA *RmABCB10* ou dsRNA controle. Estas foram posteriormente alimentadas artificialmente com sangue ou sangue acrescido de ivermectina (0.02µg/mL) durante 24 horas. RNA total foi extraído de intestino e ovário de seis fêmeas

de cada grupo e reversamente transcrito e as demais fêmeas de cada grupo foram pesadas e incubadas a 28°C e 80% de umidade. O efeito do silenciamento do *RmABCB10* e alimentação com ivermectina sobre o ingurgitamento e reprodução das fêmeas foi avaliado pela determinação da taxa de ingurgitamento (peso final/ peso final), mortalidade (% de fêmeas mortas), taxa de fertilidade (peso dos ovos/ peso final das fêmeas) e viabilidade dos ovos (% de massa de ovos eclodida). O percentual de silenciamento gênico do *RmABCB10* em intestino e ovário foi determinado por PCR quantitativo. O experimento seguiu o mesmo protocolo descrito em matérias e métodos do artigo IV.

Verificamos um percentual de silenciamento para o gene *RmABCB10* de 92% no intestino e 34% nos ovários. Nas fêmeas *RmABCB10*-silenciadas foi observado redução significativa da viabilidade dos ovos quando estas foram alimentadas com sangue acrescido de ivermectina, comparado as fêmeas alimentadas apenas com sangue (Tabela 1). Resultado semelhante foi observado quando as fêmeas desta mesma população foram alimentadas com o inibidor de transportador ABC, CsA (artigo I). Nas fêmeas que receberam o dsRNA controle não houve diferença significativa em nenhum dos parâmetros avaliados (Tabela 1). Estes resultados sugerem a participação do transportador RmABCB10 na resitência a ivermectina nesta população.

Tabela 1- Efeito do silenciamento gênico do *RmABCB10* e alimentação com ivermectina sobre fêmeas de *R. microplus* da população JUA resistente a ivermectina.

	Taxa de ingurgitamento	**Mortalidade (%)**	**Taxa de fertilidade**	**Viabilidade dos ovos (%)**
dsCT + sangue	2.00 (± 0.45)	11.7	0.24 (±0.13)	83.3
dsABC + sangue	2.08 (± 0.54)	14.3	0.28 (±0.14)	93.3
dsCT + IVM	1.72 (± 0.35)	7.7	0.22 (± 0.08)	73.3
dsABC + IVM	1.75 (± 0.46)	0	0.23 (± 0.06)	46.7*

Dados apresentados como média ± S.E. Asterisco representa diferença significativa com p≤ 0,05. IVM- ivermectina, dsCT- dsRNA gene controle, dsABC- dsRNA gene *RmABCB10*

ANEXO II: submissão dos artigos

Carta do editor com a confirmação da submissão e aceite do artigo II

Assunto: **Parasitology Research** - Manuscript ID PR-2012-0621 (SY-05)
Data: Thu, 21 Jun 2012 06:12:41 -0400 (EDT)
De: mehlhorn@uni-duesseldorf.de
Para: itabajara.vaz@ufrgs.br

21-Jun-2012

Dear Dr. da Silva Vaz, Jr:

Your manuscript entitled "**ABC transporters as a multidrug detoxification mechanism in *Rhipicephalus (Boophilus) microplus***" has been successfully submitted online and is presently being given full consideration for publication in the Parasitology Research.

Your manuscript ID is PR-2012-0621.

Please mention the above manuscript ID in all future correspondence or when calling the office for questions. If there are any changes in your street address or e-mail address, please log in to Manuscript Central at http://mc.manuscriptcentral.com/parasite and edit your user information as appropriate.

You can also view the status of your manuscript at any time by checking your Author Center after logging in to http://mc.manuscriptcentral.com/parasite .

Thank you for submitting your manuscript to the Parasitology Research.

Sincerely,
Parasitology Research Editorial Office

Assunto: Parasitology Research - Decision on Manuscript ID PR-2012-0621 (D-SW-03)
Data: Tue, 17 Jul 2012 04:21:28 -0400 (EDT)
De: mehlhorn@uni-duesseldorf.de
Para: itabajara.vaz@ufrgs.br

17-Jul-2012
Dear Dr. da Silva Vaz, Jr:

Manuscript ID PR-2012-0621 entitled "**ABC transporters as a multidrug detoxification mechanism in *Rhipicephalus (Boophilus) microplus***" which you submitted to the Parasitology Research, has been reviewed. The comments of the reviewer(s) are included at the bottom of this letter.

