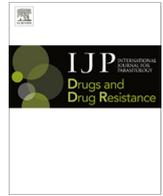




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## Development of an *in vitro* bioassay for measuring susceptibility to macrocyclic lactone anthelmintics in *Dirofilaria immitis* ☆



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### ABSTRACT

For more than 20 years, anthelmintics of the macrocyclic lactone (ML) drug class have been widely and effectively used as preventives against the canine heartworm, *Dirofilaria immitis*. However, in recent years an increased number of lack of efficacy (LOE) cases are being reported, in which dogs develop mature heartworm infections despite receiving monthly prophylactic doses of ML drugs. While this situation is raising concerns that heartworms may be developing resistance to MLs, compelling evidence for this is still lacking. Resolution of this dilemma requires validated biological or molecular diagnostic assays, but, unfortunately, no such tests currently exist. To address this need, we developed and optimized a larval migration inhibition assay (LMIA) for use with *D. immitis* third-stage larvae. The LMIA was used to measure the *in vitro* dose–response of two ML drugs (ivermectin and eprinomectin) on a known ML-susceptible laboratory strain of *D. immitis*. A nonlinear regression model was fit to the dose–response data, from which  $IC_{50}$  values were calculated; the mean  $IC_{50}$  and 95% confidence interval for IVM was 4.56  $\mu$ M (1.26–16.4  $\mu$ M), greater than that for EPR at 2.02  $\mu$ M (1.68–2.42  $\mu$ M), and this difference was significant ( $p = 0.0428$ ). The  $R^2$  value for EPR assays (0.90) was also greater than that for IVM treatment (0.71). The consistency and reproducibility of the dose–response data obtained with this assay suggests that it may be a useful technique for investigating the relative susceptibilities to ML drugs in other *D. immitis* populations.

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### 1. Introduction

The canine heartworm (*Dirofilaria immitis*) is an important filarial nematode parasite of dogs, cats, and other animals. The definitive host becomes infected through the bite of mosquitoes harboring infective third-stage larvae ( $L_3$ ), which penetrate the bite wound and invade the subcutaneous tissues. Over a period of approximately 2 months, the larval stages of *D. immitis* migrate through the subcutaneous tissues and musculature before penetrating into the vasculature. Once in the vasculature, immature adult worms migrate to the pulmonary arteries and the right side of the heart, where they mature to adults (Kotani and Powers, 1982). Tissue-migrating larval stages are not associated with any recognizable pathology; however, once in the vasculature, worms can cause significant damage and life-threatening disease (Bowman and Atkins, 2009).

Treatment of mature heartworm infection is costly and can cause serious adverse side effects, thus prevention by monthly administration of drugs that interrupt larval development is widely used (Bowman and Atkins, 2009). All compounds currently used for prevention of adult heartworm infection are members of the macrocyclic lactone (ML) drug class. In 1987, ivermectin became the first ML drug approved for use as a heartworm preventive (Campbell, 1989), and since then, products containing other ML drugs (milbemycin oxime, selamectin, and moxidectin) have been introduced. The US Food and Drug Administration (FDA) requires that all heartworm preventives achieve 100% efficacy when administered according to label directions (Hampshire, 2005). Therefore, when owner compliance with label directions is exact, no adult worms should develop in a dog administered one of these products. However, in recent years the FDA has received increasing reports of cases where mature worms develop in dogs despite evidence that monthly preventives were administered in a compliant fashion (Hampshire, 2005). These lack of efficacy (LOE) cases, are raising doubts that ML drugs achieve 100% protection (Geary et al., 2011). The two most obvious and common explanations for these LOE cases are (1) a lack of full compliance by the pet owners, which cannot be properly tested, or (2) that *D. immitis* has developed resistance to the ML drugs.

Resistance to the ML drugs is highly prevalent throughout the world in numerous species of gastrointestinal nematodes of

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livestock (Kaplan, 2004; Kaplan and Vidyashankar, 2012). Several *in vitro* bioassays have been developed and validated to detect ML resistance in many of these species (Taylor et al., 2002). One of these, the larval migration inhibition assay (LMIA) has already been successfully applied to several important species of nematode pathogens of sheep and cattle for diagnosis of ML resistance Kotze et al., 2006; Demeler et al., 2010) and appears particularly amenable for use with *D. immitis*.

