Correlation between loss of efficacy of macrocyclic lactone heartworm anthelmintics and P-glycoprotein genotype

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\textbf{A R T I C L E   I N F O}

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\textbf{A B S T R A C T}

Macrocyclic lactone (ML) molecules have been used for heartworm control for more than 25 years. However, in recent years, there have been reports of loss of efficacy of ML heartworm preventatives against \textit{Dirofilaria immitis} in some locations in the United States. Macrocyclic lactone resistance is a common problem in nematode parasites of livestock, and more recently, evidence of ivermectin resistance has been reported in the human filarial nematode \textit{Onchocerca volvulus}. In this study, four \textit{D. immitis} sample groups from the United States with different treatment histories were investigated for evidence of ML-driven genetic selection. DNA from individual adult worms and microfilariae was amplified by polymerase chain reaction to investigate a gene encoding a P-glycoprotein, a protein class known to be involved in ML pharmacology. A significant correlation of a GG–GG genotype with ivermectin response phenotype was found. Moreover, a significant loss of heterozygosity was found in a low responder group; loss of heterozygosity is commonly seen in loci when a population has been under selection. Further studies are required to confirm ML resistance in heartworm populations. However, the genetic changes observed in this study may be useful as a marker to monitor for ML resistance in \textit{D. immitis}.

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1. Introduction

Macrocyclic lactone (ML) heartworm preventatives were developed for use in uninfected dogs and cats to prevent establishment of adult \textit{Dirofilaria immitis} infections by targeting L\textsubscript{3}/L\textsubscript{4} stages. ML endectocides, such as ivermectin, milbemycin oxime, moxidectin, and selamectin are administered for heartworm chemoprophylaxis during the transmission season, which may be year-round in some locations. Macrocyclic lactones also have effects on the microfilarial stage (L\textsubscript{1}) in hosts (Bowman et al., 1992; Courtney et al., 1998; McCall et al., 1998). While the developing L\textsubscript{3}/L\textsubscript{4} stages of \textit{D. immitis} are ultra-sensitive to MLs, it appears that MLs can be microfilaricidal and also cause a cumulative insult that affects the ability of adults to reproduce and can eventually be lethal to the adult heartworms. It has been suggested that MLs can be used monthly to suppress reproduction in adult worms and remove microfilariae, thereby reducing transmission and gradually causing the attrition of adult worms – the “safety net” strategy for ML heartworm preventives (McCall, 2005).

However, we do not know how the MLs act on the different stages of this parasite. The classical view is that MLs act by opening glutamate-gated chloride channels (GluCls), which leads to paralysis of the pharynx and/or body muscles, resulting in starvation or inability of the nematodes to move, which is lethal for parasites in the gastrointestinal tract. These effects, based on observations in \textit{Caenorhabditis}...
elegans and trichostrongylid nematodes, are acute and lead to rapid paralysis and death (Geary et al., 1993; Courtney and Roberson, 1995; Blackhall, 2000). In filariae such as D. immitis, these acute effects do not occur, at least not in adults, and repeated treatments are required to produce lethality. Ivermectin does not paralyze adult filariae in vitro at the pharmacologically relevant concentrations found after treatment with the recommended dose rate (Bennett et al., 1988). Filarial nematodes may be physiologically different from C. elegans and trichostrongylid nematodes, as it is believed that the pharynx is vestigial in filariae and that nutrient uptake occurs through the cuticle (Strote et al., 1996).

There is much evidence of ML resistance in nematode parasites of ruminants (see e.g. Kaplan et al., 2004; Kaplan, 2004; Wolstenholme et al., 2004), and there have been reports of ivermectin resistance in nematode parasites of horses (Boersema et al., 2002; Hearn and Peregrine, 2003), including Cyathostomum species (Trawford et al., 2005; Molento et al., 2008), and in the human filarial nematode Onchocerca volvulus (Osei-Atweneboana et al., 2007). There are also recent reports (Hampshire, 2005) of a loss of efficacy of ML heartworm preventatives in some locations in the United States.

