AN *IN VITRO* BIOASSAY FOR MEASURING ANTHELMINTIC SUSCEPTIBILITY IN *DIROFILARIA IMMITIS*

by

CHRISTOPHER CHARLES EVANS

(Under the Direction of Ray M. Kaplan)

ABSTRACT

The canine heartworm (*Dirofilaria immitis*) is a widespread parasitic filarial nematode of veterinary importance. Anthelmintics of the macrocyclic lactone (ML) drug class have been widely and effectively used in chemoprophylaxis, but an increased number of lack of efficacy reports, in which dogs develop mature infection despite consistent preventive dosing, has raised concerns that heartworms are developing resistance. The objective of this study was to optimize an *in vitro* bioassay for measuring the susceptibility of heartworm populations to ML drugs and, using this assay, to compare the dose-responses of susceptible and suspected-resistant isolates. We developed and used a larval migration inhibition assay (LMIA) in which the ability of third-stage larvae to migrate through a 25-µm sieve following incubation in drug was measured. Fitting the dose-response data from the LMIA to a nonlinear regression model, we observed reproducible results, but found no differences in the IC_{50} values of susceptible and suspected-resistant isolates.

INDEX WORDS: anthelmintic resistance, bioassay, *Dirofilaria immitis*, *in vitro*, larval migration inhibition assay, macrocyclic lactone, third-stage larvae

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CHRISTOPHER CHARLES EVANS

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CHRISTOPHER CHARLES EVANS

Major Professor: Ray M. Kaplan

Committee: Andrew R. Moorhead
Adrian J. Wolstenholme

DEDICATION

To my mother and father. To have their love and encouragement, near and far, I am unduly blessed.

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CHAPTER 1

LITERATURE REVIEW

Overview of the heartworm

The canine heartworm (*Dirofilaria immitis*) is a parasitic nematode belonging to the family Onchocercidae (superfamily Filarioidea), which also includes some important pathogens of humans, including *Wuchereria bancrofti*, *Onchocerca volvulus*, and *Loa loa*. The heartworm itself is a serious and potentially lethal parasite, mainly of dogs. Mature worms inhabit the pulmonary arteries and, in heavy infections, the heart of a canine definitive host, and require a mosquito intermediate host for the further development and transmission of new larvae (Anderson 1992).

The earliest published report on the canine heartworm was written in 1626 by Italian Francesco Birago (Roncalli 1998). *Dirofilaria immitis* is thought to have originated in Asia, spreading along the Mediterranean Sea, and arriving in the New World with immigrants (Bowman and Atkins 2009). Its first reported appearance in the United States was made during the necropsy of a 'hydrophobic' dog, the heart of which was found to contain more than 100 large white worms (Osborne 1847). It was not until almost a decade later that the parasite was described in detail and named *Filaria immitis*, with the current generic name *Dirofilaria* applied in 1911 (Leidy 1856; Roncalli 1998). Today, the canine heartworm remains a widespread and serious threat to dogs and other animals.

Distribution and prevalence

Dirofilaria immitis infection is reported on every continent except Antarctica. Originally thought to be limited to warmer coastal regions, the parasite appears to be spreading to more temperate zones (Lok 1988). In the United States, heartworm is enzootic in every state but Alaska, with a high prevalence in the

Mississippi River basin, and along the Missouri and Ohio Rivers (McCall, Genchi et al. 2008). In 2005, a quarter of a million heartworm cases were diagnosed in the USA (increased from a survey conducted three years prior), with twice as many dogs estimated to be infected (Guerrero, Nelson et al. 2006; McCall, Genchi et al. 2008). The nationwide prevalence of heartworm infection in dogs is about 1.4% (Bowman, Little et al. 2009). Among the wild canids that harbor the parasite in the USA, only the coyote (*Canis latrans*) is thought to serve as a significant reservoir, with infection rates of 21-42% (Nelson, Gregory et al. 2003; Sacks and Caswell-Chen 2003).

The heartworm life cycle

Intermediate host

The unsheathed microfilariae of *D. immitis* present in the peripheral circulation of the definitive host may be taken up by a mosquito during a blood meal (Figure 1.1). Potential intermediate hosts for the parasite include mosquitoes of the genera *Aedes*, *Anopheles*, *Culex*, *Mansonia*, and *Psorophora*, with some 70 species reported susceptible worldwide, 13 of which inhabit the United States (Ludlam, Jachowski et al. 1970; Lok 1988). The mosquito vector is necessary for development of larvae to the infective stage and for transmission to a new definitive host. Ingesting high numbers of microfilariae in a single blood meal (15 or more, in a study on *Aedes trivittatus*) is lethal to the mosquito; however, mosquitoes seem to have a limited ability to restrict the uptake of microfilariae when feeding (Christensen 1978; Abraham 1988).

After being ingested by the mosquito and arriving in the midgut, *D. immitis* microfilariae migrate to the primary cells of the Malpighian tubules, enter them and begin an intracellular phase of development (Kartman 1953; Bradley, Sauerman et al. 1984). By 3–4 days post-infection, the microfilaria grows shorter and stouter into the 'sausage stage' (Taylor 1960). Because circulating microfilariae are relatively long and thin, it is hypothesized that this stage is necessary to bring primordial cells within the worm closer together to ensure proper larval development (Anderson 1957). Approximately 10 days after entering the mosquito, the larva undergoes the first molt. The second-stage larva (L_2) grows long and thin, molting again at about 13 days (Taylor 1960). Breaking free of the Malpighian tubules, the third-stage

larva (L_3) enters the hemocoel and migrates to the mosquito's salivary glands where it becomes infective (Anderson 1992). At 27°C, the entire period of development in the mosquito host takes about 16 days in *Aedes aegypti* (Kartman 1953). The rate of development is affected by environmental factors, including temperature and humidity (Lok and Knight 1998).

Definitive host

The infective L_3 are transmitted to their definitive host when the mosquito next takes a blood meal. The domestic dog (*Canis lupis familiaris*) is the definitive and typical host of the canine heartworm, but the parasite has also been recovered from wolves, dingoes, coyotes, foxes, felines (including the domestic cat, *Felis catus*), sea lions, harbor seals, mustelids (including the domestic ferret, *Mustela putorius furo*),

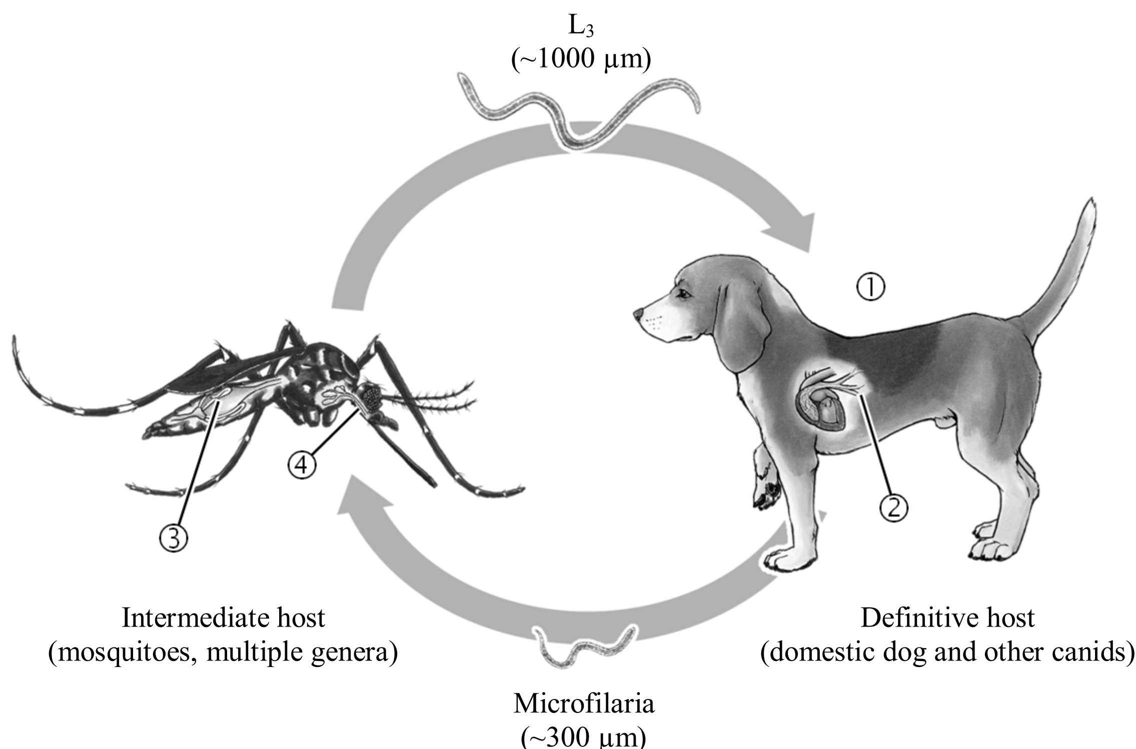

Figure 1.1: Life cycle of *Dirofilaria immitis*. ①, during mosquito feeding, infective L_3 leave the mouthparts and enter the definitive host via the bite wound. ②, migrating through muscle tissue and penetrating a vein, immature adults localize in the pulmonary arteries, where they reach sexual maturity and mate, releasing microfilariae into the peripheral circulation. ③, when ingested by a mosquito, microfilariae enter the Malpighian tubules, undergo two molts, and ④ migrate to the salivary duct, from which they may emerge during the next blood meal.

bears, red pandas, beavers, coatis, rabbits, deer, horses, primates (including humans), and others (Abraham 1988; McCall, Genchi et al. 2008).

While the infected mosquito is feeding, L_3 break out of the tip of the labellum or sometimes the mid-portion of the labium. This is thought to be stimulated by the bending of the labium as the mosquito probes and feeds (McGreevy, Theis et al. 1974). A drop of hemolymph is also released as the larvae escape, providing them with a moist environment on the host's skin until the mosquito withdraws its fascicle. The L_3 can then migrate through the bite wound, thus infecting the host (Grassi and Noe 1900; McGreevy, Theis et al. 1974). For the first three days after infection, most L_3 are recovered from the subcutaneous tissues near their entry site. Larvae molt to the fourth stage (L_4) 3–9 days post-infection, migrating through muscle fibers. In laboratory infections of dogs using an inoculation site in the hind limb, the L_4 appear to migrate to the abdomen within the first 21 days and to the thorax by 41 days, demonstrating an orientation towards their final predilection site. The final molt to the adult stage occurs 50–70 days post-infection. Immature adults are able to penetrate the jugular and other veins, allowing their entry into the heart. Adult worms reach the heart as early as 70 days post-infection, with all having entered the heart and pulmonary arteries by 90-120 days (Kume and Itagaki 1955; Kotani and Powers 1982). Here, mature worms can reach a length of 12–20 cm for males and 25–31 cm for females.

The adult heartworm is ovoviviparous, with embryos developing to microfilariae that emerge unsheathed from the female and are released into the circulatory system of the host (Abraham 1988). Microfilariae typically appear in the blood 6–9 months post-infection (Orihel 1961; Kotani and Powers 1982). In dogs, microfilaremia from a single infection with adult worms can last up to 7.5 years, with individual microfilariae surviving as long as 2.5 years in the blood (Underwood and Harwood 1939; Newton 1968). Daily and seasonal periodicity of microfilariae has been reported, appearing to correlate with the presence of vectors, with a slightly higher microfilaremia observed in the late afternoon and evening, as well as during the spring and summer months (Abraham 1988; Bowman and Atkins 2009).

Heartworm disease

In prepatent and low worm-burden infections, the canine host is usually asymptomatic. The pathophysiology associated with heartworm infection is mainly a result of the presence of adult worms in the pulmonary arteries. This causes pulmonary hypertension, which, if not treated, can lead to congestive heart failure and death. Other syndromes associated with infection are glomerulonephritis, and eosinophilic pneumonitis and granulomatosis. Additionally, a life-threatening condition called caval syndrome results from the retrograde migration of adult worms in heavily-infected animals to the right atrium and venae cavae, producing serious clinical signs. Aberrant migration of young adult worms (such as to the brain, spinal cord, peritoneal cavity, or anterior chamber of the eye) is an uncommon but possible complication of infection (McCall, Genchi et al. 2008). Feline hosts may also develop severe disease from heartworm infection, which is generally pulmonary in nature and more frequently caused by larvae or immature adults (McCall, Genchi et al. 2008; Bowman and Atkins 2009). For these reasons, the prevention and treatment of heartworm infection is a serious and important issue in pet health.