The reviewer(s) have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, I invite you to respond to the reviewer(s)' comments and revise your manuscript.

To revise your manuscript, log into http://mc.manuscriptcentral.com/parasite and enter your Author Center, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision.

You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript using a word processing program and save it on your computer. Please also highlight the changes to your manuscript within the document by using the track changes mode in MS Word or by using bold or colored text.Once the revised manuscript is prepared, you can upload it and submit it through your Author Center.

When submitting your revised manuscript, you will be able to respond to the comments made by the reviewer(s) in the space provided. You can use this space to document any changes you make to the original manuscript. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response to the reviewer(s).

IMPORTANT: Your original files are available to you when you upload your revised manuscript. Please delete any redundant files before completing the submission.

Because we are trying to facilitate timely publication of manuscripts submitted to the Parasitology Research, your revised manuscript should be uploaded as soon as possible. If it is not possible for you to submit your revision in a reasonable amount of time, we may have to consider your paper as a new submission.

Once again, thank you for submitting your manuscript to the Parasitology Research and I look forward to receiving your revision.

Sincerely,
Prof. Heinz Mehlhorn
Managing Editor, Parasitology Research
mehlhorn@uni-duesseldorf.de

Carta do editor com a confirmação da submissão do artigo IV

From: "**PLoS ONE**" <plosone@plos.org>
To: "Pedro Lagerblad Oliveira" <pedro@bioqmed.ufrj.br>
Sent: 25 Jun 2012 09:36:27 -0400
Subject: Submission Confirmation for An ATP Binding Cassette Transporter Mediates both Heme and Pesticide Detoxification in Tick Midgut Cells

Dear Dr. Oliveira,

Your submission entitled "**An ATP Binding Cassette Transporter Mediates both Heme and Pesticide Detoxification in Tick Midgut Cells**" has been received by PLoS ONE. You will be able to check on the progress of your paper by logging on to Editorial Manager as an author. The URL is http://pone.edmgr.com/.

Your manuscript will be given a reference number once an Editor has been assigned.

Thank you for submitting your work to this journal.

Kind regards,

PLoS ONE

ANEXO III: Curriculum Vitae

Paula Cristiane Pohl
Curriculum Vitae

Dados Pessoais

Nome: Paula Cristiane Pohl
Nascimento: 20/11/1982 - Tres de Maio/RS - Brasil
CPF: 98675524072
e-mail: paula@cbiot.ufrgs.br; pcpohl2012@gmail.com

Formação Acadêmica/Titulação

2008: Doutorado em Biologia Celular e Molecular. Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil.
Título: Participação dos transportadores ABC na destoxificação de acaricidas no carrapato *Rhipicephalus (Boophilus) microplus.*
Orientador: Aoi Masuda
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

2007- 2008: Mestrado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Função da THAP na embriogênese do carrapato *Rhipichephalus (Boophilus) microplus,*
Ano de obtenção: 2008
Orientador: Aoi Masuda
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

2002 - 2006: Graduação em Ciências Biológicas.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Caracterização enzimática de uma aspártico protease recombinante do carrapato *Boophilus microplus* produzida na forma ativa em *Escherichia coli.*
Orientador: Aoi Masuda
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

Formação complementar

2011: Curso de curta duração em Advances in the knowledge of parasite resistance.
Embrapa Pecuária Sudeste, EMBRAPA, Brasil.
2005: Curso de curta duração em Caracterização Estrutural de Proteinas.
Centro Nacional de Pesquisa em Energia e Materiais, CNPEM, Campinas, Brasil

2005: Curso de curta duração em Curso de Biologia Molecular Básica.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2004: Extensão universitária em E Por Falar Em Genética Segunda Edição.

Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2003- 2006: Estágio de iniciação científica
Centro de biotecnologia UFRGS, laboratório de imunologia aplicada à sanidade animal.
Orientador: Aoi Masuda
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

Atuação profissional

1. Universidade Federal do Rio Grande do Sul - UFRGS

Vínculo institucional
2003 – Atual: Vínculo atual: aluno de pós-graduação, Enquadramento funcional: aluna de doutorado, Carga horária: 40, Regime: Dedicação Exclusiva