When ML-resistant trichostrongylid parasites, such as Haemonchus contortus and Cooperia oncophora, have been compared with ML-susceptible isolates, genetic changes in GluCl subunits (Blackhall et al., 1998a; Njue et al., 2004), a GABA-gated chloride channel subunit (Feng et al., 2002; Blackhall et al., 2003), P-glycoprotein (Pgp) ABC transporters (Blackhall et al., 1998b; Xu et al., 1998; Le Jambre et al., 1999; Sangster et al., 1999), and β-tubulin (Eng et al., 2006; Mottier and Prichard, 2008) have been reported. In the free-living nematode C. elegans, the deletion of three GluCl subunits resulted in an essentially complete loss of susceptibility to ivermectin (Dent et al., 2000), indicating that GluCls are the primary site of action of ivermectin on this nematode. However, these data cannot be interpreted to imply that ML-resistance mechanisms in parasitic nematodes necessarily involve changes in GluCls.

As noted above, ivermectin resistance (Osei-Atweneboana et al., 2007) and suboptimal responses to ivermectin (Ali et al., 2002; Awadzi et al., 2004a,b) have been reported in O. volvulus, which is phylogenetically much closer to D. immitis than are trichostrongylid parasites or C. elegans. Extensive investigations have been made into genetic changes that may be associated with developing ivermectin resistance in O. volvulus. Eng and Prichard (2005) investigated a large number of candidate and noncandidate genes for association with ivermectin resistance in O. volvulus. No evidence was found for selection on GluCl or GABA genes, but significant selection on β-tubulin and a Pgp gene was observed. Further investigations have confirmed selection on β-tubulin (Eng et al., 2006; Bourguinat et al., 2007), on a Pgp gene, and on other ABC transporter genes in O. volvulus (Ardelli and Prichard, 2004, 2007; Ardelli et al., 2005, 2006a,b; Bourguinat et al., 2008). Single nucleotide polymorphisms (SNPs) for ivermectin selection have been identified for O. volvulus in β-tubulin (Eng et al., 2006) and a half-sized ABC transporter, OvPLP (Bourguinat et al., 2008) and may be useful markers for monitoring for ML resistance in this parasite.

Loss of efficacy of ML heartworm anthelmintics could have a genetic basis and indicate a developing drug resistance situation in D. immitis. Ivermectin is a substrate for Pgp (Lespine et al., 2007) and Pgps have been implicated in resistance to MLs (Xu et al., 1998; Kerboeuf et al., 2003; Prichard, 2007). Thus, Pgp was an interesting gene to investigate for a possible relationship with altered ivermectin response phenotypes in D. immitis.

2. Materials and methods

2.1. Sample details

Four groups of samples were investigated. US-lab samples were from 37 susceptible individual adult worms from dogs that were purposefully infected with infective larvae of D. immitis (Missouri HW Isolate, TRS Labs, Athens, GA). The original donor dog for the Missouri Isolate originated from an animal pound in Missouri in 2000, some years prior to initial concerns about loss of ML efficacy. No treatment history is available for the original pound donor dog prior to its acquisition by TRS Labs, and this donor dog was not treated with heartworm preventatives once acquired as a donor dog for D. immitis. This isolate was maintained as a susceptible isolate at TRS Labs without being subjected to ML treatment. US-field samples correspond to 53 individual adult worms from field dogs from Florida, Louisiana, and Texas where ML prophylaxis is commonplace. For these samples, the individual treatment history of the donor dogs is not known. US-low responders correspond to 67 microfilariae from three dogs (named Tip, Kendall, and Tootie) originally from Arkansas and Louisiana. The dogs were naturally infected client-owned animals selected because they had been on ML heartworm preventative treatment, and in each case, their veterinarian was convinced that compliance was not an issue. Also, all three dogs had a patient record that indicated that the proper amount of product had been provided to the client based on numbers and weights of target animals in the household. The dog owners were willing to transport the dogs to Auburn University for further investigation. The dogs were microfilaricemic despite the fact that they had been under ML prophylaxis. Finally, “Buster” samples correspond to 54 microfilariae from a dog, not under ML pressure that was used to provide susceptible microfilariae for the in vitro sensitivity assay described elsewhere (Blagburn, 2010). Buster was originally infected in July 2005 with the Missouri strain from TRS Labs and maintained without ML treatment.