Diagnosis of heartworm infection

Mature heartworm infections can be diagnosed using a number of methods. Observation of microfilariae in the blood using microscopy can be achieved using the modified Knott test or a filtration test (Difil-Test®, EVSCO, Buena, NJ) (Knott 1939; McCall, Genchi et al. 2008). Histochemical staining for microfilariae is another approach, but both methods require morphological identification of the filarial species when encountered (Chalifoux and Hunt 1971). A more specific diagnostic test for *D. immitis* is the immunochromatography-based blood antigen test, the most common version of which reacts with an adult female antigen of that species only (for example, the IDEXX SNAP® test). More recently, PCR tests have also been developed (Casiraghi, Bazzocchi et al. 2006). Abnormalities in the heart and pulmonary arteries associated with heartworm disease can be visualized through radiography and echocardiography, with the lattermost method allowing the detection of live adult worms (McCall, Genchi et al. 2008).

Treatment of heartworm infection

Once heartworms reach maturity in the canine host, treatment options remain, but no method yet developed for eliminating adult worms has been without considerable risk to the patient. Originally discovered in studies on human filariasis during the Second World War, thiacetarsamide was later proposed as a treatment for mature heartworm infection (Otto and Maren 1947). Over time, the more soluble analog, thiacetarsemide sodium, administered intravenously twice-daily in a two-day regimen became the standard treatment for adult heartworm infection. However, hepatotoxicity is sometimes associated with treatment, and perivascular leakage of the drug results in severe local tissue necrosis (Courtney 1988). Another arsenical compound, melarsomine dihydrochloride (Immiticide®, Merial, Duluth, GA) has more recently been applied as an adulticide. In the advised treatment regimen, melarsomine is administered intramuscularly with a single injection, followed not less than 30 days later by two injections 24h apart; the drug label, however, only calls for the latter two doses. Because treatment is highly effective and safer than with thiacetarsemide, this is currently the only approved adulticide for heartworm treatment in common use (McCall, Genchi et al. 2008; Bowman and Atkins 2009; American Heartworm Society 2010). In all adulticidal therapy, however, the death of worms results in some degree of pulmonary thromboembolism brought on by the inflammatory response to fragments of decomposing worms carried into the distal pulmonary arterioles and capillary beds (Sutton 1988). This complication may be minor to life-threatening and can be minimized by restricting exercise of the animal for a period of 30–40 days supplemented with calcium heparin and glucocorticosteroid therapy (McCall, Genchi et al. 2008).

Adulticidal activity has also been demonstrated with macrocyclic lactones (MLs) at prophylactic doses (McCall, Ryan et al. 1998). Thirty months of continuous administration of the ML ivermectin (IVM) yielded clearance of more than 95% of adult worms, but such extra-label use of the drug, sometimes called ‘slow kill’, is not recommended as a primary choice of adulticide (McCall, Guerrero et al. 2001; Nelson, McCall et al. 2005; American Heartworm Society 2010). Additionally, a study on client-owned dogs with naturally-acquired infections showed worsening of disease after two years of

IVM treatment (Venco, McCall et al. 2004). Instead, the ‘slow kill’ treatment is reserved for cases in which melarsomine use is not advised due to the age of the animal or medical complications (McCall, Genchi et al. 2008).

In dogs with a high risk of post-adulticidal pulmonary thromboembolism or suffering from caval syndrome, the surgical extraction of adult worms is a viable treatment option. This is achieved by introduction of a rigid or flexible alligator forceps into the jugular vein of the animal and, preferably with fluoroscopic or endoscopic guidance, removal of worms from the caudal vena cava, right chambers of the heart, and pulmonary artery (McCall, Genchi et al. 2008; American Heartworm Society 2010). This procedure is relatively safe because it minimizes *in situ* death of the worms and avoids arsenic toxicity, though adulticidal chemotherapy is recommended following the surgery to eliminate the few remaining worms (Morini, Venco et al. 1998; American Heartworm Society 2010). The need for local anesthesia of the animal along with specialized training and equipment are downsides that complicate the surgical extraction method (Bowman and Atkins 2009).

Chemoprophylaxis of heartworm infection

Considering the inherent health risks and high veterinary costs associated with treating mature heartworm disease, prevention is a far more attractive approach, especially with the availability of safe and effective chemoprophylactics. In 1947, the microfilaricidal effects of diethylcarbamazine (DEC) were reported, and among the first filarial species tested with this drug was *D. immitis* (Hewitt, Kushner et al. 1947). The mechanism of action remains unclear; no *in vitro* effect is observed at clinically-relevant concentrations in microfilariae (Johnson, Mackenzie et al. 1988) or L_3 (Jordan 1958), yet the drug’s *in vivo* action against microfilariae is rapid (Hawking and Laurie 1949). Current evidence indicates that DEC interferes with the host’s arachidonic acid metabolic pathway, increasing the adhesion of microfilariae to endothelial cells and granulocytes (Maizels and Denham 1992). However, clear ultrastructural damage is done to microfilariae treated with DEC *in vitro*, suggesting that clearance is not exclusively host-mediated (Alves, Brayner et al. 2005). The prophylactic is administered daily at 5.5 mg/kg bodyweight in the form of a

chewable tablet (Filaribits®, Pfizer Animal Health, New York, NY; Nemacide®, Boehringer Ingelheim, St. Joseph, MO), but missed doses can compromise the animal's protection against heartworm larvae. In addition to the need for daily dosing, anaphylactoid reactions are seen when DEC is used in microfilaremic dogs (Courtney 1988; McCall, Genchi et al. 2008). With the advent of MLs for heartworm prevention, DEC fell into limited use and is no longer marketed in the United States, though it remains an important drug in lymphatic filariasis control programs (WHO 2010). For a time, prior to the introduction of ML drugs, twice-yearly administration of thiacetarsamide at the adulticidal dosage was an alternative to DEC for heartworm prophylaxis in cases where the latter was not advised (American Heartworm Society 1983).

Since their introduction, the MLs have proven to be safe and effective anthelmintics and are now the most widely-used heartworm preventives. The ML drug class includes the avermectins and milbemycins, which are both similar to antibacterial macrolides and antifungal polyenes but do not exhibit antibacterial or antifungal properties (Rugg, Buckingham et al. 2005). In 1974, microbial screening studies (part of a collaborative program by Merck, Sharp and Dohme in the United States and the Kitisato Institute in Japan) found that the fermentation products of *Streptomyces avermitilis* possessed anthelmintic effects in a murine infection model with the gastrointestinal nematode *Nematospiroides dubius* while exhibiting little or no toxicity to the vertebrate host. The name avermectin was applied to the isolated compound responsible for these effects, which was found to have activity against a wide variety of nematodes, insects, and arachnids (Omura and Crump 2004). The fermentation of *S. avermitilis* yields four homologous pairs of similar compounds (A_1, A_2, B_1, and B_2) with a major and minor component in each pair at a ratio of about 4:1 (for example, abamectin is >80% B_{1a} and <20% B_{1b}) (Lasota and Dybas 1991).

Ivermectin

The semisynthetic compound IVM (22,23-dihydroavermectin B_1; Figure 1.2) was released to the animal health market for use in cattle in 1981 and, owing to its high therapeutic index and broad spectrum of activity, has since become the biggest-selling antiparasitic drug in the world (Shoop and Soll 2002). IVM

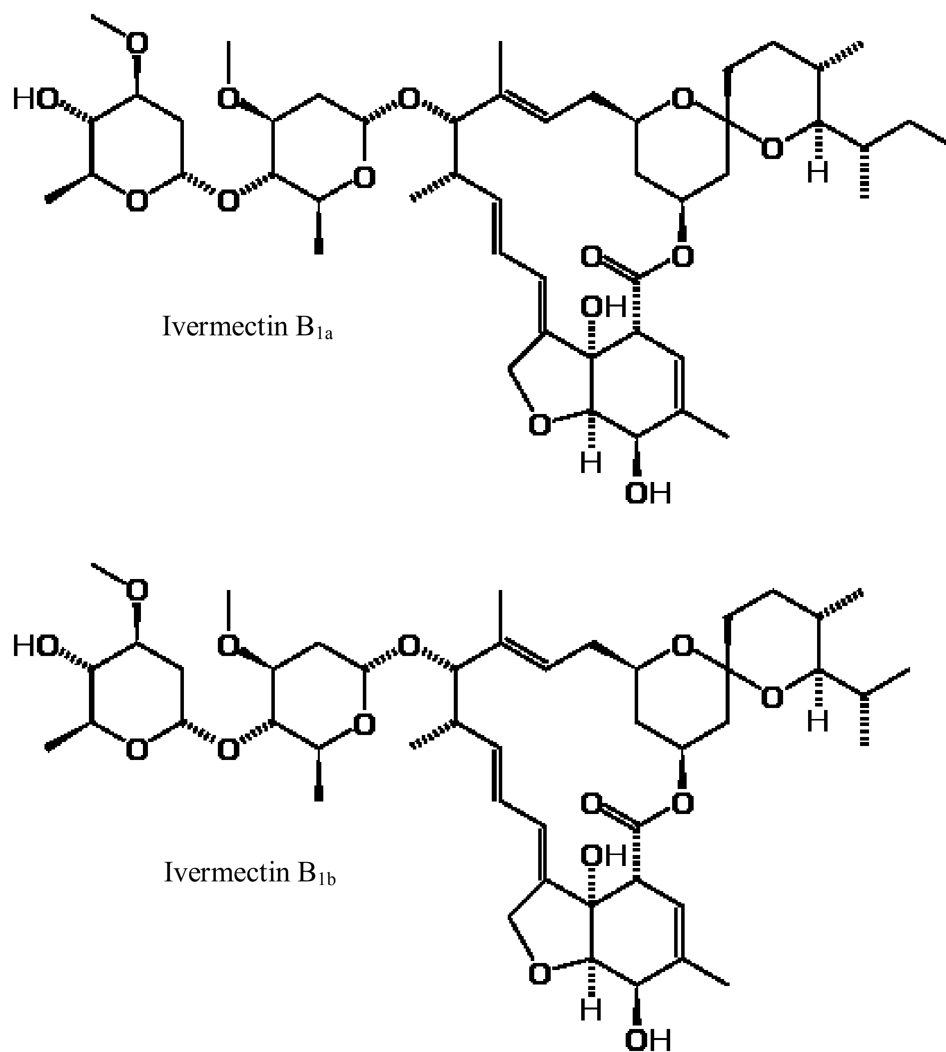

Figure 1.2: Chemical structure of ivermectin. The macrocyclic lactone ivermectin is composed of a mixture of the major component (ivermectin B_{1a}) and the minor component (ivermectin B_{1b}). The milbemycins share a similar 16-carbon lactone ring with the avermectins but lack the disaccharide moiety at C13 position.

was initially marketed for the control of nematodes and arthropods parasitizing livestock, and was first approved by the FDA for use in horses in 1983 as Eqvalan® (Merck and Co., Rahway, NJ) (Campbell and Blair 1983; Campbell, Fisher et al. 1983). The activity of avermectin B_{1a} against *D. immitis* in dogs and ferrets was known during the early development of this drug class (Campbell and Blair 1978). Later, IVM was found to prevent larval maturation in dogs treated with as single dose of 50 µg/kg up to two months post-infection (Blair and Campbell 1980). Monthly doses as low as 1 µg/kg (for 19–20 months) were found to be 100% effective in preventing the development of naturally-acquired heartworms (McCall, Cowgill et al. 1983; McCall, Dzimianski et al. 1986). Based on the results of these and several other studies, a monthly 6 µg/kg dosage of IVM was selected for HEARTGARD-30® (Merck and Co., Rahway, NJ) when it was released in 1987; this relatively high dose was chosen to ensure complete prevention of heartworm maturation in dogs (Campbell 1989; Bowman and Atkins 2009). Indeed, this dosage was found to be completely effective against two-month-old heartworm infections (McCall, Dzimianski et al. 1986). Even at three and four months post-infection, prolonged treatment with the prophylactic dose was 97.7% and 95.1% effective, respectively, against heartworm development (McCall, Guerrero et al. 2001).

IVM (0.05 mg/kg) was found to be 100% effective against *D. immitis* L_3 in dogs when treated as soon as one day post-infection (Blair, Williams et al. 1982) and it retains some activity against older larvae and adults (McCall, Guerrero et al. 2001). A single administration of the prophylactic dose was shown to be completely effective against two-month-old larvae (McCall, Dzimianski et al. 1986). This 'reach-back' effect has been reported in all ML preventives and helps ensure protection even when doses are delayed or missed (McCall 2005). Microfilariae are also eliminated during prolonged IVM treatment; at the prophylactic dose, only a single microfilaria was observed in one out of five dogs with four-month-old infections after four months of treatment (McCall, McTier et al. 1995). The potency of the drug against larvae during the first two months in the canine host indicates that the tissue-dwelling L_3 and L_4 are the most susceptible stages and, therefore, the best targets for treatment.