Atividades: participação em projetos de pesquisa
2011- Atual: Programas de Pós-graduação da UFRGS, UENF, FIOCRUZ e UFRJ: Estímulo a formação de recursos humanos em parasitas e vetores
2010- Atual: Papel de transportadores ABC na resistência ao acaricida ivermectina no carrapato *Rhipicephalus (Boophilus) microplus*
2008 – 2011: Vacina para controle do carrapato bovino Boophilus microplus: Colaboração entrea UFRGS e UENF (Brasil) com o Laboratório de Imunologia da UDELAR (Uruguai) , Instituto Nacional de Ciência e Tecnologia de Entomologia Molecular
2007 – 2010: Estudo da fisiologia do carrapato bovino para identificação de alvos para seu controle
2006 – 2009: Caracterização da Atividade Enzimática e da Imunogenicidade da "Tick Heme-binding Aspartic Proteinase" do carrapato *Boophilus microplus*
2006 – Atual: Embriogênese do carrapato bovino *Boophilus microplus*: interação do Programa de pós-graduação de Biologia Celular e Molecular da UFRGS e Programa de pós-graduação em Biociências e Biotecnologia da UENF.
2003- 2007: O carrapato bovino e a biologia de vetores: integração entre a pesquisa básica e a procura de alvos para o desenvolvimento de estratégias de controle
2003- 2005: Produção e análise da imunogenicidade de proteínas recombinantes do *Boophilus microplus*

2. Universidade de São Paulo - USP

Vínculo institucional
2009- Atual: Enquadramento funcional: aluno especial,
Regime: Parcial

Produção bibliográfica

Artigos completos publicados em periódicos

1. POHL, Paula Cristiane, Klafke, Guilherme M., Carvalho, Danielle D., Martins, João Ricardo, Daffre, Sirlei, da Silva Vaz, Itabajara, MASUDA, Aoi. ABC transporter efflux

pumps: A defense mechanism against ivermectin in *Rhipicephalus (Boophilus) microplus*. International Journal for Parasitology. , v.41, p.1323 - 1333, 2011.

2. PARIZI, L. F., POHL, Paula Cristiane, MASUDA, Aoi, VAZ JUNIOR, Itabajara da Silva. New approaches toward anti-*Rhipicephalus (Boophilus) microplus* tick vaccine. Revista Brasileira de Parasitologia Veterinária. , v.18, p.1 - 7, 2009.

3. POHL, Paula Cristiane, SORGINE, Marcos, LEAL, Alexandre Trindade, LOGULLO, Carlos, OLIVEIRA, Pedro L, VAZ JR, Itabajara da Silva, MASUDA, Aoi. An extraovarian aspartic protease accumulated in tick oocytes with vitellin-degradation activity. Comparative Biochemistry and Physiology. B, Biochemistry & Molecular Biology. , v.151, p.392 - 398, 2008.

4. LEAL, Alexandre Trindade, POHL, Paula Cristiane, FERREIRA, Carlos Alexandre Sanchez, SILVA, Maria C L Nascimento, SORGINE, Marcos, LOGULLO, Carlos, OLIVEIRA, Pedro L, FARIAS, Sandra E, VAZ JUNIOR, Itabajara da Silva, MASUDA, Aoi. Purification and antigenicity of two recombinant forms of *Boophilus microplus* Yolk pro-Cathepsin (BYC) expressed in inclusion bodies. Protein Expression and Purification. , v.45, p.107 - 114, 2006.

5. LEAL, Alexandre Trindade, SEIXAS, Adriana, POHL, Paula Cristiane, FERREIRA, Carlos Alexandre Sanchez, LOGULLO, Carlos, OLIVEIRA, Pedro L, FARIAS, Sandra Estrazulas, TERMIGNONI, Carlos, VAZ JR, Itabajara da Silva, MASUDA, Aoi. Vaccination of bovines with recombinant Boophilus Yolk pro-cathepsin. Veterinary Immunology and Immunopathology. , v.114, p.341 - 345, 2006.

6. FREITAS, Daniela, POHL, Paula Cristiane, VAZ JUNIOR, Itabajara da Silva. Caracterização da resistência para acaricidas no carrapato Boophilus microplus. Acta Scientiae Veterinariae. , v.33, p.109 - 117, 2005.

Trabalhos publicados em anais de eventos (resumo)

1. POHL, Paula Cristiane, Lara, F. A., OLIVEIRA, Pedro L, VAZ JUNIOR, Itabajara da Silva, MASUDA, Aoi. ABC Transporter Mediate Heme Detoxification In Rhipicephalus (Boophilus) microplus Midgut Cells In: XL Reunião Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular - SBBq, 2011, Recife, PE. Livro de Resumo da Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular - SBBq. , 2011. v.40. p.8553 – 8553.