2.2. In vitro sensitivity assay

Microfilariae were collected from the US-low responder group (Tip, Kendall, and Tootie) and from Buster (susceptible in the in vitro microfilariae assay). In vitro sensitivity assays on microfilariae from each dog were performed using a concentration of ivermectin that was lethal to 95% (ivermectin-LC95) of susceptible microfilariae or a concentration of 2 × the LC95 of ivermectin against susceptible...
microfilariae. The concentration for $2 \times$ ivermectin-LC$_{95}$ corresponded to 72 $\mu$g/mL.

2.3. Molecular biology

Genomic DNA from individual adult worms was extracted with a Dneasy kit (Qiagen Inc, Mississauga, Canada). Genomic DNA from individual microfilariae was extracted using a QIAamp DNA kit (Qiagen), followed by amplification of the full genome with a Repli-g screening kit (Qiagen). Since only a few sequences from *D. immitis* are available in GenBank, bioinformatic analysis was performed based on *O. volvulus*, *Brugia malayi*, *C. elegans*, or *H. contortus* sequences to enable amplification of a 620-bp segment of a *D. immitis* Pgp gene. The sequence was deposited in GenBank (accession number HM596853).

Polymerase chain reaction (PCR) used primers Pgp-1-sense 5′gga caa tta tcc ggt ggt ca3′ and Pgp-1-antisense 5′tcg caa att tcc ttc cac tt3′. Denaturation was performed at 94°C for 45 s, annealing at 56°C for 45 s and extension at 68°C for 2 min for 35 cycles. PCR amplification was confirmed by electrophoresis through a 1% agarose gel. PCR products were sequenced using the 3730XL DNA Analyser system (McGill University/Genome Quebec Innovation Centre). High Fidelity Platinum® Taq DNA polymerase (Invitrogen) was used in the PCR to avoid the introduction of errors during amplification. Each chromatogram was analyzed with Sequencher™4.7 software (Gene Codes Corporation, Ann Arbor, MI). This program allows discrimination at each nucleotide to select only secondary peaks that were >90% of the major nucleotide peak on the chromatogram. This high level of discrimination provided confidence in determining the significance of any secondary peak (Sequencher® 4.0 User Manual for Windows; 1991–2010 Gene Codes Corporation, Inc.).

2.4. Statistical analysis

Genotype frequencies of SNPs in US-low responders were compared with the genotype frequencies of SNPs of the other groups using Fisher’s exact test. A linear regression was performed to assess the correlation between the Pgp genotype of microfilariae from Buster and US-low responders and their corresponding ivermectin-LC$_{95}$% phenotype using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

2.5. Deviation from Hardy-Weinberg equilibrium

The *F* coefficient was calculated for 10 different SNPs from three genes for the US-lab, US-field, and US-low responder populations. Of the 10 SNPs, two were investigated from a $\beta$-tubulin gene (accession number: HM596854), six from a heat shock protein gene (accession number: HM596851), and two from a Pgp gene (accession number: HM596853), based on a previous analysis performed to determine genetic baseline information of *D. immitis* (Bourguinat et al., 2011). Buster samples were not included in this population analysis as the Buster microfilariae were collected from a single dog.

3. Results

3.1. General findings

Two common SNPs were found in the Pgp fragment analyzed. One was located at position 11 (A11G) of that fragment, while the second was at position 618 (A618G). The A11G SNP was in a coding region (Fig. 1) just before the second adenosine-5′-triphosphate-(ATP) binding domain and resulted in an amino change from lysine to arginine. The A618G SNP was located in a non-coding region. Based on sequences from *O. volvulus*, *B. malayi*, and *C. elegans*, the fragment analyzed would start approximately at position 1200 of the consensus Pgp amino acid sequence.