Evidence suggests that ML drugs exert their anthelmintic effects on nematodes by binding to glutamate-gated chloride channels (GluCl<sub>s</sub>) and hyperpolarizing motor neurons, resulting in paralysis and death (Wolstenholme and Rogers, 2005). This paralytic action of the ML drugs is the rationale behind the development of the LMIA, which measures the ability of larvae to migrate across a fine mesh sieve following incubation with increasing concentrations of drug. Motility of the infective L<sub>3</sub> of heartworms is critical for both the initial infection process, as well as for migration through the subcutaneous and muscular tissues of the canine host in order to gain access to the vasculature. Furthermore, the L<sub>3</sub> is one of the targets (along with the fourth-stage larvae) of monthly preventive treatment with ML drugs. Since L<sub>3</sub> can be produced in large numbers in properly equipped laboratories, this appears to be the ideal life cycle stage of *D. immitis* for use in an *in vitro* bioassay, and the LMIA appears to be the most suitable bioassay for quantifying the susceptibility of L<sub>3</sub> to the paralytic effects of ML drugs.

In this study, we optimized the LMIA for use with *D. immitis* L<sub>3</sub>. We then measured the ML susceptibility of a laboratory heartworm strain in the LMIA using both ivermectin and eprinomectin. This generated reproducible dose–response data, to which we applied a variable-slope nonlinear regression model, enabling us to calculate IC<sub>50</sub> values as a means of characterizing the *in vitro* susceptibility to these drugs. Validation of the LMIA for identifying reduced susceptibility in heartworm populations would provide an extremely valuable tool for monitoring the emergence and spread of anthelmintic resistance in *D. immitis*.

## 2. Materials and methods

### 2.1. Parasites

An ML-susceptible strain of *D. immitis* (2005 Missouri strain) maintained and passaged in beagle dogs at the University of Georgia (Athens, GA) was used in this study. This strain was originally collected in 2000 from a dog at a Missouri animal pound, and while this donor dog was not treated with any MLs thereafter, prior treatment records are not available. Third-stage larvae were obtained by feeding microfilaremic blood to *Aedes aegypti* mosquitoes (black-eyed Liverpool strain) using artificial blood feeders, as previously described (McCall, 1981). Fourteen days after feeding, L<sub>3</sub> were obtained by gently crushing the infected mosquitoes, rinsing them onto a 32 µm mesh sieve set in either a Petri dish or a Baermann apparatus, and soaking them in warm Hanks' balanced salt solution. The *Brugia pahangi* L<sub>3</sub> (FR3 strain) used in some of the optimization experiments were obtained in the same manner, and are also maintained in beagle dogs at the University of Georgia.

### 2.2. Drug solutions

ML stock solutions (10 mM) were prepared by dissolving powdered ivermectin or eprinomectin (Sigma, St. Louis, MO) in DMSO (>99.5%; Sigma). Stock solutions were diluted with additional DMSO into 100× working solutions ranging from 0.0312 to 2.0 mM. These were then added to culture media at a rate of 1% (v/v) to yield the concentrations used in the LMIA (0.312–20 µM

in 1% DMSO). For each drug, the maximum concentration tested was restricted by its solubility in 1% DMSO.

### 2.3. Larval migration assay optimization

#### 2.3.1. Media

RPMI-1640 (Lonza BioWhittaker, Basel, Switzerland) media were prepared with 100 U/ml penicillin (Gibco-Invitrogen, Carlsbad, CA), 100 mg/ml streptomycin (Gibco-Invitrogen), 40 µg/ml gentamicin (Sigma). For use in the assays, ivermectin or eprinomectin in the concentrations detailed above were added to the RPMI, or only DMSO (1%) in the no-drug controls. NCTC/IMDM (NI) media were prepared from equal parts NCTC medium (Sigma) and IMDM (Sigma) with L-glutamine (200 mM; Sigma) added to a concentration of 2 mM and with 100 U/ml penicillin (Gibco-Invitrogen, Carlsbad, CA), 100 mg/ml streptomycin (Gibco-Invitrogen).

#### 2.3.2. DMSO concentration

The LMIA was performed with the Missouri strain of *D. immitis* using a 48 h incubation period and 2 h migration period. Fifty larvae per well were cultured in a 24-well plate in RPMI-1640 with antibiotics, as described above, with DMSO concentrations of 0%, 1%, and 2%. Assay results were scored as described for the optimized LMIA. Each concentration was tested in triplicate in three separate experiments.