2. Rodrigues, F. S., Ferreira, v., PARIZI, L. F., POHL, Paula Cristiane, MASUDA, Aoi, VAZ JÚNIOR, Itabajara da Silva. Cloning and expression of an ABC transporter from Rhipicephalus (Boophilus) microplus In: XL Annual Meeting of SBBq, 2011, Foz do Iguaçu, PR. Livro de Resumo da Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular - SBBq. , 2011. v.40. p.45 – 45.

3. POHL, Paula Cristiane, KLAFKE, G., DAFFRE, S., VAZ JUNIOR, Itabajara da Silva, MASUDA, Aoi. A new ivermectin detoxification pathway in Rhipicephalus (Boophilus)

microplus In: XIII Internacional Congress of Acarology, 2010, Recife, PE. Abstract book of the XIII International Congress of Acarology, 2010. , 2010. v.13. p.210 – 211.

4. POHL, Paula Cristiane, Lara, F. A., VAZ JUNIOR, Itabajara da Silva, MASUDA, Aoi An ABC Transporter of Rhipichephalus (Boophilus) microplus In: XXXVIII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular - SBBq, 2009, Águas de Lindóia - São Paulo. Livro de resumos da XXXVIII Reunião Anual da SBBq, 2009. , 2009. v.38. p.8055 – 8055.

5. ARAUJO, L. A. L., Lara, F. A., VAZ JUNIOR, Itabajara da Silva, POHL, Paula Cristiane, MASUDA, Aoi. Clonagem e sequenciamento do gene de um transportador ABC de Boophilus microplus In: Clonagem e sequenciamento do gene de um transportador ABC de Boophilus microplus, 2009, Porto Alegre-RS. Livro de Resumos do XXI Salão de Iiniciação Científica da UFRGS. , 2009. v.21. p.176 – 176.

6. POHL, Paula Cristiane, SORGINE, Marcos, LEAL, Alexandre Trindade, LOGULLO, Carlos, OLIVEIRA, Pedro L, VAZ, Itabajara S, MASUDA, Aoi. Functional characterization and VT-degradation activity of Tick Heme-binding Aspartic Protease (THAP) In: VI International Conference on Ticks and Tick-borne Pathogens, 2008, Buenos Aires- Argentina. VI International Conference on Ticks and Tick-borne Pathogens-Book of Proceedings. , 2008. v.6. p.187 – 187.

7. COSTA, E., CAMPOS, E., ANDRADE, C., POHL, Paula Cristiane, FACANHA, A., MASUDA, Aoi, VAZ JR, Itabajara da Silva, LOGULLO, Carlos. PARTIAL BIOCHEMYSTRY AND MOLECULAR STUDY ABOUT INORGANIC PYROPHOSPHATASE OF CATTLE TICK RHIPICEPHALUS MICROPLUS EMBRYOS In: XXXVII Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology (SBBq) and XI Congress of the Pan American Association for Biochemistry and Molecular Biology (PABMB), 2008. Abstracts of the XI Congress of the Panamerican Association for Biochemistry and Molecular Biology (PABMB). , 2008. v.11. p.8812 – 8812.

8. Grunwald, POHL, Paula Cristiane, VAZ JUNIOR, Itabajara da Silva, MASUDA, Aoi PURIFICAÇÃO E CARACTERIZAÇÃO IMUNOGÊNICA DA THAP RECOMBINANTE In: XX Salão de Iniciação Científica e XVII Feira de Iniciação Científica, 2008, Porto Alegre- RS. Livro de Resumos do XX Salão de Iniciação Científica. , 2008. v.20. p.330 – 330.

9. POHL, Paula Cristiane, LOGULLO, Carlos, OLIVEIRA, Pedro L, VAZ JR, Itabajara da Silva, MASUDA, Aoi. THAP, an aspartic protease involved in tick embryogenesis: detection of the gene and protein expression In: XXXVII SBBq and XI PABMB, 2008, Águas de Lindóia, São Paulo. Abstracts of the XI Congress of the Panamerican Association for Biochemistry and Molecular Biology (PABMB). , 2008. v.11. p.8199 – 8199.

10. Almeida, P. G., POHL, Paula Cristiane, MASUDA, Aoi, VAZ JUNIOR, Itabajara da Silva.Clonagem do cDNA completo de um transportador ABC de Rhipicephalus (Boophilus) microplus In: XIX Salão de Iniciação Científica da UFRGS, 2007, Porto

Alegre-RS. Livro de Resumos do XIX Congresso de Iniciação Científica da UFRGS. , 2007. v.19. p.197 - 197

11. POHL, Paula Cristiane, VAZ, Itabajara, MASUDA, Aoi.Clonagem e caracterização imunogênica da THAP recombinante In: XIX Salão de Iniciação Científica da UFRGS, 2007, Porto Alegre. Livro de Resumos do XIX Congresso de Iniciação Científica da UFRGS. , 2007. v.19. p.413 – 413.