In addition to its effects against heartworms, IVM is active against almost all gastrointestinal worms of the canine host at dosages of 200 µg/kg, but the idiosyncratic sensitivity of some lines of collies and

other breeds to doses higher than 100 µg/kg has prevented the drug's application against them (Pulliam, Seward et al. 1985; Shoop and Soll 2002). Because the heartworm prophylactic dose is too low to kill gastrointestinal nematodes, to provide broad-spectrum activity against these parasites, pyrantel pamoate (5 mg/kg) was added to the chewable formulation of IVM (HEARTGARD® Plus, Merial, Duluth, GA) and introduced to the veterinary market in 1993 (Dzimianski, Roberson et al. 1992; Shoop, Michael et al. 1996; Guerrero, McCall et al. 2002).

Selamectin

Another avermectin widely used as a heartworm preventive is selamectin (SEL), a semisynthetic monosaccharide oxime derivative of doramectin (Conder and Baker 2002). SEL was developed specifically to provide protection against the cat flea (*Ctenocephalides felis*) while maintaining activity against heartworm larvae (Banks, Bishop et al. 2000; Bishop, Bruce et al. 2000). Introduced in 2000, the drug is marketed as topical solution (Revolution®, Pfizer Animal Health, New York, NY) at a monthly dosage of 6–12 mg/kg body weight for dogs and cats, and is safe for use in avermectin-sensitive collies (Novotny, Krautmann et al. 2000; Conder and Baker 2002).

A reach-back effect against older worms is also observed with SEL, as a single dose of 6 mg/kg was shown to be 100% effective against heartworm larvae up to two months post infection in dogs (McTier, Shanks et al. 2000). SEL treatment is also highly effective against certain gastrointestinal worms and ectoparasites (McTier, Siedek et al. 2000; Guerrero, McCall et al. 2002).

Milbemycin oxime

Along with the avermectins, the structurally similar milbemycins belong to the ML drug class and are used in heartworm prevention. These differ from the avermectins principally in that they lack a disaccharide moiety at the C13 position. The milbemycins were first discovered in 1967, noted for their acaricidal activity, and represent a mixture of similar compounds produced by the actinomycete *Streptomyces hygroscopicus* subspecies *aureolacrimosus* (Ide, Okazaki et al. 1993). The major

metabolites yielded in fermentation are milbemycin A_3, A_4, and D, with the lattermost marketed in Japan for some years as a heartworm preventive. The 5-oxime derivatives of A_3 and A_4 (milbemycin oxime; MBO) were later developed in efforts to improve upon the safety, efficacy, and production of natural milbemycins (Jung, Saito et al. 2002). MBO at a dosage of 0.5 mg/kg was released in 1990 as a monthly oral heartworm preventive for cats and dogs (Interceptor®, Novartis Animal Health, Greensboro, NC).

Monthly doses of MBO as low as 0.25 mg/kg were reported to be 100% effective in laboratory heartworm infections (Bradley 1989). Additionally, multiple treatments at the prophylactic dose as late as 60 days post-infection were found to be completely effective (Grieve, Frank et al. 1991).

Moxidectin

Moxidectin (MOX), also a milbemycin, is a semisynthetic derivative of nemadectin (also known as F-alpha), which itself originated from the fermentation of *Streptomyces cyaneogriseus* subspecies *noncyanogenus* (Rock, DeLay et al. 2002). Oral administration of the drug (once monthly, 1 µg/kg) was 100% effective against heartworm development in both naturally-infected (McCall, McTier et al. 1992) and experimentally-infected dogs (treated two months post-infection with 0.5 µg/kg) (McTier, McCall et al. 1992). Building on studies demonstrating the efficacy of a timed-release IVM bolus against endo- and ectoparasites in small ruminants, a sustained-release formulation of MOX microspheres was developed. When administered subcutaneously to dogs 180 days pre-infection, doses of 0.17 mg/kg were 100% effective against heartworm development (Lok, Knight et al. 2001). At this dosage, ProHeart® 6 (Fort Dodge Animal Health, Fort Dodge, IA) was approved by the FDA in 2001 for use every six months as a heartworm preventive. An unusual number of adverse event reports in dogs prompted a voluntary recall of the product from the US market in 2004, with a return in 2008 (Scheid 2008). Sustained-release MOX was shown to be safe in IVM-sensitive collies and microfilaremic dogs (Blagburn, Paul et al. 2001). A topically-applied formulation of MOX (2.5 mg/kg) was added to an existing imidacloprid product (for fleas) and marketed as the monthly heartworm and flea preventive Advantage Multi® (Bayer HealthCare, Shawnee Mission, KS), approved by the FDA in 2006.

Pharmacokinetics of the macrocyclic lactones

The effective use of a drug relies on an appreciation of its pharmacokinetic disposition within the animal being treated, and this is evidenced by the various dosages and formulations developed for the prevention of heartworm disease. While DEC therapy must regularly maintain larvicidal concentrations in the animal, monthly doses of MLs periodically kill larvae prior to their entrance into the bloodstream (Lok, Knight et al. 2001). Within the MLs, differences in drug bioavailability exist. At equal dosages (250 µg/kg administered orally), significantly higher plasma levels were observed for MOX compared to IVM; a peak plasma concentration (C_{max}) of 234.0 ng/ml was measured for MOX and 132.6 ng/ml for IVM, while a terminal half-life of about 26 days was measured for MOX and about 3 days for IVM (Al-Azzam, Fleckenstein et al. 2007). This is because MOX is much more lipophilic than IVM, and thus more likely to persist in the treated animal's fatty tissue (Hennessy and Alvinerie 2002). Characteristics of the treated animal (including diet, bodyweight, and sex) also affect drug bioavailability. Topically-applied SEL (6 mg/kg) reaches a C_{max} of 12.72 ng/ml in male dogs and 22.65 ng/ml in female dogs, with a terminal half-life of about 12 days in both (Dupuy, Derlon et al. 2004). Thus, considerations of efficacy must be made in light of each drug's particular pharmacokinetic disposition.

Mode of action of the macrocyclic lactones

The MLs came to be such successful drugs in large part because of their effect on a broad range of nematodes, insects, and acarines combined with minimal toxicity to vertebrates. However, these drugs are not effective against all developmental stages of nematodes, nor are they effective against flukes and tapeworms (Martin, Robertson et al. 2002). Since the introduction of the MLs, numerous studies have attempted to elucidate their mechanism of action, and this model is still being developed.

Early in its use as an anthelmintic, IVM was found to increase chloride conductance in neuronal membranes and this effect was blocked by picrotoxin. Because picrotoxin inhibits the action of γ-aminobutyric acid- (GABA-) gated chloride channels, which are involved in nematode locomotion, IVM was hypothesized to act as a GABA agonist (Pong and Wang 1982; Olsen and Tobin 1990; McIntire,

Jorgensen et al. 1993). While this would explain the lack of activity against flukes, tapeworms, and mammals (in which GABA receptors are either absent or, in the lattermost, protected by the blood-brain barrier), conflicting evidence has suggested that this is not a principal mode of action for the MLs (Scott and Duce 1987; Wolstenholme and Rogers 2005).

Later work revealed another ligand-gated chloride channel now thought to be the target of ML activity. Expression of *Caenorhabditis elegans* mRNA in *Xenopus* oocytes led to the discovery of a glutamate-gated chloride channel (GluCl) with an IVM-sensitive α subunit and a glutamate-sensitive β subunit (Arena, Liu et al. 1991; Arena, Liu et al. 1992; Cully, Vassilatis et al. 1994). Consistent with this, GluCls are unique to invertebrates and are found in all phyla susceptible to the MLs (Wolstenholme 2010). A number of GluCl subunits have been identified in *C. elegans* and other organisms, each composed of four membrane-spanning domains (M1-M4), an extracellular domain containing the ligand-binding site, and a cytoplasmic loop between M3 and M4. Five of these subunits associate in the membrane to form complete ion channels, either as homo- or heteropentamers, such that the M2 domains are arranged as the pore lining (Cully, Vassilatis et al. 1994; Martin, Robertson et al. 2002). In nematodes, GluCls are present in various motor neurons and the pharyngeal muscle tissue. These are also the sites to which a fluorescent IVM derivative has been shown to localize in *Ascaris suum* (Martin, Kusel et al. 1992). ML binding disrupts neurotransmission by inducing slow but irreversible opening of these channels leading to chloride influx, hyperpolarization of cells, and, consequently, the death of the worm by paralysis or starvation (Martin, Robertson et al. 2002; Wolstenholme and Rogers 2005). At low concentrations, ML binding seems to potentiate channel opening in the presence of otherwise insufficient levels of L-glutamate (Cully, Vassilatis et al. 1994); recombinant GluCls are shown to be activated by IVM at concentrations as low as 0.1 nM (McCavera, Rogers et al. 2009). MLs do not compete for the L-glutamate binding site but act at a separate location, between M1 and M3 of two adjacent subunits, forming hydrogen bonds with each as well as with the M2 domain of the latter subunit. This site lies within the lipid bilayer, proximal to the extracellular side, where IVM effects local and global conformational changes when bound (Hibbs and Gouaux 2011). Evidence suggests that the ML binding

site is similar to that for general anesthetics in vertebrate $GABA_A$ receptors (Wolstenholme 2010). Furthermore, certain amino acids lining a water-filled pocket at this site have been reported to be critical for high-affinity ML binding (Kwon, Yoon et al. 2010; Lynagh and Lynch 2010).

Among nematodes, the adult filariae are noted for their insusceptibility to MLs. The reason for this is unknown, but it may be that the major processes targeted by the MLs (pharyngeal pumping and motility) are not essential for the survival of macrofilariae in the host (Wolstenholme and Rogers 2005). Filarial worms possess incomplete pharynxes, with nutrient uptake thought to occur through the cuticle, and, unlike gastrointestinal nematodes, they do not depend on musculature to maintain their position in the host (Chen and Howells 1981; Howells and Chen 1981). However, adult females do depend on uterine musculature for the release of microfilariae, and, indeed, reduced microfilarial output is associated with ML treatment in filarioids (Tompkins, Stitt et al. 2010).

The *in vitro* application of MLs to microfilariae does not reduce motility at pharmacologically relevant concentrations (Tagboto and Townson 1996; Tompkins, Stitt et al. 2010). It has, however, been demonstrated that GluCls in the microfilarial excretory-secretory (ES) system bind MLs, thereby inhibiting the release of protein. Based on these data it is hypothesized that MLs act by inhibiting the release of immunomodulatory ES products, thus facilitating clearance by the host immune system (Moreno, Nabhan et al. 2010). This model draws support from the current understanding that filarial parasites can suppress various immune factors and that this is a significant facet of their relationship with the host (Hoerauf, Satoguina et al. 2005). Furthermore, studies with *Acanthocheilonema viteae* microfilariae demonstrated that, while this filarioid is unaffected by nanomolar concentrations of IVM *in vitro*, it is subject to cell-mediated cytotoxicity when treated in the presence of *Mastomys* serum (Rao, Chandrashekar et al. 1987). This effect was prevented by heat-inactivation of serum, indicating the potential significance of a host immune component. Nonetheless, the precise mechanism with which the MLs exert their effect on nematodes remains unclear and the subject of active research.

Anthelmintic resistance

Resistance in a nematode population can be defined as a heritable trait occurring when a greater number of individuals tolerate anthelmintic doses than in a normal, susceptible population (Prichard, Hall et al. 1980). Anthelmintic resistance has been observed since the 1950s, initially in the treatment of *Haemonchus contortus* infection with phenothiazine (Drudge, Leland et al. 1957). With the introduction of new anthelmintic classes (benzimidazoles, imidazothiazoles/tetrahydropyrimidines, and MLs) resistance has followed (Kaplan 2004). Because the effect of a drug on individual parasites is a function of host physiology, the parasite's age and location within the host, and many other factors, identifying resistant phenotypes can be greatly complicated. Still, practical definitions may follow; for instance, reduction of < 95% in fecal egg counts indicates resistance in some gastrointestinal nematode infections (Coles, Jackson et al. 2006). As such, resistance to MLs has been reported in a number of nematode species, beginning with IVM-resistant *H. contortus* in South Africa (van Wyk and Malan 1988). Despite the tremendous efficacy of this drug class upon its introduction to the veterinary market, evidence for resistance in target organisms has been increasingly common and the implications increasingly severe. ML resistance in gastrointestinal nematodes of the genera *Teladorsagia*, *Ostertagia*, *Trichostrongylus*, *Cooperia*, *Nematodirus*, *Parascaris* and others has been reported in the field (Waghorn, Leathwick et al. 2006; Craig, Diamond et al. 2007; Sutherland, Damsteegt et al. 2008; Edmonds, Johnson et al. 2010). Resistance in the filaria is also suspected following observations of suboptimal efficacy in *O. volvulus* (Awadzi, Boakye et al. 2004; Osei-Atweneboana, Eng et al. 2007; Osei-Atweneboana, Awadzi et al. 2011). However, owing in part to the complex life cycles and culture requirements of filarial parasites, resistance has not yet been confirmed in any species.