#### 2.3.3. Migration period

Migration tubes were prepared using a method modified from Wagland et al. (1992) and set in 400 µl RPMI-1640 plus antibiotics in the wells of a 48-well culture plate. A square of 20 µm mesh was secured over the bottom of each glass migration tube and held in place by a polymer collar fit around the tube. Tubes were suspended 2 mm above the bottom of the plate by a rubber o-ring fit around each collar. In a 100 µl volume, 50 *B. pahangi* L<sub>3</sub> were introduced to each of three migration wells. Larvae were allowed to migrate while incubating at 37 °C and 5% CO<sub>2</sub>. For the first 3:45 h, the migration tubes were transferred to new wells in the plate every 15 min, and the tubes were finally removed 24 h after the beginning of the experiment. The number of L<sub>3</sub> to migrate over each time interval was then determined by counting the larvae in each well.

#### 2.3.4. Incubation period

The LMIA was performed with the Missouri strain of *D. immitis* using either a 48 or 72 h incubation period and 2 h migration period. Fifty larvae per well were cultured in a 24-well plate in RPMI-1640 medium with 1% DMSO. Four independent experiments were performed, with each group tested in triplicate.

#### 2.3.5. Mesh size

The LMIA was performed with the Missouri strain of *D. immitis* using a 48 h incubation period and 2 h migration period. Fifty larvae per well were cultured in a 24-well plate in RPMI-1640 medium with eprinomectin (0.625–50 µM) in 1% DMSO or in a DMSO-only control. Larvae were allowed to migrate through nylon mesh of either 25 or 30 µm pore size. Three independent experiments were performed, with each group tested in triplicate.

To determine the effect of larval diameter on migration, 25 *D. immitis* L<sub>3</sub> per well were cultured in a 24-well plate as above. Larvae that successfully migrated in the LMIA ( $n = 12$ ) and those that failed to migrate ( $n = 12$ ) were measured using an eyepiece micrometer on an inverted compound microscope (IX51; Olympus, Center Valley, PA) at 400× magnification.

#### 2.4. Optimized protocol for larval migration inhibition assay

After collection from mosquitoes, *D. immitis* L<sub>3</sub> were washed in RPMI-1640 and added to individual wells of a 24-well tissue culture plate (30 larvae per well) in 1 ml RPMI-1640 containing antibiotics and drug. Assay plates were then incubated at 37 °C and 5% CO<sub>2</sub> for 48 h. Following this incubation period, acrylic migration tubes were placed into a new 24-well migration plate (Fig. 1); migration tubes were set in rows of six on a horizontal acrylic bar, as described by Demeler et al. (2010), and bottomed with 25 µm nylon mesh screens (Sefar, Inc., Heiden, Switzerland). With the migration tubes in place, 1.4 ml of culture medium warmed to 37 °C was added over the tube in each well; drug concentrations in the media added to each well corresponded to those in the incubation plate. Using a dissecting microscope, all 30 larvae in each well of the incubation plate were collected and gently transferred in a volume of 200 µl to the corresponding migration tube. Once all the L<sub>3</sub> were transferred to migration tubes, they were placed back into the incubator at 37 °C and 5% CO<sub>2</sub> for a 2 h migration period during which motile larvae may pass through the mesh screen (Supplementary video 1). After 2 h, the migration tubes were gently removed and the larvae that had migrated into each well of the migration plate were enumerated during examination with an inverted compound microscope. Three experiments were performed for each drug (ivermectin and eprinomectin) with concentrations tested in triplicate for each experiment.

#### 2.5. Statistics

Differences in migration following incubation for 48 and 72 h (without drug) and differences in migration through meshes of different size were calculated using a one-tailed unpaired *t*-test. Correlation between larval diameter and the ability to migrate was calculated as a point-biserial correlation coefficient, with significance assessed by application of a two-tailed *t*-test.

Percent inhibition of migration was calculated for each well based on the number of larvae failing to migrate corrected for the percent of larvae that did not migrate in the no-drug control wells. Sigmoidal dose–response curves were calculated in GraphPad Prism® version 5 (GraphPad Software, Inc., San Diego, CA)

using a variable slope nonlinear regression model. A four-parameter logistic equation was applied using global curve-fitting, with the bottom of the curves constrained to zero. For each treatment, the IC<sub>50</sub> and IC<sub>95</sub> (defined as the concentration effecting inhibition halfway between zero and the maximal response, and 95% of the maximal response, respectively) were calculated. *R*<sup>2</sup> values and 95% confidence intervals were calculated by GraphPad Prism®. Differences between the IC<sub>50</sub> values of IVM and EPR-treated groups were calculated using the extra sum-of-squares *F* test.