The genotypes from the SNPs at positions 11 and 618 were combined for diplotype analysis (Fig. 2). For example, the AA–GG genotype corresponded to genotype AA
at position 11 and genotype GG at position 618. Of the nine possible combined genotypes, seven were found in the whole sample population (AA–AA, AA–GG, AA–AG, AG–AG, AG–AG, GG–AG and GG–GG). Genotypes were unevenly distributed among the groups. For instance, genotype GG–AG was only found in Buster microfilariae. GG–GG was not found in US-lab samples, and AG–AG and AA–AA were absent from US-field samples. Interestingly, the genotype GG–GG was significantly higher in US-low responder samples compared with US-lab samples ($P = 0.000007$), US-field samples ($P = 0.0001$) and Buster ($P = 0.04$) (Table 1), and this genotype was absent in the US-lab samples analyzed.

### 3.2. In vitro sensitivity assay and genetic analysis

The US-low responder group includes microfilariae isolated from three dogs that showed reduced sensitivity to MLs. In the in vitro assay (Table 2), the ivermectin $L_{C95}$ killed 95% of the microfilariae from Buster. Only 8% and 39% (in two different experiments at different time points) of microfilariae from US-low responder dog Tip died after exposure to the ivermectin $L_{C95}$. By exposing microfilariae from Tip to ivermectin at $2 \times$ the $L_{C95}$ on these two occasions, only 24.1% and 50.4% died, respectively. Fifty-six percent and 79% of microfilariae from Kendall and Tootie, respectively, died after exposure to ivermectin at the $L_{C95}$.

### Table 1

$P$-values from Fisher’s exact test for comparison of each combined genotype between the US-low responder group and the other groups.

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>US-lab</td>
<td>0.17</td>
<td>0.0001</td>
<td>0.118</td>
<td>0.23</td>
<td>0.30</td>
<td>0.99</td>
<td>0.000007</td>
</tr>
<tr>
<td>US-field</td>
<td>0.18</td>
<td>0.006</td>
<td>0.0008</td>
<td>0.44</td>
<td>0.67</td>
<td>1</td>
<td>0.0001</td>
</tr>
<tr>
<td>Buster</td>
<td>0.09</td>
<td>0.50</td>
<td>0.30</td>
<td>0.30</td>
<td>0.45</td>
<td>0.20</td>
<td>0.04</td>
</tr>
</tbody>
</table>

### Table 2

Results of in vitro assays for microfilariae sensitivity to ivermectin.

<table>
<thead>
<tr>
<th>Ivermectin</th>
<th>US-low responders</th>
<th>Susceptible</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Tip</td>
<td>Tip</td>
</tr>
<tr>
<td>$L_{C95}$</td>
<td>5.0/62.2</td>
<td>13.0/33.3</td>
</tr>
<tr>
<td>%</td>
<td>8.0</td>
<td>39.0</td>
</tr>
<tr>
<td>$L_{C95} \times 2$</td>
<td>14.2/58.8</td>
<td>16.3/32.3</td>
</tr>
<tr>
<td>%</td>
<td>24.1</td>
<td>50.5</td>
</tr>
<tr>
<td>Buster</td>
<td>59.0/62.0</td>
<td>95.2</td>
</tr>
</tbody>
</table>

Data are shown as number of dead microfilariae/total microfilariae (mean of three replicate experiments).
However, 99.2% and 100% of microfilariae from these two dogs, respectively, died after exposure to $2 \times$ the ivermectin LC$_{95}$.

Interestingly, microfilariae from Tip and Kendall were only genotyped as either GG–GG or AA–GG at the Pgp SNPs (Fig. 3). The frequency of the GG–GG genotype was 51.3%, 28.0%, 20.7%, and 18.5% in microfilariae collected from Tip, Kendall, Tootie, and Buster, respectively, and as noted above, was 0% in the susceptible US-lab strain.

A significant correlation ($r^2 = 0.978; P = 0.0024$) (Fig. 4) was found between the GG–GG Pgp genotype of microfilariae from Tip, Kendall, Tootie, and Buster and their ivermectin-LC$_{95}$ phenotype.