Currently, the bulk of evidence for anthelmintic resistance in *D. immitis* lies in lack of efficacy (LOE) reports, along with some recently published controlled studies (Blagburn, Dillon et al. 2011; Snyder, Wiseman et al. 2011). The FDA began receiving heartworm preventive LOE complaints in 1998, proceeding to monitor these events and standardize reporting guidelines. A sharp increase in reports was seen in 2000, reaching as many as 1,503 in the year 2003 (Hampshire 2005). Some explanations for

prophylaxis failure exist outside the development of resistance, including compliance failure; pet owners may not administer the preventive correctly or veterinarians may not be encouraging proper use. The rejection of oral treatments by the pet or even physiological idiosyncrasies may also be responsible. The consistent and proper administration of heartworm preventives is not always practiced, and this may account for some or all LOE cases (Rohrbach, Odoi et al. 2011). However, the FDA determined that 20–35% of LOE reports had a "high" likelihood of drug failure and this range was observed for all commercial preventives (Hampshire 2005).

Heartworm preventives are approved by the FDA for 100% efficacy in preventing development to the adult stage (Hampshire 2005), so any failure is cause for concern. A recent controlled study (in which compliance failure should not be a factor) examined the efficacy of commercially available IVM and MBO preventives on a laboratory heartworm infection (Snyder, Wiseman et al. 2011). Given a single treatment 30 days post-infection, one adult worm was recovered from one dog out of fourteen in each treatment group. Another study assessing the efficacy of four ML preventives, administered as a single dose 30 days post-infection, also found incomplete protection (Blagburn, Dillon et al. 2011). Here, adult worms were recovered in seven out of eight dogs in all but one of the treatment groups. Both studies used the MP3 strain of *D. immitis*, which was isolated in June 2006 in northeast Georgia from a dog with no record of ML exposure (Blagburn, Dillon et al. 2011). Persistent microfilaremia has also been observed in a particular case following multiple high-dose ML treatments (Bourguinat, Keller et al. 2011). While these reports of suboptimal efficacy may expose some potential inadequacies in current treatment protocols, they do not necessarily indicate true resistance in heartworms.

The development of resistance

The emergence of ML resistance in other nematode species and the serious impact being realized in parasite control encourage the study of how this phenomenon arises and what precautionary measures can be taken. The selection of anthelmintic resistance in heartworms would involve many varied factors, including parasite biology at the individual and population levels, parasite genetics, and treatment

practices. These will be addressed sequentially below, but many aspects of selection pressure are interrelated.

Parasite biology

Aspects of a nematode's life cycle and biology can affect the rate at which anthelmintic resistance occurs and may explain why some species develop resistance so rapidly. A high rate of reproduction is conducive to the spread of parasites harboring resistance alleles. Certainly, the widely-resistant *H. contortus* is noted for a high reproduction rate and rapid generational turnover (Prichard 2005). *Dirofilaria immitis* also exhibits a high reproduction rate (greater than 10,000 microfilariae per female per day has been observed *in vitro*) indicating that resistance alleles could be rapidly inherited when they occur (Tamashiro and Palumbo 1985). Acting contrary to this, however, is the long lifespan of the heartworm once it establishes itself in the definitive host. This would ensure that susceptible progeny continue to be produced in untreated hosts, effectively diluting resistance alleles in the population at large.

Another factor affecting the development and maintenance of anthelmintic resistance is the relative reproductive fitness of resistant worms compared to their susceptible counterparts. If the cost of resistance alleles to the parasite negatively affects its ability to survive and reproduce, then reversion to a susceptible state is expected when selection pressure is lifted (Prichard 2005). Because reversion of resistant gastrointestinal nematodes has not been observed when drug pressure is removed, the cost of resistance is thought to be low (Waller, Dobson et al. 1988; Kaplan 2004). Furthermore, the high efficacy levels of MLs against heartworm larvae ensure that survival and reproduction would likely be limited to the most resistant individuals within a population.

Parasite genetics

Anthelmintic resistance is defined as a heritable trait and, as such, it must arise from the selection of existing alleles in a susceptible population or the production of new alleles from genetic mutation or

recombination. A feature of nematodes that forms the basis of this development is their high level of genetic polymorphism. This improves the opportunity for resistance alleles to arise and, in the presence of adequate pressure, to be selected. In a recent study of multiple populations of *D. immitis*, the genetic heterogeneity of this species was confirmed, with implications that support the potential emergence of resistance (Bourguinat, Keller et al. 2011).

Given the genetic variability in nematode species, the frequency of preexisting resistance alleles in unselected populations helps drive the rate at which resistant phenotypes will emerge. Because the genes responsible for ML resistance are uncertain, this phenomenon cannot be adequately measured. In *O. volvulus*, however, an ATP-binding cassette (ABC) transporter gene polymorphism associated with drug treatment pressure is found in unselected worms, suggesting that this potential resistance allele is already present in the worm population (Ardelli and Prichard 2004). A better-established β-tubulin polymorphism conferring benzimidazole resistance was also found at moderate levels in an unselected *W. bancrofti* population (Schwab, Boakye et al. 2005).

Even when resistance alleles occur in the parasite, expression of a resistant phenotype may depend on multiple genes. Indeed, current evidence suggests that ML resistance is polygenic (Ardelli and Prichard 2004; McCavera, Walsh et al. 2007). This may convolute the development of resistance or the level of resistance may reflect an accumulation of certain alleles. Developing the resistant phenotype may be further complicated if these alleles are recessive. When a resistance allele is dominantly expressed, the phenotype is expected to arise more often in the nematode's progeny. In the case of *H. contortus*, ML resistance was reported to be dominant—an observation supported by its rapid spread in this species (Le Jambre, Gill et al. 2000).

Treatment practices

In addition to the inherent traits mentioned above, selection pressure for drug resistance in heartworms must also be a function of treatment practices. Perhaps the most critical consideration in the application of anthelmintics is parasite refugia; that is, the proportion of parasites not exposed to drug at the time of

treatment. Nematodes may avoid the effects of drug because of their particular host, location within the host, or by living outside the host (van Wyk 2001; Kaplan 2004). In the case of heartworms, the great majority of the population resides in the definitive host because the vector stage is relatively brief with a low worm capacity (Prichard 2005). In itself, that would suggest a high selection pressure during treatment, but a large proportion of worms still find refugia in wild canids and companion animals not on preventives (Nelson, Gregory et al. 2003; Sacks and Caswell-Chen 2003; Merial Limited 2011). This would tend to slow the development of ML resistance by diluting out worms that might survive treatment. The frequency of drug treatment is another factor affecting the selection of resistance. Most ML preventives are administered on a monthly basis, and this fairly persistent treatment regimen is expected to place a high selective pressure on the worms. But because of the large numbers of untreated hosts, as mentioned above, frequent treatment is not expected to be a significant source of selection pressure (Prichard 2005).

One of the principal advantages of ML heartworm preventives is the low dose at which they achieve 100% efficacy. However, if a dosage achieves high, but incomplete, efficacy, a strong selection for resistant parasites exists. Preventing the emergence of resistance in heartworms depends to some degree on the complete larvicidal activity of the MLs and avoiding their use on less susceptible parasite stages. The 'slow kill' treatment method for mature infections is not advised because of the selection pressure it is believed to exert on juveniles, adults, and microfilariae. Still, current guidelines suggest ML therapy in mature heartworm infection for three months prior to adulticide administration with the goal of allowing immature adults to reach a stage susceptible to adulticides and removing late L_4 before they reach a stage susceptible to neither drug (American Heartworm Society 2010). Similar to 'slow kill' therapy, some stages of the parasite are exposed to sublethal drug concentrations and, in theory, retain the ability to pass on resistance to their progeny. For this reason, the use of MLs in any mature heartworm infection is controversial.

The molecular basis of resistance

Efforts to elucidate how resistance manifests itself in nematodes at the molecular level may prove essential to slowing or reversing its development, either through the design of new anthelmintics or refining the treatment strategies for existing drugs. No known ML-resistant isolates of *D. immitis* exist, so an understanding of potential resistance mechanisms relies on evidence from other species. ML resistance in nematodes is currently thought to arise from mutations in the target proteins and/or changes in drug distribution within the parasite.

As discussed earlier, the most likely targets of ML treatment are ligand-gated chloride channels, and it has been proposed that mutations in the genes that encode these proteins may confer anthelmintic resistance (McCavera, Walsh et al. 2007). By comparing resistant and susceptible populations of *H. contortus* a number of genes have been implicated, including the GABA-gated chloride channel genes *Hco-lgc-37* and *Hco-ggr-3*, and the GluCl subunit genes *Hco-glc-5* and *Hco-avr-14* (Blackhall, Pouliot et al. 1998; Blackhall, Prichard et al. 2003; Rao, Siddiqui et al. 2009). The lattermost is a widely conserved gene in nematodes and has been associated with ML resistance in multiple species (Dent, Smith et al. 2000; Njue, Hayashi et al. 2004). Resistance-associated alleles of the *Hco-glc-5* and *Hco-lgc-37* genes were found to affect the normal feeding and motility exhibited by *H. contortus* (behaviors affected by ML treatment), supporting their putative link to resistance (Wolstenholme and Rogers 2005; Beech, Levitt et al. 2010). In field isolates of the parasite, however, these alleles have not been associated with resistance, casting some doubt on their relevance (Beech, Levitt et al. 2010). Some channel subunits known to be IVM-sensitive do not appear to be under selection, as is the case for *Hco-glc-2* (Blackhall, Pouliot et al. 1998). A goal of this area of research is to identify specific polymorphisms associated with resistance, but the variety of alleles selected in worms of various backgrounds greatly complicates this endeavor.

Resistant nematodes are also believed to modulate the distribution of MLs during treatment, preventing the drugs from reaching their active sites. MLs are known to be substrates of P-glycoprotein (P-gp), a transmembrane protein of the ABC transporter family involved in drug efflux (Alvarez, Merino et al. 2006). In mammals, P-gp prevents IVM from crossing the blood-brain barrier and causing toxicity

in the central nervous system (Schinkel, Smit et al. 1994). In fact, it is a defect in this protein that leads to the IVM sensitivity observed in certain collies (Mealey, Bentjen et al. 2001). The expression of P-gp in *H. contortus* was found to be increased in ML-selected parasites, with increased susceptibility following treatment with verapamil, a P-gp inhibitor (Xu, Molento et al. 1998). This implicates ML removal by P-gp as a mechanism of resistance in nematodes. A number of P-gp genes in *O. volvulus* have also been associated with ML selection, supporting its relevance to filarial parasites (Prichard and Roulet 2007). Recent findings have correlated a GG-GG genotype for two single nucleotide polymorphisms (SNPs) in a *D. immitis* P-gp gene with a reduced microfilarial response to MLs (Bourguinat, Keller et al. 2011). In the future, this may serve as a genetic marker for resistance in heartworms. Because of the accumulating evidence of drug transporter involvement, it has been proposed that P-gp inhibitors be co-administered with MLs to improve drug availability within the parasites and restore susceptibility to currently resistant populations (Lespine, Alvinerie et al. 2008). Whatever the results of such a treatment strategy, insights into resistance mechanisms are sure to be gained.

The role of a polymorphism in β-tubulin is already established in benzimidazole resistance, but research has also shown this protein to be involved in resistance to MLs (Mottier and Prichard 2008). Following the use of IVM against *O. volvulus* in the field, selection for a β-tubulin allele in this parasite was observed, with three amino acid changes from the allele found in ML-naïve worms (Eng and Prichard 2005; Eng, Blackhall et al. 2006). How these changes relate to a resistant phenotype is still unclear, but a degeneration of amphidial neurons and the microtubules within them has been implicated from observations in resistant *H. contortus* (Freeman, Nghiem et al. 2003). The amphids are a pair of channels at the anterior end of nematodes, housing sensory neurons exposed to the environment, and it has been proposed that they may be a route of entry for MLs—a pathway theoretically blocked by amphidial deformation (Wolstenholme and Rogers 2005). In *H. contortus*, a β-tubulin polymorphism at codon 200 has also been linked to ML-resistance (Eng, Blackhall et al. 2006). This is the same codon under selection in benzimidazole resistance, and, though IVM selects for heterozygosity at this position and the benzimidazoles for homozygosity, there is a possibility that selection for one may confer resistance to the

other, with practical implications for treatment strategies that use both anthelmintic classes (Prichard and Roulet 2007).

It should be noted that the avermectins and milbemycins, while similar, do exhibit differences. The *in vitro* effects of IVM and MOX on *C. elegans* were shown to differ in some regards, including the effects on motility, development, and pharyngeal pumping in the nematode (Ardelli, Stitt et al. 2009). This might indicate that resistance to one ML could occur in a manner different from resistance to another. However, resistance to multiple MLs was observed at similar levels in nematodes only selected for resistance to one (Molento, Wang et al. 1999; Ranjan, Wang et al. 2002). This reported cross-resistance suggests that one ML should not be replaced by another as a means of curbing selection pressure.