### 3. Results

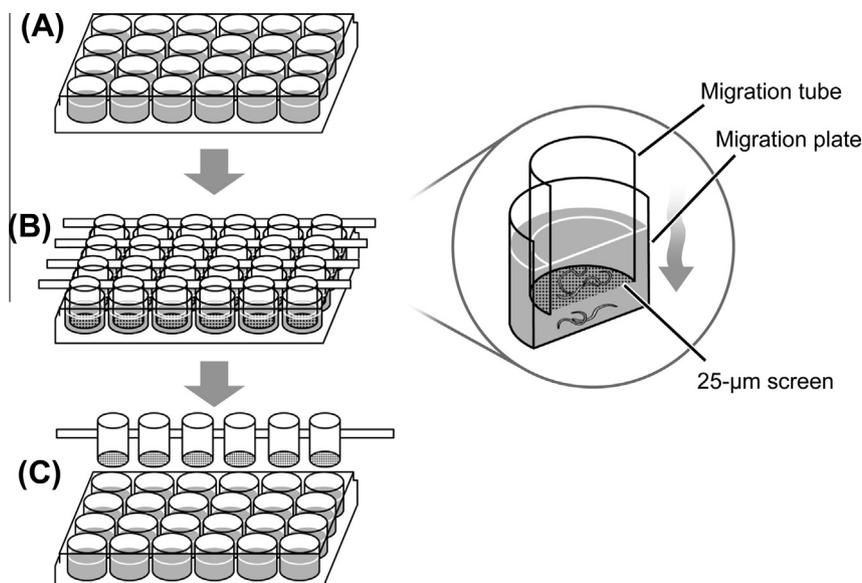
#### 3.1. Migration period

*B. pahangi*, a filarioid parasite closely related to *D. immitis* was used in some preliminary experiments with the LMIA, since this parasite is easier to cycle and was readily available through the FR3. In these early experiments, we observed that almost all larvae that successfully migrated over 24 h did so within the first 2 h of incubation (mean ± SD = 99.2 ± 6.56%; Fig. 2). To ensure a convenient yet sufficiently long migration period, the migration rate of *D. immitis* L<sub>3</sub> over 1 h was determined, followed immediately by an additional 1 h in a separate migration plate. Significantly more larvae migrated over the combined 2 h (83%) period than 1 h (77%; *p* = 0.037), therefore a 2 h migration period was used for this LMIA.

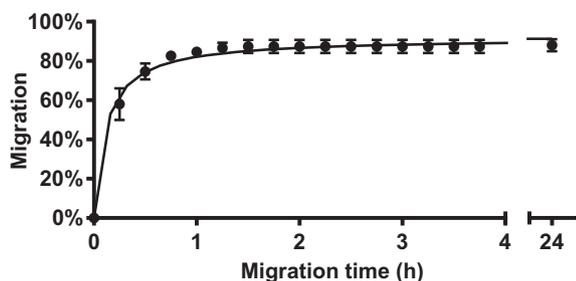
#### 3.2. Media and DMSO content

To determine the optimal culture medium for use in the *D. immitis* LMIA, RPMI- and NI-based media with added antibiotics were compared. Six groups of 50 L<sub>3</sub> were incubated in each medium with no difference observed in migration rates (*p* = 0.81; data not shown). The RPMI-based medium was chosen for future assays because of its ease of preparation and convenience.

To solubilize macrocyclic lactone anthelmintics in an aqueous media it is necessary to use DMSO, but this solvent is known to negatively affect nematode viability. To determine an appropriate solvent concentration, larvae were incubated in media containing 1%, 2%, or no DMSO and their migration rates assessed. While no



**Fig. 1.** Larval migration inhibition assay procedure. (A) Larvae are incubated in media with or without drug for 48 h in the 24-well incubation plate. (B) Larvae are then transferred into migration tubes, which are suspended over the migration plate. The larvae are then allowed to migrate for 2 h through a 25 µm mesh and are collected in the migration plate. (C) Migration tubes are gently removed from the migration plate and the numbers of larvae that successfully migrated are enumerated.



**Fig. 2.** Rate of filarial larval migration. Fifty untreated *Brugia pahangi* L<sub>3</sub> were allowed to migrate across a 20  $\mu$ m mesh in each migration tube ( $n = 3$ ). Tubes were transferred to new wells every 15 min for the first 3:45 h and finally removed 24 h after the beginning of the experiment.