### 3.3. Deviation from Hardy–Weinberg equilibrium

There was an excess of homozygosity in the US-low-responder group (Fig. 5). For the 10 SNPs, US-lab and US-field groups had an average $F$ coefficient of $-0.1211$ and $-0.013$ respectively, whereas US-low-responders had an average $F$ coefficient of 0.8847. There was a clear deviation from Hardy–Weinberg equilibrium in the low responder population.

### 4. Discussion

From different reports on veterinary trichostrongyloid nematodes, and more recently, on *O. volvulus*, which is phylogenetically closely related to *D. immitis*, it has become clear that genetic selection occurs on ABC transporter genes with repeated ML treatment (Ardelli and Prichard, 2004, 2007, 2008; Ardelli et al., 2005, 2006a,b; Blackhall et al., 1998a,b; Bourguinat et al., 2008; Eng and Prichard, 2005; Xu et al., 1998). With recent reports of dogs with *D. immitis* showing loss of ML efficacy, it is important to identify a reliable genetic marker to monitor for the possible selection and spread of ML resistance.

This study compared *D. immitis* with different ML treatment histories in the United States and different response profiles in an *in vitro* susceptibility assay. In this regard, we compared worms that had been maintained without exposure to MLs and were susceptible to MLs with field samples that were likely (or their ancestors were likely) to have been exposed to MLs, and other samples that showed low responses to ivermectin and had come from dogs that
Fig. 5. F-coefficient or deviation from Hardy–Weinberg equilibrium. Comparison of 10 SNPs in three genes (beta-tubulin (tub), heat shock protein 60 (hsp), P-glycoprotein (pgp)) in three different groups of *D. immitis* from USA. $F = +1$, 100% homozygous; $F = -1$, 100% heterozygous. The number next to the gene corresponds to the position of the SNP in the segment analyzed.

had become infected despite being maintained on heartworm preventatives. A strong correlation of the GG–GG genotype with ivermectin response phenotype was found. The data clearly shows evidence of ML selection on the Pgp gene in *D. immitis*. One of the SNPs causes an amino acid change from lysine to arginine between transmembrane domain TM12 and the ATP-binding domain and may have implications for Pgp function, but further study of the expressed protein would be required to determine this.

The results also showed a significant loss of heterozygosity in the US-low responder group, an effect commonly seen in loci when a population has been under selection (Hedrick, 2005). The excess of homozygosity in this group could be a consequence of non-random mating in the population. Non-random mating can be caused by inbreeding, population substructure, or drug selection. The probability that inbreeding occurred in this population is very low, as these samples were obtained from three dogs that were not from the same area. Moreover, it is possible to exclude population substructure as the cause of non-random mating because samples from Florida, Louisiana, and Texas did not differ significantly in allele frequency (Bourguinat et al., 2011). The excess homozygosity in the US-low-responder group is most likely due to ML selection.

Based on the extremely high correlation of the GG–GG genotype with ivermectin response phenotype, this Pgp genotype may be useful as a genetic marker in the field, to assay for low responding *D. immitis* before treatment and to follow the spread of *D. immitis* populations that are ML sub-optimal responders in dogs. However, further work is required to confirm the utility of this diplotype as a reliable marker for sub-optimal responsiveness.

MLs have been used for more than 25 years for heartworm prevention with remarkable success. However, it must be recognized that ML resistance is well known in other nematode parasites (Kaplan et al., 2004; Kaplan, 2004; Wolstenholme et al., 2004; Osei-Atweneboana et al., 2007; Gasbarre et al., 2009a,b). If ML resistance is confirmed in other *D. immitis* populations, alternative treatments may be required to maintain control of heartworm disease in some regions. It is important at this stage to survey for possible ML resistance in *D. immitis* in regions of the southern United States, where the highest incidence of loss of ML efficacy has been reported.

Conflict of interest

The research was supported by Novartis Animal Health. RS is an employee of the sponsor. However, the sponsor of the research exercised no influence over the conduct of the research. The other authors declare no conflicts of interest that could influence the conduct or results of this study.

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