Considering the various influences driving anthelmintic resistance in nematodes, including the disposition of the heartworm at the molecular and ecological levels and the treatment strategies in use, the potential for ML resistance developing in heartworms may exist. This scenario has been considered unlikely by some (Prichard 2005) while other reports claim heartworm resistance has already appeared (Bourguinat, Keller et al. 2011). Without new anthelmintics entering the market, the control of heartworms may be presented with serious challenges. Whatever the case, developing the means of identifying and monitoring the emergence of resistant worms would be very helpful in assessing the current situation and informing prophylaxis strategies.

Assays for resistance

Anthelmintic resistance is a widespread problem in many nematode species, and, while timely changes in treatment protocols may slow its development, it is often too late once the level of resistance manifests itself as treatment failure (Kaplan 2004; Demeler, Küttler et al. 2010a). For this reason, assays that recognize low levels of resistance in worm populations would be valuable tools in an effort to restrain it. Some assays have already been developed and validated for the detection of resistance in gastrointestinal nematodes, prominent among them the fecal egg count reduction test (FECRT); this assay uses the *in vivo* egg production of parasites before and after treatment as the parameter of drug efficacy (Coles, Bauer et

al. 1992). While successful for testing the parasites of ruminants, the FECRT is not applicable to *D. immitis*, nor is any *in vivo* assay because of the difficulty, cost, and time associated with raising worms in the definitive host.

Another measure of drug susceptibility in geohelminths is the maturation of larvae from the egg to the L_3, and this is assessed in the larval development assay (LDA). The LDA benefits from the relatively low concentrations of MLs required to inhibit maturation *in vitro*, providing a sensitive measure of worm health (Gill, Redwin et al. 1995; Demeler, Küttler et al. 2010a). However, this assay is not applicable to filarioids, which lack a corresponding free-living stage.

An *in vitro* assay that has already been applied to the heartworm involves the assessment of microfilarial motility. In this test, the motility of microfilariae following *in vitro* drug treatment is scored microscopically and efficacy is presented as the proportion of immotile worms (Blagburn, Spencer et al. 2008). Advantages of this approach are the use of the easily-obtained microfilarial stage and a relatively simple protocol; however, the assignment of motility scores introduces operator bias. A validation of this assay has not yet been published. A related technique involves the use of a micromotility meter, with the benefit of eliminating subjective motility scoring. This device measures rapid changes in the diffraction of light passing through worms in culture, indirectly measuring their movement. The assay has been applied with some success in discriminating resistant and susceptible *Cooperia oncophora* populations (Demeler, Küttler et al. 2010a), but with mixed results in assessing filarioid drug responses (Satti, Van de Waa et al. 1988).

A promising test for use with *D. immitis* is the larval migration inhibition assay (LMIA). The protocol for this assay involves an *in vitro* drug treatment period followed by a migration period, in which larvae are allowed to pass through a fine mesh, with the proportion of larvae inhibited from migrating being the measure of drug susceptibility. By the end of the assay, motile worms are separated from the immotile so they only need to be enumerated, thus removing the operator bias associated with scoring motility (Kotze, Le Jambre et al. 2006). The first LMIAs measured the ability of larvae to migrate out of agar blocks after drug treatment, mimicking the migration of gastrointestinal nematodes through mucus to the gut wall

(Douch, Harrison et al. 1983). This was inspired by a collection method for such worms in which total ingesta is suspended in agar and larvae migrate into a surrounding bath of saline solution (van Wyk and Gerber 1978). The difficulty and inconsistency involved in preparing agar blocks containing viable larvae encouraged the development of a mesh-based LMIA (Sangster, Riley et al. 1988; Wagland, Jones et al. 1992). The advantages of this assay were its simplified setup, minimal damage to the larvae, and the consistency afforded by using nylon mesh. Over the years, further modifications to the LMIA were made in efforts to provide improved drug responses for various nematode species.

One such modified assay was described by Kotze et al. that used a low viscosity agar layer to add a further level of discrimination for larval inhibition (2006). In this LMIA, about 60 L_3 were aliquoted into each well of a 96-well microtiter plate, to which dilutions of drug were added. Larvae were incubated in the presence of drug for 24h and then transferred to a filter plate (in the bottom of which were 20-µm mesh screens overlaid with 0.125% agar) set on top of a corresponding microtiter plate, into which sufficiently motile larvae migrated over the course of 48h. Migration rates for larvae in each drug concentration were expressed as the percentage of larvae migrating in untreated controls.

From these data, dose-response curves can be plotted, which reflect the susceptibility of a normally-distributed worm population to the anthelmintic in question. This allows the calculation of the IC_{50} for each population, that is, the concentration of drug at which 50% of the measured response (such as migration through a mesh) is inhibited. A resistance ratio (RR) can then be expressed as a measure of the difference in susceptibility between nematode populations. RRs are equal to the IC_{50} of a resistant parasite strain divided by the IC_{50} of a susceptible strain. Therefore, greater RRs denote greater differences in the responses of the two strains, which is a desirable property for drug resistance assays.

More recently, Demeler et al. reported success with a geohelminth LMIA using mesh migration wells without an agar layer (2010b). This protocol involved a 24h incubation period followed by a 24h migration period, after which migrated larvae were counted while the mesh-bottomed migration wells were inverted, rinsed, and the remaining larvae enumerated. Thus, migration was calculated as a percentage of the total worms counted for each well.

Motility is not an effective measure of drug susceptibility in all nematode species; the motility of *H. contortus* correlates relatively well with ML-resistance (Gill, Redwin et al. 1991), but this is not the case with some *Teladorsagia (Ostertagia) circumcincta* strains (Gill and Lacey 1998). Furthermore, not all MLs are equally suited to differentiating resistant and susceptible worms in the LMIA; compared to ivermectin and abamectin, eprinomectin showed the highest RRs in the assay described above (Kotze, Le Jambre et al. 2006). The ideal ML for use in the LMIA, therefore, may not be one that is used clinically against the parasite in question. For these reasons, any *in vitro* assay for relative anthelmintic susceptibility requires careful and thorough validation. In *D. immitis*, microfilariae depend on motility to penetrate the Malpighian tubules of the arthropod host (Kartman 1953; Bradley, Sauerman et al. 1984) as does the infective L_3 for its entry into the definitive host and subsequent tissue migration (McGreevy, Theis et al. 1974; Kotani and Powers 1982). These active stages of the species seem well-suited to motility-dependent *in vitro* assays like the LMIA. The L_3 and L_4 are the target stages of ML prophylaxis (Blair, Williams et al. 1982; McCall, Guerrero et al. 2001), and are theoretically the most relevant stages for assessing the drug efficacy between populations. While the L_3 can be collected from lab-raised mosquitoes in large numbers, the difficulty in obtaining the L_4 precludes its use in a high-throughput bioassay. For the purpose of measuring drug susceptibility in heartworm populations, the L_3 is an attractive stage of the parasite to use in an LMIA.

Conclusions

The canine heartworm is a parasite responsible for serious illness in companion animals around the world, affecting quality of life and sometimes resulting in death. Though many preventive products are available for cats and dogs, they mainly represent a single class of anthelmintic—the MLs. Widespread resistance to this drug class is well-documented in gastrointestinal nematodes and lack of efficacy reports for heartworm preventives raise concern that these parasites may too develop resistance. Such a scenario demands the validation of a reliable and sensitive assay for diminished susceptibility in heartworm populations so that this phenomenon can be monitored before reaching the level of drug failure.

Assays for resistance have been developed and validated for other nematode species, mostly gastrointestinal parasites of small ruminants. The known effect of MLs on larval motility presents the use of heartworm L_3 in a motility-based assay, like the LMIA, as an attractive option. The aims of this study are to optimize the LMIA for use with *D. immitis*, generate consistent dose-response curves for a known ML-susceptible population and multiple suspected-resistant isolates, and to compare the susceptibility of the various isolates to EPR and IVM treatment as measured by their IC_{50} values. Such an assay, if effective, would be of great use in the detection of emerging anthelmintic resistance.

CHAPTER 2

INTRODUCTION

The canine heartworm (*Dirofilaria immitis*) is a parasitic filarial nematode and a serious parasite of dogs, cats, and other mammals. Infective larvae are transmitted by mosquitoes and adult parasites find residence in the heart and pulmonary arteries of their definitive host, where they can generate life-threatening pathology, including pulmonary hypertension and congestive heart failure (McCall, Genchi et al. 2008). Treatment of mature infection is costly and hazardous, requiring either surgical removal of the worms or injection of arsenical compounds, and so, monthly preventives that interrupt larval development are widely used (Bowman and Atkins 2009). While taking different forms, these heartworm preventives are all members of the macrocyclic lactone (ML) drug class and are all approved for 100% efficacy by the FDA at relatively low doses.

With the rise in lack of efficacy (LOE) reports, in which mature heartworms developed despite the administration of monthly preventives, the complete larvicidal activity of the MLs was called into question (Hampshire 2005). More recently, controlled studies on laboratory infections have confirmed that single doses of multiple preventive products do not provide total protection (Blagburn, Dillon et al. 2011; Snyder, Wiseman et al. 2011). While most LOE cases can be explained by some form of compliance failure, the rapid spread of ML resistance in other nematodes (particularly gastrointestinal parasites of small ruminants; Kaplan 2004) gives reason to investigate whether the same is appearing in heartworms. Diminished ML efficacy has even been reported in the related filarial parasite *O. volvulus* (Awadzi, Boakye et al. 2004; Osei-Atweneboana, Awadzi et al. 2011). When anthelmintic resistance manifests itself as drug failure, it has often progressed to a level too difficult to effectively contain, and so a sensitive assay is needed to detect decreases in parasite susceptibility while it still occurs at a low level.

A number of *in vitro* bioassays have been developed to detect resistance in various geohelminth species, but one assay that appears especially amenable to use with *D. immitis* is the larval migration inhibition assay (LMIA). Already applied successfully to trichostrongylids, the LMIA measures the fitness of larvae in their ability to migrate across a fine mesh following a drug treatment period (Kotze, Le Jambre et al. 2006; Demeler, Küttler et al. 2010b). The infective third-stage larvae (L_3) of heartworms depend on their ability to migrate through the muscle tissues of the vertebrate host for access to their predilection site, and this is the stage targeted by ML chemoprophylaxis (Abraham 1988). MLs are thought to affect nematodes by binding glutamate-gated chloride channels (GluCls) and hyperpolarizing motor neurons, resulting in paralysis (Wolstenholme and Rogers 2005). Therefore, motility seems to be a relevant and sensitive measure of drug efficacy, and it is this parameter that is tested by the LMIA. From the data generated by this assay, dose-response curves can be calculated and the IC_{50} values (concentrations at which 50% of migration is inhibited) can be compared between parasite populations.

In this study, we optimized the LMIA for use with *D. immitis* L_3. Treated *in vitro* with the MLs ivermectin (IVM) or eprinomectin (EPR), the relative susceptibilities of a laboratory strain of heartworm and three suspected-resistant isolates were compared. The ability to identify reduced susceptibility in heartworm populations will be valuable in determining the existence of genuine anthelmintic resistance in this species and in monitoring its potential emergence.

CHAPTER 3

MATERIALS AND METHODS

Maintenance of parasites

Dirofilaria immitis adults (2005 Missouri strain) were maintained in beagle dogs at the University of Georgia (Athens, GA) and passed through mosquitoes (*Aedes aegypti*, black-eyed Liverpool strain) to produce L_3. Throughout their life cycle, mosquitoes were kept at approximately 27°C, 90% RH.

Mosquitoes were hatched from eggs by overnight immersion in deionized water containing 300 mg of powdered mosquito food (rat chow, brewer's yeast, and lactalbumin). From a glass beaker on a magnetic stir plate, mosquito larvae were aliquoted in three volumes into glass jars containing about 900 ml deionized water and 50 mg mosquito food; the total volume of mosquito larvae was calculated so that about 200 larvae were dispensed into each jar. Larvae received mosquito food once daily on the second (100 mg), third (300 mg), fourth (400 mg), and fifth (500 mg) days after hatching. On the seventh day, pupae were passed through a pupal separator (as described by Focks, Fay et al. 1980) with male pupae discarded and female pupae retained; the incomplete separation of sexes in this method allows some males to be retained so that their population in the lab may be regenerated. The pupae, collected in polystyrene cups and housed in mesh-topped paper buckets, were provided with sugar cubes and moist cotton pads placed on the mesh above them as a source of food and water once adults emerged.