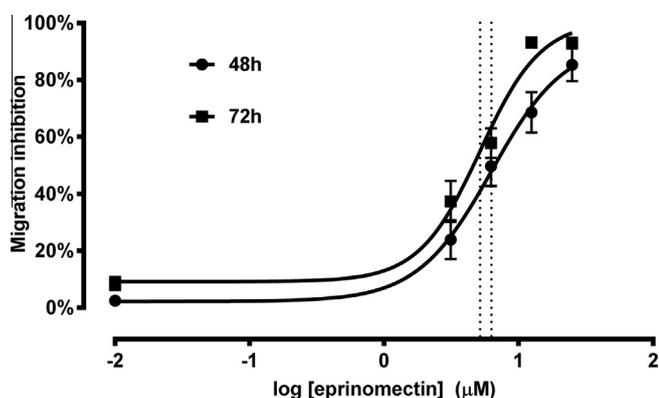
decrease from control migration rates was observed in the 1% DMSO group ( $p = 0.39$ ), a significant decrease was noted when larvae were exposed to 2% DMSO ( $p = 0.018$ ; data not shown). Based on these data, 1% DMSO was used in the LMIA culture medium for all further experiments.

### 3.3. Incubation period

To allow sufficient exposure of the larvae to the drug without inducing an excessive reduction in migration of the untreated control larvae, it was necessary to optimize the incubation period. Comparison of the LMIA performed using 48 h or 72 h incubation periods showed that migration rates were significantly lower following the longer incubation ( $p = 0.011$ ; Fig. 3). Furthermore, the  $\log_{IC_{50}}$  for each assay was not significantly different ( $p = 0.51$ ); therefore, a 48 h incubation period was selected for use in all future assays.

### 3.4. Larval diameter and mesh size

The pore size of the mesh sieve is a discriminating parameter for parasite migration in the LMIA. We observed that the diameter of L<sub>3</sub> varied somewhat, so we wanted to confirm that this variable would not affect migration rates in the LMIA. Larvae that had either migrated or failed to migrate through a 25  $\mu$ m mesh were measured using an ocular micrometer in an inverted compound microscope (Fig. 4). The largest *D. immitis* L<sub>3</sub> diameter measured was 25  $\mu$ m (mean  $\pm$  SEM = 20.6  $\pm$  0.268  $\mu$ m). No correlation between migration and larval diameter was observed ( $p = 0.95$ ), providing



**Fig. 3.** Effect of drug incubation period on larval migration. The LMIA was performed using 48 and 72 h incubation periods with eprinomectin. To more accurately compare the effect of incubation time on migration, data are not corrected for control inhibition. Dotted lines indicate  $\log_{IC_{50}}$  values. Each data point represents mean  $\pm$  SD,  $n \geq 12$  from four independent assays.

evidence that migration rates were not a function of variation in larval diameter.

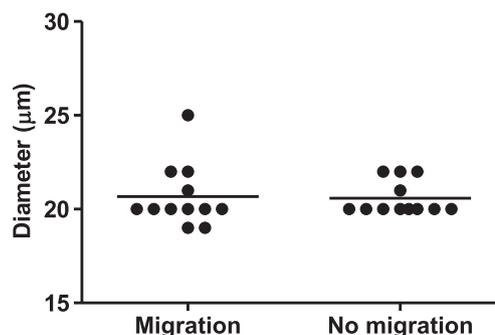
Mesh size was selected so that it was no smaller than the largest larval diameter, yet remained fine enough to restrict the passage of immotile larvae. Given the commercial availability of mesh with a variety of pore sizes, migration rates through 25 and 30  $\mu$ m mesh sieves were compared. No reduction in migration was observed for the smaller mesh size compared to the larger (three independent experiments,  $p = 0.38$ ) for worms incubated without drug. However, migration of drug-treated larvae at the highest concentration tested was significantly reduced with the 25  $\mu$ m mesh ( $p = 0.034$ ; data not shown). A small percentage of larvae were observed to migrate through the 25  $\mu$ m mesh at the highest drug concentrations tested, but it was confirmed visually that this was due to larval motility, and not the incidental passage of immotile larvae. In contrast, with the 30  $\mu$ m mesh, small numbers of immotile larvae were found to pass through the mesh. Given these data, the 25  $\mu$ m mesh size was selected as being optimal for use in this assay.