12. SIMIONATTO, S., MARCHIORO, S. B., GALLI, V., POHL, Paula Cristiane, HARTWIG, D., DINIZ, T., DELLAGOSTIN, O. A. Clonagem, purificação e caracterização imunológica de antígenos recombinantes de Mycoplasma hyopneumoniae In: 24º Congresso Brasileiro de Microbiologia, 2007, Brasília. Anais do 24º Congresso Brasileiro de Microbiologia. , 2007.

13. POHL, Paula Cristiane, SORGINE, Marcos, VAZ JR, Itabajara da Silva, MASUDA, Aoi. Partial characterization of a recombinant aspartic proteinase involved in Boophilus microplus embryogenesis In: IX Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular do Centro dew Biotecnologia da UFRGS, 2007, Porto Alegre. Livro de resumos da IX reunião anual do PPGBCM da UFRGS. , 2007. v.9.

14. POHL, Paula Cristiane, SORGINE, Marcos, VAZ JÚNIOR, Itabajara da Silva, MASUDA, Aoi. Recombinant expression and partial characterization of an aspartic proteinase from Boophilus microplus In: 8th International Veterinary immunology Simposium, 2007, Ouro Preto- MG. Program and Book of abstracts of the International Veterinary Immunology Symposium. , 2007. v.1. p.101 - 101

15. SEIXAS, Adriana, LEAL, Alexandre Trindade, POHL, Paula Cristiane, FERREIRA, Carlos Alexandre Sanchez, LOGULLO, Carlos, OLIVEIRA, Pedro L, FARIAS, Sandra Estrazulas, TERMIGNONI, Carlos, VAZ JR, Itabajara da Silva, MASUDA, Aoi
Bovine vaccination with a recombinant Boophilus Yolk pro-Cathepsin (rBYC) In: XXXV Reunião Anual da Sociedade de Bioquímica e Biologia Molecular, 2006, Águas de Lindóia, São Paulo. Livro de Resumos da XXXV Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2006. , 2006. v.35. p.8272 – 8272.

16. POHL, Paula Cristiane, LEAL, Alexandre Trindade, SORGINE, Marcos, MEDRANO, F. J., VAZ JR, Itabajara da Silva, MASUDA, Aoi. Enzymatic characterization of a recombinant aspartic proteinase from Boophilus microplus In: XXXV Reunião Anual da Sociedade de Bioquímica e Biologia Molecular, 2006, Águas de Lindóia, São Paulo.
Livro de Resumos da XXXV Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2006. , 2006. v.35. p.8087 – 8087.

17. POHL, Paula Cristiane, LEAL, Alexandre Trindade, SORGINE, Marcos, VAZ JR, Itabajara da Silva, MASUDA, Aoi. Expressão e Caracterização de uma Aspártico Protease Recombinante do Boophilus microplus In: XVIII Salão de Iniciação Científica, 2006, Porto Alegre. Livro de Resumos do XVIII Salão de Iniciação Científica. , 2006. v.18. p.439 - 439

18. LEAL, Alexandre Trindade, SEIXAS, Adriana, POHL, Paula Cristiane, FERREIRA, Carlos Alexandre Sanchez, LOGULLO, Carolos, OLIVEIRA, Pedro, FARIAS, Sandra Estrazulas, TERMIGNONI, Carlos, VAZ JUNIOR, Itabajara da Silva, MASUDA, Aoi
Vaccination of bovines with a recombinant Boophilus microplus pro-Cathepsin (BYC) In: I Simpósio Brasileiro de Acarologia, 2006, Viçosa, MG. Anais do I Simpósio Brasileiro de Acarologia, 2006.. , 2006. v.1. p.160 – 160.

19. POHL, Paula Cristiane, LEAL, Alexandre Trindade, SORGINE, Marcos, VAZ JUNIOR, Itabajara da Silva, MASUDA, Aoi. Atividade enzimática de uma proteinase aspártica recombinante do Boophilus microplus In: XIX Congresso Brasileiro de Parasitologia, 2005, Porto Alegre. Revista de Patologia Tropical - edição especial - resumos. , 2005.