Eleven days after the mosquitoes hatched (four days after sorting pupae), blood was drawn from a *D. immitis*-infected beagle dog via the jugular vein and collected in heparin tubes (Becton Dickinson, Franklin Lakes, NJ). On the twelfth day, adult mosquitoes were fed on the microfilaremic canine blood, diluted to a concentration of 2,500 to 5,000 ml^{-1} with uninfected canine blood, promoting a survivable, yet productive, worm burden for each mosquito. The microfilaremic blood

was collected a day before feeding to simulate the delay experienced when receiving heartworm isolates shipped from other sources, controlling for any damage done to the microfilariae during that time; though no loss of fitness has been observed over this time. Mosquitoes were fed blood via artificial glass feeders for 3h; these allowed water at 37°C to be circulated through a water jacket in each, while mosquitoes fed through a paraffin film on 5 ml of blood per feeder. Every 3-4 days, as cotton pads dried out, they were exchanged for moist pads, on which adults eventually laid eggs, which were retained for future use.

Media

Heartworm culture medium was prepared from RPMI-1640 (Lonza BioWhittaker, Basel, Switzerland) with penicillin (10,000 U/ml; Gibco-Invitrogen, Carlsbad, CA) added at a rate of 100 U/ml, streptomycin (10,000 mg/ml; Gibco-Invitrogen) at a rate of 100 mg/ml, and gentamicin (10 mg/ml; Sigma, St. Louis, MO) at a rate of 40 µg/ml. NCTC/IMDM (NI) medium was prepared from equal parts NCTC medium (Sigma) and IMDM (Sigma) with L-glutamine (200 mM; Sigma) added to a concentration of 2 mM and penicillin/streptomycin added as above.

Collection of parasites

Fourteen days after feeding mosquitoes on microfilaremic blood, the L_3 were isolated for use in the LMIA. Adult mosquitoes were collected into a mesh drawstring bag using a vacuum chamber and, having been temporarily paralyzed by impact within the bag, were emptied into a glass mortar and crushed carefully with a pestle. Mosquitoes were then washed with cold Hanks' balanced salt solution (HBSS) into a 32-µm mesh sieve set in a Petri dish and washed again by transferring the sieve to another dish filled with cold HBSS; thus insect debris was removed while the L_3 were inhibited from migrating by the temperature of the medium. The sieve contents were then rinsed into another 32-µm sieve set in a Baermann apparatus filled with HBSS warmed to about 37°C into which the L_3 migrated. A 25-µm mesh screen was fastened beneath the sieve to allow only sufficiently motile larvae to settle to the bottom. After at least 30min, L_3 were collected in 50-ml tubes by releasing a clamp at the bottom of the apparatus.

L_3 were counted in Petri dishes under a dissecting stereomicroscope and pipetted into microfuge tubes (in a volume of about 200 µl) at a rate of 30 larvae per tube. For inclusion in the assay, larvae were to appear active, undamaged, and developed to the third-stage. To each tube, 1 ml of the RPMI-based culture medium warmed to 37°C was added. Allowing at least 30min for larvae to settle, they were transferred in a volume of 100 µl from each microfuge tube to the wells of a 24-well culture plate, to which 1 ml of culture medium with drug was added. Larvae were then incubated at 37°C, 5% CO_2.

Source of parasite isolates

The control *D. immitis* isolate used in this study (2005 MO strain) was originally isolated from an infected dog housed by an animal shelter in Missouri in 2000 and has since been propagated in laboratory dogs. This isolate is assumed to be representative of typical ML-susceptible heartworm populations.

Suspected-resistant *D. immitis* isolates were maintained in beagle dogs at Auburn University (Auburn, AL), each originally obtained from dogs that developed mature infections despite regular administration of commercial ML preventives. The 'Tootie' isolate was collected from a 3-year-old female Labrador retriever in West Monroe, LA; the 'Jojo' isolate was collected from a 10-year-old male toy poodle in Earle, AR; the 'Cash' isolate was collected from a 4-year-old male German shepherd dog, also in Earle, AR.

Heparinized microfilaremic blood was shipped overnight from Auburn University at ambient temperature. Upon receipt, the microfilariae were diluted with uninfected canine blood and fed to mosquitoes as described above.

Drug solutions

Stock solutions (10 mM) were prepared by adding powdered IVM or EPR (Sigma) to dimethyl sulphoxide (DMSO; Sigma) and vortexing for 1–2min. These were diluted into a range of 100X working solutions in DMSO (from 0.0312 mM to 2 mM), added at 1% (v/v) to culture medium for use in L_3 drug incubation (from 0.312 µM to 100 µM). Control wells received culture medium with 1% DMSO.

Larval migration inhibition assay

In the optimized LMIA (Figure 3.1) L_3 were incubated in 24-well plates in the presence of drug at 37°C, 5% CO_2 for 48h. After this period, the 24-well migration plate was prepared by setting migration tubes into the corresponding wells of the plate. The polycarbonate migration tubes have 25-µm nylon mesh fixed to the base of each with a water-resistant epoxy and are set in rows of six by a horizontal bar. The bottoms of these tubes are suspended several millimeters above the bottom of the migration plate, allowing larvae to migrate out and collect in the latter. To provide an environment into which larvae can migrate, and ensure the free movement of liquid across the mesh, 170 µl of culture medium warmed to

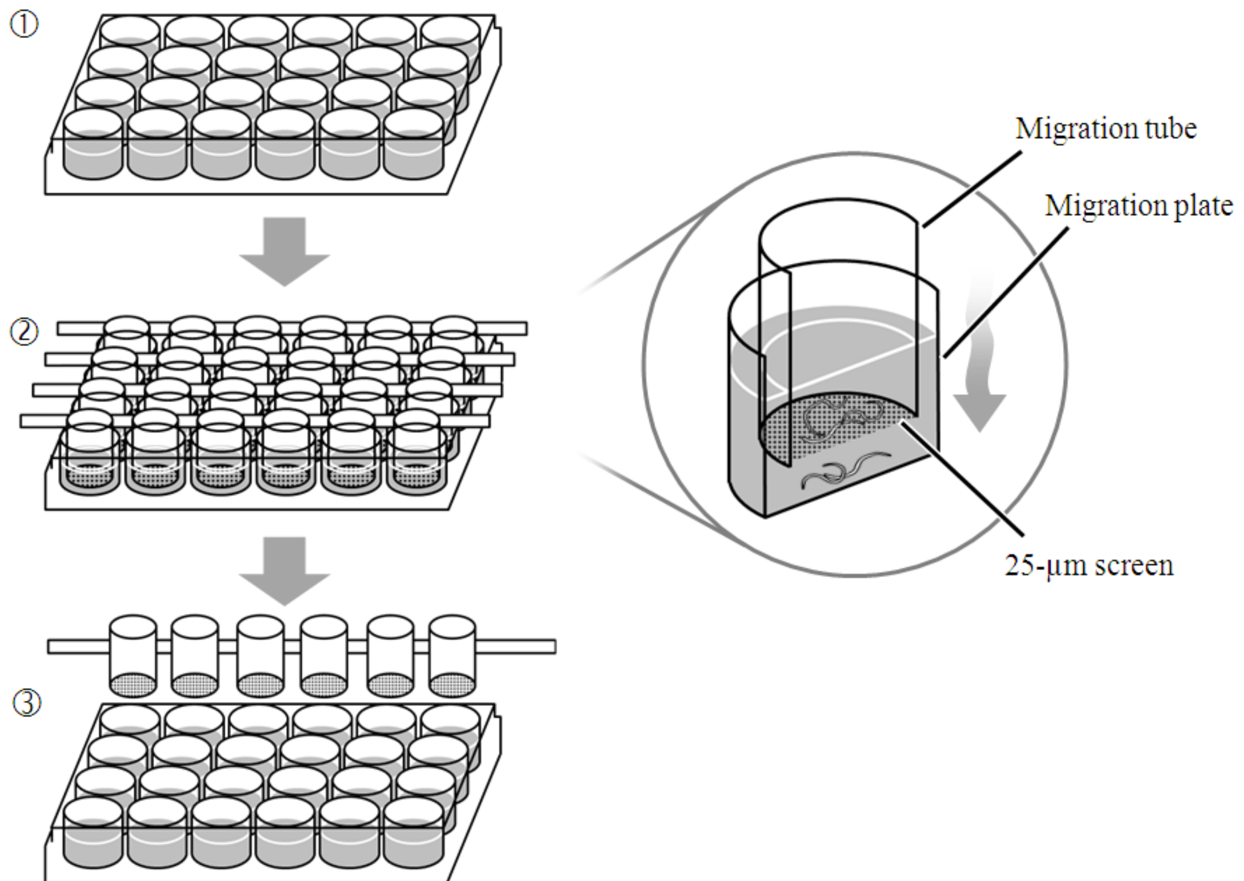

Figure 3.1: Larval migration inhibition assay procedure. ①, larvae were treated with drug solutions for 48h in the 24-well incubation plate. ②, larvae were then transferred into migration tubes which were suspended over the migration plate. Over 2h, the larvae were allowed to migrate through a 25-µm screen and collect in the migration plate. ③, migration tubes were gently removed from the migration plate and the number of larvae that successfully migrated were enumerated.

37°C was added over each of the migration tubes into the migration plate. Drug concentrations matched those in the incubation plate. In 200 µl, all 30 larvae in each well of the incubation plate were gently transferred over the corresponding migration tube under a dissecting microscope. The L_3 were then allowed to migrate for 2h at 37°C, 5% CO_2 (Figure 3.2). After the migration period, the tubes were gently removed and the larvae that had migrated into each well were enumerated using an inverted compound microscope at 40X magnification. Three experiments were performed for each isolate and anthelmintic, with drug treatments performed in triplicate for each experiment. Not all concentrations were tested in each experiment.

Data analysis

Differences in larval migration rates after 1h and 2h were calculated using a one-tailed paired t-test. Differences in control migration following incubation for 48h and 72h were calculated using a one-tailed unpaired t-test, as were differences in migration through meshes of different size. 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.

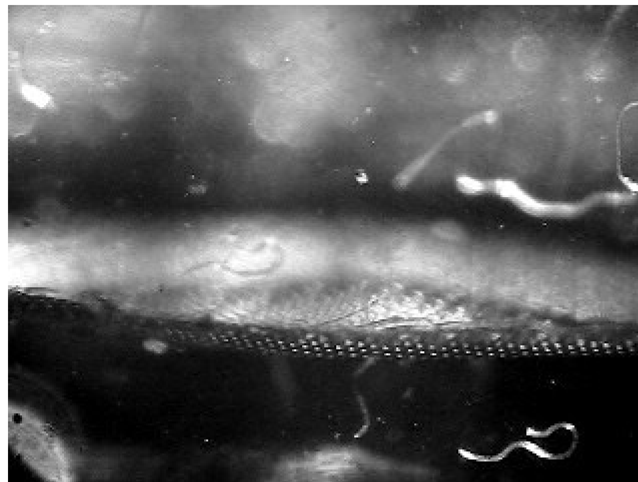

Figure 3.2: Migration of *Dirofilaria immitis* L_3. This image was taken approximately 45 seconds after larvae were introduced to a migration tube and shows a side-on view of their position above the 25-µm mesh, passing through the mesh, and below the mesh. 40X magnification.

In the LMIA, the number of L_3 that migrated out of the initial 30 was counted. In each experiment, data were corrected for control inhibition by dividing each value by the mean number of L_3 migrating in the control group. Percent inhibition was calculated by subtracting the corrected proportion of migration from one and multiplying by 100.

Using the corrected data, sigmoidal dose-response curves were plotted with GraphPad Prism® version 5 (GraphPad Software, Inc., San Diego, CA) using a variable slope nonlinear regression model. A four-parameter logistic equation was applied, with the bottom constrained to zero and percent migration inhibition plotted against the log-transformed concentration of each drug treatment. Controls were plotted with 0.01 μM assigned as the x-value. For each treatment, the IC_{50} was calculated by Prism®, defined as the concentration producing inhibition halfway between zero and the maximal y-value. Differences in the IC_{50} between control and suspected-resistant isolates were calculated by the software using the extra sum-of-squares F test, with the degree of these differences calculated as a resistance ratio (suspected-resistant IC_{50} divided by susceptible IC_{50}).

CHAPTER 4

RESULTS

Media

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_3 were incubated in each medium with no difference observed in migration rates ($p = 0.81$). The RPMI-based medium was chosen for future assays because of its relative availability and ease of preparation. To assess the value of adding an antifungal agent, L_3 were cultured for 3 days in medium with or without 0.5 µg/ml amphotericin B (Sigma). No difference in the activity of larvae was observed and so, for the sake of simplicity and consistency, amphotericin B was excluded from future culture media.

DMSO is added to media to improve drug solubility, but it negatively affects 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 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 ($p = 0.018$). Because 1% DMSO was the highest concentration tested that did not reduce control migration rates, this was used in the LMIA culture medium.

Migration period

In previous experiments with *Brugia pahangi*, we observed that 90% of the L_3 that migrated over 24h in the LMIA did so within the first 45min (Figure 4.1). To ensure a short yet sufficient migration period, the migration of *D. immitis* L_3 over 1h was determined, followed immediately by an additional 1h in a

separate migration plate. Significantly more larvae migrated over the combined 2h period than 1h (p = 0.037), therefore a 2h migration period was used for this LMIA.