### 3.5. Optimized larval migration inhibition assay

The mean migration inhibition rates in the control wells were consistently less than 10% (mean  $\pm$  SD = 7.78  $\pm$  7.75%) in most assays, indicating that *D. immitis* L<sub>3</sub> retain high levels of motility after 48 h of incubation in RPMI media containing 1% DMSO. However, mean inhibition at the highest drug concentrations tested infrequently exceeded 90% (mean  $\pm$  SD = 80.7  $\pm$  13.9%). Dose–response curves were plotted for the ML-susceptible Missouri heartworm strain treated with either ivermectin or eprinomectin (Fig. 5) at concentrations ranging from 0.312 to 20  $\mu$ M. The  $IC_{50}$  values for *D. immitis* L<sub>3</sub> exposed to ivermectin and eprinomectin were 4.56 and 2.02  $\mu$ M, respectively, and these values were significantly different from each other ( $p = 0.0428$ ). The calculated  $IC_{95}$  values were less reliable, characterized by very large 95% confidence intervals, especially for ivermectin (Table 1). The fit of each curve to the global nonlinear regression model was expressed as an  $R^2$  value, and higher values were consistently found with eprinomectin, suggesting that eprinomectin is a better choice for use in this *in vitro* bioassay.

## 4. Discussion

The emergence of *D. immitis* that are resistant to macrocyclic lactone anthelmintics would represent a serious threat to canine health. However, there still is no consensus on the issue of whether ML resistance has emerged or if LOE cases are sufficiently



**Fig. 4.** Effect of larval diameter on migration through 25 and 30  $\mu$ m mesh sieves. The diameters of larvae that successfully migrated in the LMIA and those that failed to migrate were measured. Horizontal line indicates mean,  $n = 12$ .

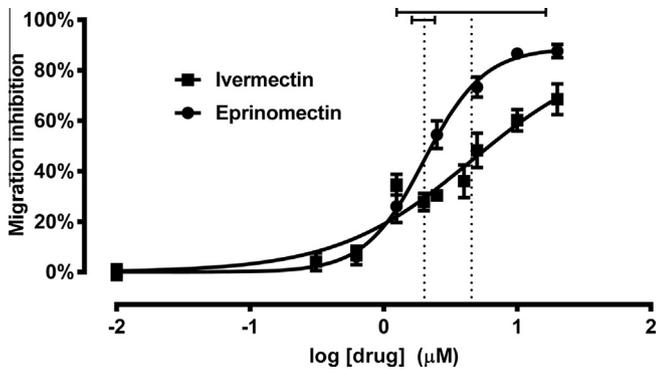


Fig. 5. Larval migration inhibition assay dose–response curves. Data from the ML-susceptible Missouri (2005) *D. immitis* strain are plotted for both ivermectin (squares) and eprinomectin (circles). Dotted lines indicate mean  $\log IC_{50}$  values, with solid horizontal bars representing the 95% confidence interval of each. Each data point represents the mean  $\pm$  SD for three independent assays.

explained by other factors. The major impediment to resolving this issue is the absence of a validated diagnostic test for measuring resistance in *D. immitis*. Here we report the development and optimization of an *in vitro* bioassay for measuring the *in vitro* susceptibility of *D. immitis* L<sub>3</sub> to ML anthelmintics. Using an established ML-susceptible laboratory isolate, we demonstrated the ability to reproducibly measure the *in vitro* dose–response characteristics of *D. immitis* L<sub>3</sub> to ivermectin and eprinomectin.

Considering the biology and epidemiology of *D. immitis* and its extreme sensitivity to ML drugs, the emergence of resistance in this parasite has historically been considered unlikely (Prichard, 2005). However, increasingly common LOE reports have raised concerns that ML resistance is developing in heartworms (Hampshire, 2005). In addition, several recent studies have reported genetic structural differences and/or sequence changes in some heartworm isolates leading these authors to conclude that resistance is present (Bourguinat et al., 2011a,b). Single nucleotide polymorphisms (SNPs) have been reported to occur in a gene encoding a P-glycoprotein, a class of proteins implicated in reduced ML efficacy against the filarioid *Onchocerca volvulus* (Prichard and Roulet, 2007), and associated with ML-resistance in *Haemonchus contortus* (Xu et al., 1998). Examining multiple *D. immitis* isolates, Bourguinat et al. (2011b) reported a correlation between the frequency of a pair of SNPs and reduced *in vitro* ML efficacy on microfilariae in an isolate-specific manner. In one case, a dog originating from Louisiana demonstrated persistent microfilaremia following multiple high-dose ML treatments (Bourguinat et al., 2011a). A high frequency of the aforementioned genotype was observed in this population of microfilariae, prompting the authors to cite ML resistance as the cause of this anomaly. However, even after numerous high-level doses of ML drugs, only 45% of the recovered microfilariae carried the SNPs suspected to be associated with resistance. Therefore, the significance of these SNPs as markers for resistance remains unclear. Though serious