20. POHL, Paula Cristiane, LEAL, Alexandre Trindade, SORGINE, Marcos, VAZ, Itabajara, MASUDA, Aoi. Clonagem e expressão da THAP recombinante do carrapato Boophilus microplus In: IX Encontro Gaúcho de Imunologia, 2005, Porto Alegre. Anais do IX encontro gaúcho de imunologia. , 2005. v.1. p.3 - 3

21. POHL, Paula Cristiane, LEAL, Alexandre Trindade, SORGINE, Marcos, VAZ JÚNIOR, Itabajara da Silva, MASUDA, Aoi. Clonagem e expressão da THAP recombinante do carrapato Boophilus microplus para estudos enzimáticos e de imunogenicidade In: XVII Salão de Iniciação Científica, 2005, Porto Alegre. Livro de Resumo do XVI Salão de Iniciação Científica da UFRGS. , 2005.

22. POHL, Paula Cristiane, LEAL, Alexandre Trindade, SORGINE, Marcos, VAZ, Itabajara, MASUDA, Aoi. Complete amino acid sequence of the recombinant THAP of the Boophilus microplus: cloning and expression In: XXXIV Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2005, Águas de Lindóia. Livro de Resumos da XXXXIV Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular. , 2005. v.34. p.45 – 45.

23. LEAL, Alexandre Trindade, FERREIRA, Carlos Alexandre Sanchez, LOGULLO, Carlos, SORGINE, Marcos, FARIAS, Sandra E, VAZ, Itabajara S, MASUDA, Aoi
Influence of thioredoxin fusion protein in solubilization, refolding and antigenicity of an aspartic proteinase of Boophilus microplus expressed in inclusion bodies In: XXXIV Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2005, Águas de Lindóia. Livro de Resumos da XXXIV Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2005. , 2005. v.34. p.45 – 45.

24. POHL, Paula Cristiane, LEAL, Alexandre Trindade, SORGINE, Marcos, VAZ JÚNIOR, Itabajara da Silva, MASUDA, Aoi. Produção da THAP recombinante do carrapato Boophilus microplus para a caracterização enzimática e imunogênica In: VII Reunião Anual do Programa de Pós-graduação em Biologia Celular e Molecular UFRGS, 2005, Porto Alegre. Livro de Resumos da VII Reunião Anual do Programa de Pós-graduação em Biologia Celular e Molecular UFRGS. , 2005. v.7. p.75 – 75.

25. LEAL, Alexandre Trindade, POHL, Paula Cristiane, LOGULLO, Carlos, SORGINE, Marcos, FARIAS, Sandra Estrazulas, VAZ JR, Itabajara da Silva, MASUDA, Aoi. Boophilus microplus Yolk Pro-Cathepsin Cloning, Expression and Analysis of

Immunogenicity In: XXXIII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2004, Caxambu. Resumos da XXXIII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular. , 2004. v.v. 33.. p.1 – 1.

26. LEAL, Alexandre Trindade, POHL, Paula Cristiane, FERREIRA, Carlos Alexandre Sanchez. Clonagem, expressão, purificação e antigenicidade de uma aspártico proteinase recombinante de Boophilus microplus. In: VI Reunião Anual do Programa de Pós-graduação em Biologia Celular e Molecular UFRGS, 2004, Porto Alegre. Livro de Resumos da VI Reunião Anual do Programa de Pós-graduação em Biologia Celular e Molecular UFRGS. , 2004. v.6. p.50 – 50.

27. POHL, Paula Cristiane, LEAL, Alexandre Trindade, SORGINE, Marcos, VAZ JR, Itabajara da Silva, MASUDA, Aoi. Restauração da seqüência completa da THAP recombinante do carrapato Boophilus microplus. In: XVI Salão de Iniciação Científica da UFRGS, 2004, Porto Alegre. Livro de Resumo do XVI Salão de Iniciação Científica da UFRGS. , 2004. v.v. 16. p.471 – 471.

28. POHL, Paula Cristiane, LEAL, Alexandre Trindade, SORGINE, Marcos, VAZ JR, Itabajara da Silva, MASUDA, Aoi. Seqüência completa de aminoácidos da THAP recombinante do carrapato Boophilus microplus: clonagem e expressão In: VI Reunião Anual do Programa de Pós-graduação em Biologia Celular e Molecular UFRGS, 2004, Porto Alegre. Livro de Resumos da VI Reunião Anual do Programa de Pós-graduação em Biologia Celular e Molecular UFRGS. , 2004. v.6. p.65 - 65