Incubation period

To allow sufficient time for the *in vitro* drug treatment to affect larvae without an excessive reduction in control migration, an optimal incubation period needed to be determined. The LMIA was performed with either a 48h or 72h incubation (four independent experiments each) and control migration rates were found to be lower following the longer incubation (p = 0.011). Furthermore, the logIC_{50} for each assay was not significantly different (p = 0.51), and so we selected a 48h incubation period for all future assays.

Mesh size

Mesh pore size is a discriminating parameter for motility in the LMIA and was selected so that it was larger than the average *D. immitis* L_3 diameter (about 20 µm), yet small enough to restrict the passage of immotile larvae. Therefore, migration rates through 25-µm and 30-µm mesh screens were compared. No

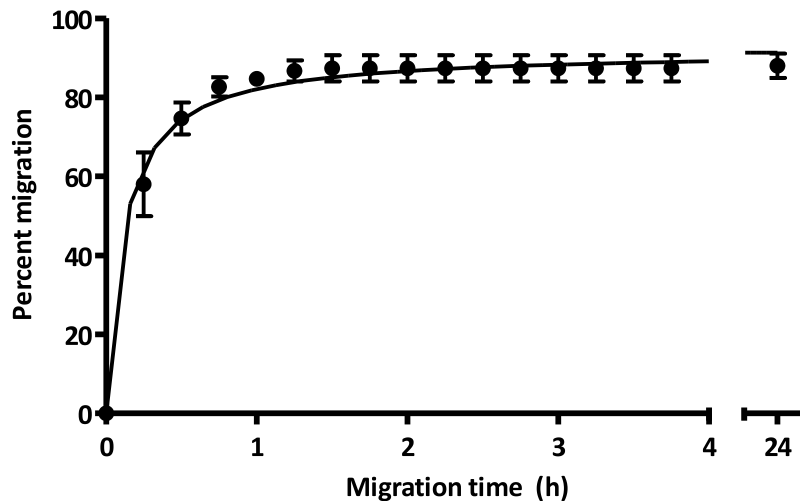

Figure 4.1: Migration of *Brugia pahangi* L_3 over time. Fifty untreated larvae were introduced to 20-µm mesh migration tubes in triplicate. The tubes were transferred to new migration wells every 15 minutes for the first 3:45h and finally removed at 24h.

reduction in control migration was observed for the smaller mesh size (three independent experiments, $p = 0.38$), however, migration of larvae treated at the highest drug concentration (50 µM EPR) was significantly lower in the 25-µm mesh ($p = 0.034$). While migration was still observed at the highest drug concentrations, it was confirmed visually that this was due to larval activity and not the incidental passage of immotile worms through the mesh. So that migration rates could be maximized in controls and minimized at the highest drug treatment, the 25-µm mesh size was selected for use in this assay.

To ensure that the diameter of L_3, which is a slightly variable feature, did not affect migration rates in the LMIA, larvae that had either migrated or failed to migrate were measured using an inverted compound microscope (Figure 4.3). No correlation between migration and larval width was observed ($p = 0.95$), so migration rates were assumed not to be a function of variation in larval diameter.

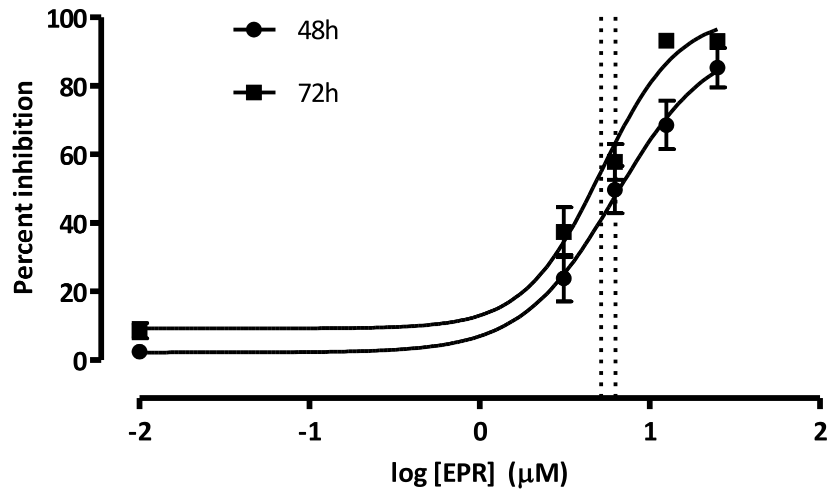

Figure 4.2: Effect of incubation period on dose-response curves. The LMIA was performed using both 48h and 72h drug treatment periods. Data are not corrected for control inhibition. Control inhibition was greater after 72h incubation ($p = 0.011$). The IC_{50} values (indicated by dashed lines) were not significantly different ($p = 0.51$). Each data point represents mean ± SD, $n \geq 12$ from four independent assays.

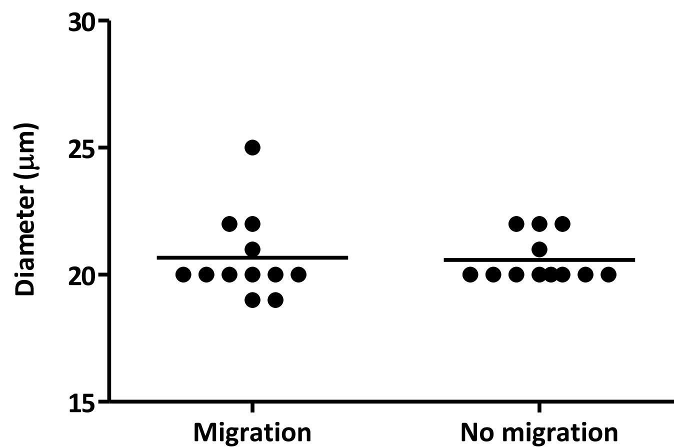

Figure 4.3: Correlation of larval diameter and migration. The diameters of larvae that successfully migrated in the LMIA and those that failed to migrate were measured microscopically. No significant correlation was observed ($p = 0.95$). Horizontal line indicates mean, $n = 12$.

Larval migration inhibition assay

For all *D. immitis* isolates, mean larval migration was regularly greater than 90% in control wells, indicating that L_3 exhibit high levels of motility after 48h incubation. Mean inhibition at the highest concentrations, however, infrequently exceeded 90%. Dose-response curves were plotted for the control isolate (2005 MO strain) and each of the suspected-resistant isolates (Tootie, Jojo, and Cash) treated with EPR (Figure 4.4) and IVM (Figure 4.5) at concentrations ranging from 0.312 µM to 20 µM. The IC_{50} values were calculated for all isolates and no differences were observed between the control isolate and the suspected-resistant isolates for each anthelmintic (Table 4.1). The highest resistance ratio calculated was very low, at 1.20 for the EPR-treated Cash isolate. The fit of each curve to the nonlinear regression model was expressed as an R^2 value, and the responses of EPR-treated larvae appeared to fit better than the IVM-treated larvae of their respective isolates.

Table 4.1: Dose-response data for the larval migration inhibition assay. The IC_{50} (µM) of the susceptible control isolate (2005 MO strain) was compared to that of each suspected-resistant isolate (Tootie, Jojo, and Cash) using the extra sum-of-squares F test with *p*-values reported. Resistance ratio (RR) = suspected-resistant IC_{50} / susceptible IC_{50}.

	Dirofilaria immitis isolate			
	Control	Tootie	Jojo	Cash
IC_{50} (EPR)	2.02	1.57	1.99	2.42
95% CI	1.68 – 2.42	1.03 – 2.41	1.49 – 2.64	1.89 – 3.10
R^2	0.90	0.84	0.83	0.88
p -value	n/a	0.37	0.93	0.22
RR	n/a	0.78	0.98	1.20
IC_{50} (IVM)	4.56	2.21	2.52	2.74
95% CI	1.26 – 16.40	0.96 – 5.10	1.97 – 3.23	2.30 – 3.27
R^2	0.71	0.72	0.81	0.83
p -value	n/a	0.35	0.19	0.23
RR	n/a	0.49	0.55	0.60

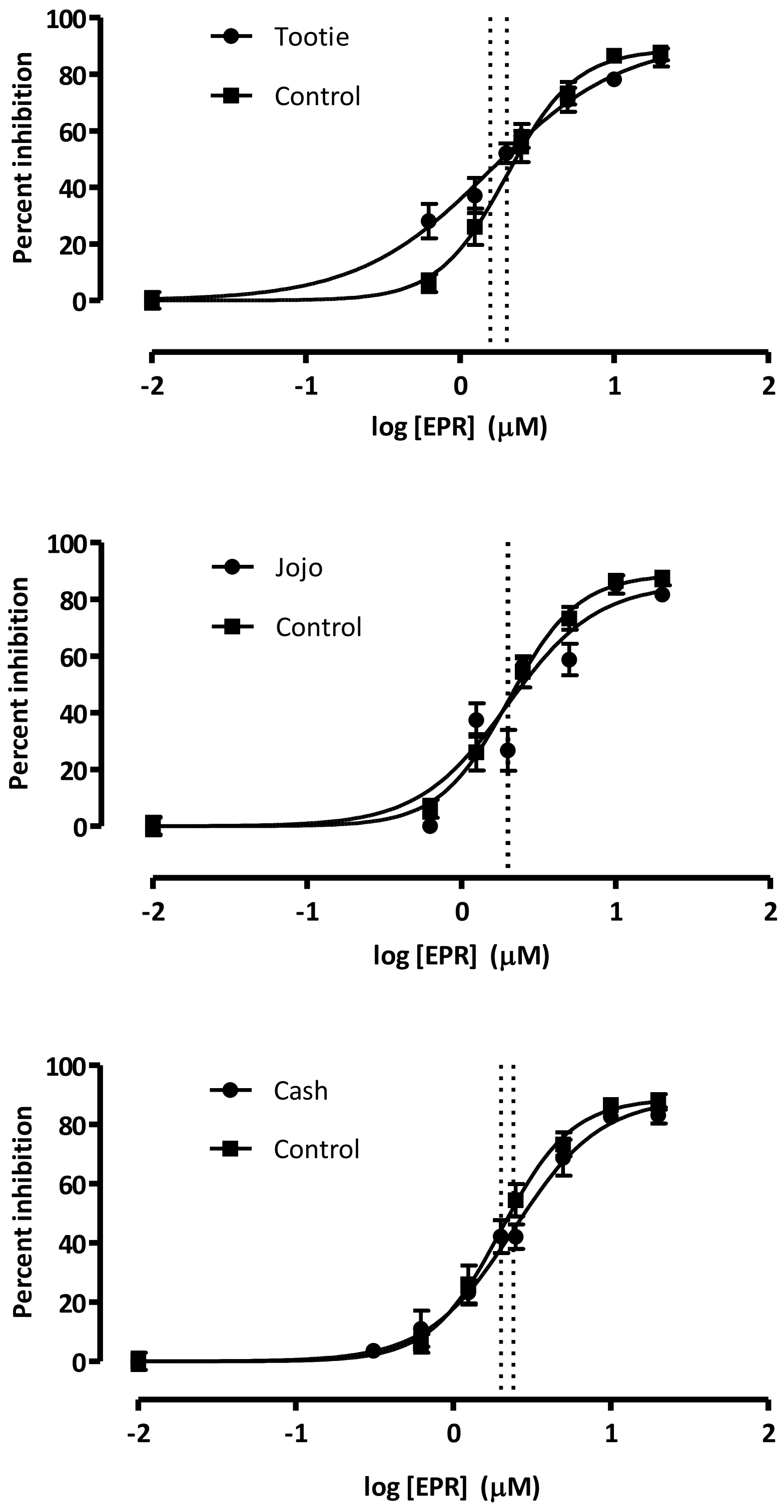

Figure 4.4: Eprinomectin larval migration inhibition assay dose-response curves. Data from the susceptible *D. immitis* isolate (Control) are plotted with each of the tested suspected-resistant isolates (Tootie, Jojo, and Cash). Dashed lines indicate logIC_{50} values. Each data point represents the mean ± SD from three independent assays.