Table 1

Larval migration inhibition assay results.  $IC_{50}$  and  $IC_{95}$  values of the ML-susceptible Missouri (2005) *D. immitis* strain are shown with their 95% confidence intervals (CI) for both ivermectin and eprinomectin. The  $R^2$  values are presented as a measure of how well dose–response data fit the nonlinear regression model used in this study. Three independent assays were performed for each group.

|                      | Treatment  |              |
|----------------------|------------|--------------|
|                      | Ivermectin | Eprinomectin |
| $IC_{50}$ ( $\mu$ M) | 4.56       | 2.02         |
| 95% CI               | 1.26–16.4  | 1.68–2.42    |
| $IC_{95}$ ( $\mu$ M) | 142        | 9.20         |
| 95% CI               | 7.02–2890  | 5.28–16.0    |
| $R^2$                | 0.71       | 0.90         |

concerns of ML-resistance in *D. immitis* have existed for several years, no definitive studies that demonstrate this resistance under controlled conditions have yet been reported, and no assays for the detection of resistance have been validated.

The LMIA developed in this study was modified from those applied to gastrointestinal nematodes of ruminants for assessing drug-resistance status. Several other *in vitro* bioassays also exist for diagnosing ML resistance in gastrointestinal nematodes, the most commonly used being the larval development assay (LDA). However, the dissimilar biology of the filarioids prevents the application of this assay, and thus the LMIA was selected as the most viable and applicable technique. Extensive modification of the assay was required for optimal performance with filarial nematodes, which are, by comparison, highly sensitive to environmental exposure. In particular, four aspects of the *D. immitis* LMIA were the focus of optimization: (1) incubation time was adjusted to allow sufficient ML exposure while maintaining control parasite viability; (2) the medium and antibiotics were optimized for filarioid culture conditions; (3) the mesh size (25  $\mu$ m) was selected for optimal discrimination of motile vs. non-motile *D. immitis* L<sub>3</sub>; and (4) individual L<sub>3</sub> were counted and placed in wells of the assay plate rather than estimated numbers being aliquoted in known volumes of liquid, as is typically done in protocols for ruminant nematodes. This was done to reduce well-to-well variability given the relatively small numbers of *D. immitis* L<sub>3</sub> that could be readily obtained for each experiment.

The optimized assay reported here permitted a consistent high-level migration in untreated control parasites and yielded consistent, reproducible dose–response data. This permitted us to calculate accurate  $IC_{50}$  values for each drug examined. However, even at the highest ML concentrations that could be solubilized, complete inhibition of larval migration was rarely observed; the mean inhibition at 20  $\mu$ M was 88.8% (SD = 6.89%) for eprinomectin and 70.0% (SD = 14.0%) for ivermectin. Therefore, it was not possible to accurately measure  $IC_{95}$  values, leading to very wide 95% confidence intervals. Higher ML concentrations were not used owing to their poor solubility at the solvent level used (1% DMSO). Increasing the solvent level permits the use of higher ML concentrations, but this adversely affects parasite viability. Since it is critical that the untreated control parasites maintain high levels of viability throughout the entire experiment, this was not feasible. Any reduction in parasite viability in the no-drug control wells will increase variability and non-specific error, decreasing the consistency and accuracy of the data. The  $IC_{50}$  usually is the most robust and accurate measure of the overall susceptibility of a population because data is collected at concentrations both above and below this value. This was also true of our data, thus the  $IC_{50}$  was selected as the parameter of choice. Furthermore, with regard to the planned use of this assay, if an LOE case is not due to compliance failure, then all parasites in that dog would have established in the presence of regular ML prophylaxis. Consequently, all worms infecting the dog would be resistant, leading to reduced heterogeneity in the dose–response of the F2 generation. Thus, the  $IC_{50}$  is the most logical and appropriate parameter for evaluating the susceptibility of any suspected-resistant *D. immitis* isolate with an *in vitro* assay. In contrast, there is usually high variability at the upper plateau of the dose–response, especially when 100% inhibition is not achievable. This tends to make the  $IC_{95}$  a highly unstable parameter that is prone to variability-induced errors of interpretation.