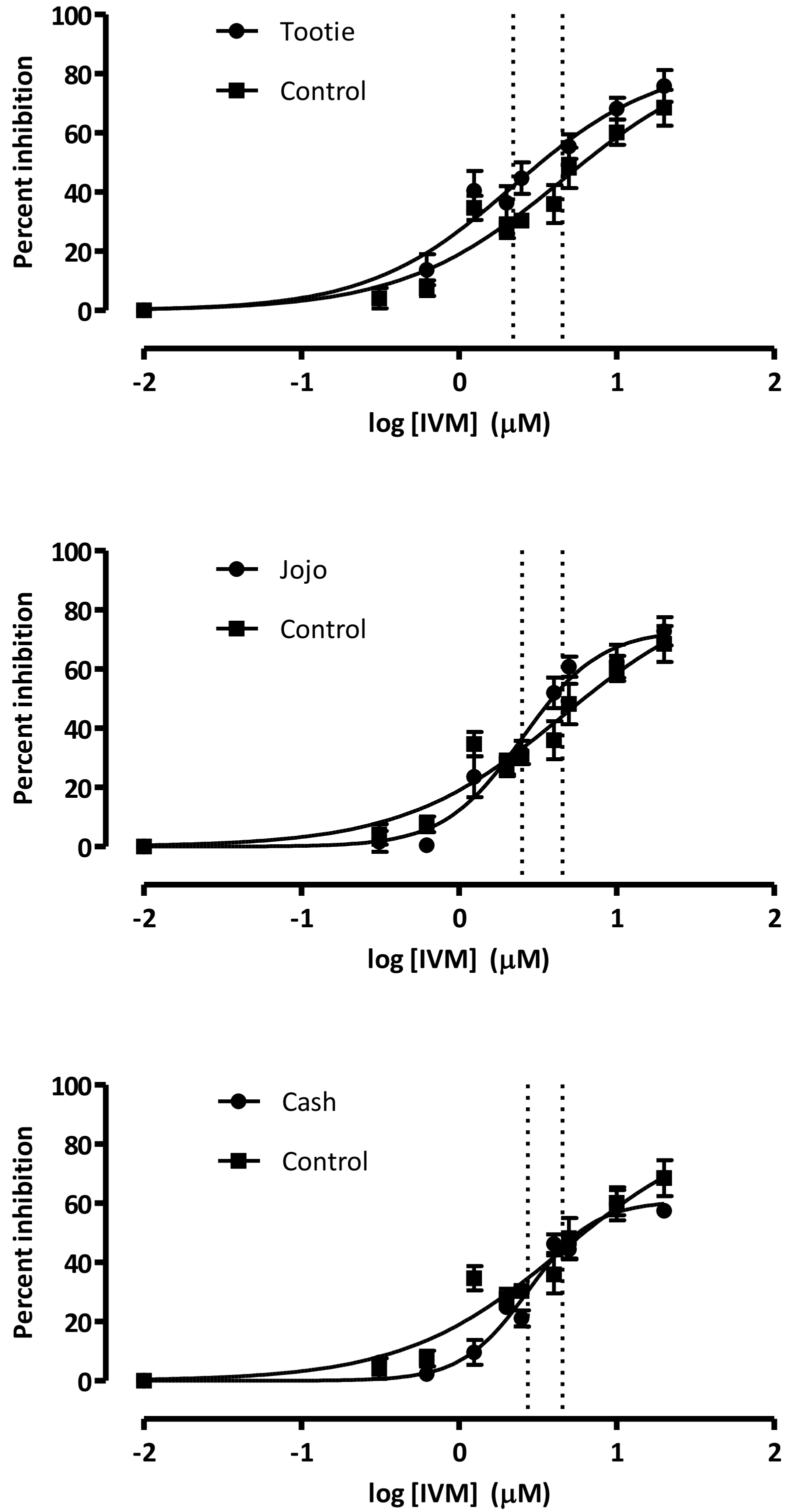

Figure 4.5: Ivermectin larval migration inhibition assay dose-response curves. Data from the susceptible *D. immitis* isolate (Control) are plotted with each of the tested suspected-resistant isolates (Tootie, Jojo, and Cash). Dashed lines indicate $\log IC_{50}$ values. Each data point represents the mean ± SD from three independent assays.

CHAPTER 5

DISCUSSION

In this study we developed a larval migration inhibition assay for measuring the effects of ML anthelmintics on *D. immitis* L_3. This motility-based bioassay was derived from that described by Sangster, et al. (1988) and modified by Kotze, et al. (2006) and Demeler, et al. (2010b). With the ability of infective larvae to migrate through a mesh screen as the discriminating parameter, we measured the effect of a range of drug concentrations, developing dose-response curves for each parasite isolate and anthelmintic tested. To our knowledge, this is the first application of the LMIA to a filarial nematode. The objective was to compare the responses of a control heartworm isolate (2005 MO strain) and multiple suspected-resistant isolates (Tootie, Jojo, and Cash) to ML treatment in order to validate the LMIA as a method for detecting susceptibility differences between heartworm populations. While a nonlinear regression model fit the dose-response data reasonably well, using the IC_{50} as a representative measure of drug susceptibility we found no differences between susceptible and suspected-resistant heartworm isolates (Figure 4.1). Given these findings, and the unknown resistance status of the 'suspected-resistant' isolates, the suitability of the LMIA to detecting differences in ML susceptibility in this species remains inconclusive.

Heartworm preventive lack of efficacy reports have spurred concern over the development of resistance to MLs, the only prophylactics widely used against this parasite (Hampshire 2005). This has been fomented by recent studies indicating less than total protection by commercial preventives (Blagburn, Dillon et al. 2011; Snyder, Wiseman et al. 2011). Presently, the emergence of genuine drug resistance in heartworms is well-debated, yet a validated assay for measuring this phenomenon has not been developed. Our study has described a reproducible *in vitro* bioassay applicable to the comparison of

drug susceptibility between heartworm populations. To this end, we have tested the EPR and IVM susceptibility of three suspected-resistant heartworm isolates originating from dogs that, despite regular administration of monthly preventives, developed mature infections. With access to more suspected cases of drug-resistant heartworm infection, our aim is to subject each to the LMIA and compare the dose-response data to our known-susceptible control strain, monitoring for evidence of significantly reduced ML susceptibility.

The LMIA is useful for measuring nematode responses to anthelmintic treatment when paralysis is a primary *in vitro* effect. The current model of ML activity indicates that the drugs exert a paralytic effect on worms by irreversibly activating GluCls and interrupting neurotransmission processes (Martin, Robertson et al. 2002; Wolstenholme and Rogers 2005); therefore, the LMIA seems to be an appropriate test. The infective third-stage larva of *D. immitis* was selected as the stage of choice for this assay because it exhibits a natural ability to migrate through host tissue and is one of the main targets of ML preventives. Like all filarioids, heartworms lack a free-living stage and are, consequently, highly sensitive to environmental exposure. This presents special problems for *in vitro* culture, and maintaining the health of these parasites over the course of several days required extensive modifications to the LMIA protocols described for use with relatively robust geohelminths. However, as indicated by the high control migration rates we observed, the culture methods we developed in this study appear sufficient for the assay. The IC_{50} was selected as the parameter of choice for comparing responses because it could be calculated most accurately with the data generated by the LMIA. Inhibition did not usually reach 100% in the assay, leaving the upper plateau more poorly defined and precluding the calculation of reliable IC_{90}s or IC_{95}s because of the response variability encountered.

Using the LMIA, we found that the IC_{50} values of the three suspected-resistant isolates tested did not significantly differ from that of the control isolate with either EPR or IVM treatment. This may suggest that the assay is not capable of discerning more and less susceptible heartworm populations or that the Tootie, Jojo, and Cash isolates are not any more resistant than our control. Currently, no confirmed ML-resistant heartworm isolates exist and so, until such a population is identified, we must use parasites only

Table 5.1: Resistance ratios for the larval migration inhibition assay. Published resistance ratios (RRs) are reported for nematodes tested with the LMIA following treatment with a macrocyclic lactone. EPR = eprinomectin, IVM = ivermectin, ABA = abamectin.

Parasite species	Drug	RR	Reference
H. contortus	EPR	16.4 – 28.1	Kotze et al. 2006
	IVM	1.7 – 5.7	
	ABA	3.5 – 11.0	
	IVM	8.4	Demeler et al. 2010b
C. oncophora	IVM	8.3	Demeler et al. 2010b
	IVM	4.5 – 5.8	El-Abdellati et al. 2010

circumstantially suspected to be resistant to test these hypotheses. Only in one instance did we calculate a resistance ratio greater than one (Cash, EPR treatment), and this was only marginally greater. While direct comparisons cannot be made, the resistance ratios reported for gastrointestinal nematodes treated with MLs in the LMIA were much higher (Table 5.1). Resistant strains are well-established in *Haemonchus contortus* and *Cooperia oncophora* and the LMIA has been found effective in discriminating them from susceptible isolates. Measuring these differences, however, is also dependent upon how well the data generated by the assay fit the statistical model being used. The goodness-of-fit of dose-response data collected from our LMIA to a variable slope nonlinear regression model was moderately high for EPR treatment, with an R^2 range of 0.83 – 0.90, but noticeably lower for IVM treatment, with an R^2 range of 0.71 – 0.83. This disparity is not unusual, as *in vitro* responses in the LMIA have been reported to vary with the ML used (Kotze, Le Jambre et al. 2006). Indeed, the use of multiple MLs in this assay will allow the selection of the analog best suited to discriminating heartworm populations of differing susceptibility. The most useful drug for such an *in vitro* assay may be one that is not used clinically for the prevention of heartworm—in this case, EPR. Treating with the appropriate drug, the IC_{50} of a resistant heartworm isolate is expected to be several times greater than that of a susceptible isolate, and thus well within the detection limit of this assay.

While the complete migration of untreated larvae in the control wells is ideal, because of the inherent variability in nematode responses, it is not expected. The control migration rates we observed were, nonetheless, fairly high with a mean greater than 90% for all isolates, thus enabling us to reasonably estimate the lower plateau of our dose-response data. At the highest concentrations of drug tested, however, total inhibition was rarely observed. The mean inhibition at 20 μM (the highest concentration we reported) was 86% in assays with EPR treatment and 69% in assays with IVM treatment; this was a significant difference ($p = 3.7 \quad 10^{-7}$). Our limited ability to define the upper plateau inevitably affects the fit of our data to the chosen model. Concentrations of drug higher than 20 μM were tested, and effected slightly greater levels migration inhibition, but were prone to precipitating out of the medium. In such cases, the drug concentrations to which larvae were exposed could not be assumed to be accurate and so responses from concentrations higher than 20 μM were not included in data analysis. The MLs are highly hydrophobic and their solubility is enhanced by including the solvent DMSO in larval culture media. While drug solubility should increase with solvent levels, DMSO is also toxic to the parasites, and so the drug levels tested in our study were limited to those that remain soluble in 1% DMSO, a level not significantly affecting larval migration. Future work may require methods for improving solubility in media at low solvent concentrations.

Even at highly soluble dilutions, the ML concentrations used in this assay were relatively high. The preventive dose of IVM (6 μg/kg) yields peak plasma levels in the dog of approximately 3 ng/ml (Daurio, Cheung et al. 1992), while the present study demonstrated IC_{50} values of 1.43 – 3.66 μg/ml. The disparity in these drug levels, augmented by our difficulty in achieving the 100% inhibition seen at such low doses clinically, challenges the assumption that the *in vitro* and *in vivo* responses of the heartworm to MLs are comparable. It should be noted that *in vitro* assays do not attempt to replicate conditions within the host, but merely act as a convenient surrogate within which relevant responses can be measured. As such, the levels of drug efficacy are not expected to be identical, but the disparity observed in this case is great enough to suggest that the drug may even be working through a different mode of action in the two settings. Indeed, the host immune response may be integral to the anthelmintic activity of the MLs. A

recent study on *Brugia malayi* microfilariae has demonstrated the localization of IVM to the excretory-secretory (ES) apparatus and the subsequent inhibition of protein release from the ES pore at nanomolar concentrations of the drug (Moreno, Nabhan et al. 2010). IVM was hypothesized to inhibit the function of ES-associated musculature and prevent the release of immunomodulatory proteins, allowing the host to clear the parasite. This model is supported by earlier works that implicate a cell-mediated immune response in the clearance of filarioid species during ML treatment (Rao, Chandrashekar et al. 1987; Zahner and Schmidtchen 1994). While this remains the focus of active research, it may become the basis of future *in vitro* bioassays.

While this study found no significant differences in the responses of a control heartworm isolate and three suspected-resistant isolates, it describes the development and optimization of an *in vitro* assay producing data that fits a dose-response curve relatively well. Future work with the LMIA will involve testing more heartworm isolates implicated in prophylaxis failure and testing more ML compounds for their ability to discriminate between populations of different susceptibilities. If no resistant heartworm populations exist, however, the results are not expected to differ greatly from those we obtained in the present study. The dose-response characteristics derived from this assay may make it amenable to anthelmintic screening. Additionally, the assay may be applied to address the possible emergence of drug resistance in other filarioid species; in fact, our LMIA was first optimized for use with the highly motile *Brugia pahangi* L_3. Given our concern that the *in vitro* effect of the MLs on larval motility may not be relevant in our assay, we intend to further modify the LMIA to incorporate a host immune component. We hypothesize that this may more closely replicate *in vivo* anthelmintic activity and allow us to better characterize the ML susceptibility of heartworms.

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