Macrocyclic lactone resistance in *H. contortus* and *Cooperia oncophora* is well-established, and the LMIA has proven effective in discriminating resistant from susceptible isolates (Kotze et al., 2006; Demeler et al., 2010). Measuring these differences is also dependent upon how well dose–response data fit the statistical model being used, and in this study we found noticeably higher

$R^2$  values and smaller confidence intervals for  $IC_{50}$  in assays with eprinomectin than with ivermectin. This disparity is not unusual, as *in vitro* responses in the LMIA have been reported to vary with the ML compound used (Kotze et al., 2006). This group also found that eprinomectin yielded superior results as compared to ivermectin in the LMIA. Gill and Lacey (1998) first reported that the optimal ML compound for use in an *in vitro* assay varies among assay type and parasite species, and thus needs to be determined empirically by experimentation for each assay-species combination. This finding demonstrates that the drug used in a particular *in vitro* bioassay has broad relevance and applicability for the entire anthelmintic class it represents. For example, in the LDA, the analog that provides the greatest discrimination between ML-susceptible and ML-resistant isolates of *H. contortus* and *Trichostrongylus* spp. is avermectin aglycone (Gill and Lacey, 1998; Kaplan et al., 2007), a drug that has poor efficacy *in vivo* and has not been developed as an anthelmintic product. Consequently, it is unimportant that eprinomectin is not used clinically for the prevention of heartworm infection. Future work will involve performing the LMIA with suspected-resistant *D. immitis* isolates, and the testing of additional ML compounds to determine whether eprinomectin or another analog is the best choice for discriminating heartworm populations of differing drug susceptibilities.

The suitability of the LMIA for assessing ML susceptibility in filarioids must also be considered further. The LMIA is a motility-based assay and the ML anthelmintics are known to cause paralysis in gastrointestinal nematodes of ruminants (Boisvenue et al., 1983; Folz et al., 1987). This rationale was the basis of the assay's development and validation as a diagnostic test for ML-resistance in ruminant nematodes. Given the extremely high ML sensitivity of *D. immitis* L<sub>3</sub> and L<sub>4</sub> stages, for which motility and migration are critical in both the initial infection of the canine host and subsequent tissue migration, we assumed that the LMIA would be the ideal assay for detecting ML resistance in this parasite. However, we observed that the ML concentrations required to induce an *in vitro* paralytic response were quite high. The preventive dose of ivermectin (6 µg/kg), which yields 100% clinical efficacy, produces peak plasma levels in the dog of approximately 3 ng/ml (Daurio et al., 1992). In contrast, the present study demonstrated an  $IC_{50}$  value of 4.56 µM (3.99 µg/ml) for ivermectin, and even at 20 µM concentrations (17.5 µg/ml ivermectin; 18.3 µg/ml eprinomectin) we failed to effect total migration inhibition. *In vitro* concentrations more than 6000 times higher than those achieved *in vivo* could not come close to reaching the level of 100% inhibition. The disparity in drug level and the limited achievable efficacy challenge our assumption that the *in vitro* and *in vivo* responses of *D. immitis* to MLs are comparable and lead us to believe that the drug may be acting through at least two different mechanisms. Indeed, the host immune response may be integral to ML activity in *D. immitis* as indicated by research in other filarioids. *In vitro* treatment of *Brugia malayi* microfilariae with ivermectin was reported to interfere with protein secretion, which may unmask this parasite to the host immune system and allow its clearance (Moreno et al., 2010). Furthermore, studies with *Acanthocheilonema viteae* microfilariae reported that, while this filarioid is unaffected by nanomolar concentrations of ivermectin *in vitro*, it is subject to cell-mediated cytotoxicity when treated in the presence of host serum (Rao et al., 1987). Similar observations were reported for *Litomosoides carinii*, though for this parasite, treatment with ivermectin before exposure to immune cells appeared to elicit a cytotoxic response when worms were later cultured in the absence of drug (Zahner and Schmidtchen, 1994). These observations, both recent and old, suggest that the ML mechanism of action in filarioid parasites (clade III) may differ from that in the phylogenetically distinct gastrointestinal nematodes (clade V). If this is true, then

it follows that *in vitro* bioassays developed for detecting ML resistance in gastrointestinal nematodes will not be effective in filarioids like *D. immitis*. Further research will investigate these issues, aiming to improve our understanding of the *in vivo* action of the MLs. We hope that further elucidation of the mechanism by which ML drugs kill these parasites will give us new insight into the effective measurement of ML susceptibility and, by extension, the diagnosis of resistance.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijpddr.2013.05.001>.

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