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Heartworm and *Wolbachia*: Therapeutic implications

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Abstract

A safer, more effective adulticidal treatment and a safe method for reducing microfilaremia and breaking transmission of heartworm disease early in the treatment are needed. The present study evaluated efficacy of ivermectin (IVM) and doxycycline (DOXY) alone or together (with or without melarsomine [MEL]) in dogs with induced adult heartworm infection and assessed the ability of microfilariae from DOXY-treated dogs to develop to L_3 in *Aedes aegypti* mosquitoes and subsequently to become reproductive adults in dogs. Thirty beagles were each infected with 16 adult heartworms by intravenous transplantation. Six weeks later, dogs were ranked by microfilarial count and randomly allocated to 6 groups of 5 dogs each. Beginning on Day 0, Group 1 received IVM (6 mcg/kg) weekly for 36 weeks. Group 2 received DOXY (10 mcg/(kg day)) orally Weeks 1–6, 10–11, 16–17, 22–25, and 28–33. Groups 3 and 5 received IVM and DOXY according to doses and schedules used for Groups 1 and 2. At Week 24, Groups 3 and 4 received an intramuscular injection of MEL (2.5 mg/kg), followed 1 month later by two injections 24 h apart. Group 6 was not treated. Blood samples were collected for periodic microfilaria counts and antigen (Ag) testing (and later immunologic evaluation and molecular biology procedures). Radiographic and physical examinations, hematology/clinical chemistry testing, and urinalysis were done before infection, before Day 0, and periodically during the treatment period. At 36 weeks, the dogs were euthanized and necropsied for worm recovery, collection of lung, liver, kidney, and spleen samples for examination by immunohistochemistry and conventional histological methods.

All dogs treated with IVM + DOXY (with or without MEL) were amicrofilaremic after Week 9. Microfilarial counts gradually decreased in dogs treated with IVM or DOXY, but most had a few microfilariae at necropsy. Microfilarial counts for dogs treated only with MEL were similar to those for controls. Antigen test scores gradually decreased with IVM + DOXY (with or without MEL) and after MEL. Antigen scores for IVM or DOXY alone were similar to controls throughout the study. Reduction of adult worms was 20.3% for IVM, 8.7% for DOXY, 92.8% for IVM + DOXY + MEL, 100% for MEL, and 78.3% for IVM + DOXY. Mosquitoes that fed on blood from DOXY-treated dogs had L_3 normal in appearance but were not infective for dogs.

Preliminary observations suggest that administration of DOXY + IVM for several months prior to (or without) MEL will eliminate adult HW with less potential for severe thromboembolism than MEL alone.

Keywords: Canine heartworm; *Dirofilaria immitis*; Ivermectin; Doxycycline; Melarsomine dihydrochloride; *Wolbachia*

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1. Introduction

All current evidence suggests that *Wolbachia* (Sironi et al., 1995), which are bacteria found in all individuals of the filarial species that are known to harbor these bacteria, are endosymbionts (i.e., the bacteria is essential for the filarial worms' survival) (Taylor et al., 2005a). Tetracyclines inhibit the early development of filarial infections in animal models and block embryogenesis in human filariae and heartworms, presumably by elimination of the *Wolbachia* from the worm (Bosshardt et al., 1993; Bandi et al., 1999; Hoerauf et al., 1999, 2001, 2000a,b, 2003a,b; McCall et al., 1999; Langworthy et al., 2000; Townson et al., 2000; Genchi et al., 2001; Casiraghi et al., 2002; Rao and Weil, 2002; Rao et al., 2002; Chirgwin et al., 2003; Volkmann et al., 2003; Taylor et al., 2005a,b; Smith and Rajan, 2000). In contrast, filarial species that do not harbor *Wolbachia* are not affected by tetracyclines (Hoerauf et al., 1999; McCall et al., 1999). *Wolbachia*-derived proteins have been implicated in the inflammatory response (Brattig et al., 2001, 2004; Cross et al., 2001; Taylor et al., 2001; Bazzocchi et al., 2003; Grandi et al., 2005; Hise et al., 2004; Kramer et al., 2005), innate and adaptive immune responses (Bazzocchi et al., 2000, 2007; Punkosdy et al., 2003; Simón et al., 2003; Brattig et al., 2004; Kramer et al., 2003, 2005; Morchón et al., 2004) and tolerized immunological phenotype (O'Connor et al., 2003; Brattig et al., 2004; Turner et al., 2006; Morchón et al., 2007a,b) associated with human filariasis and heartworm disease in dogs.

Protracted monthly prophylactic doses of ivermectin (IVM) provide ''safety-net'' activity against developing heartworms and a ''slow-kill'' effect on adult heartworms, with earlier inhibition of embryogenesis (McCall, 2005; McCall et al., 1995, 1996, 1998, 2001). The unique drug effects of IVM on 3–8-month-old heartworms are related to the age of the worms at initiation of treatment. The earlier treatment is started, the shorter the survival time of the worms and the more stunted are the worms. Worms that are fully grown when treatment is started are not shorter after prolonged monthly treatment, but worm mass is reduced by at least 20% due to the death of all uterine stages of microfilariae and the ''wasting away'' condition of the worms (McCall, 1993, unpublished data).

Melarsomine dihydrochloride (MEL) is the only drug approved in the United States by the Food and Drug Administration as an adulticide for heartworms. Two intramuscular injections (2.5 mg/kg given 24 h apart) or three injections (2.5 mg/kg followed 1-month later by two injections 24 h apart) are the two treatment protocols available for MEL. The American Heartworm Society (AHS) recommends the three-injection protocol because it is safer and more efficacious. The AHS further indicates that it may be beneficial to administer a macrocyclic lactone preventative for up to 6 months prior to the adulticide, when the presentation does not demand immediate attention (Nelson et al., 2005).

Death of adult filarial worms, whether due to treatment or natural causes, results in an exacerbated inflammatory response. We hypothesize that this heightened response is due in part to the release of *Wolbachia* following death and disintegration of the worms.

The study presented here describes an evaluation of the efficacy of IVM and DOXY, alone or together (with or without MEL) in dogs with induced, adult infection with *Dirofilaria immitis* and assessment of the ability of microfilariae from DOXY-treated dogs to develop to 3rd stage in *A. aegypti* mosquitoes and then to become reproductive adults in dogs. This article is part of a complex study that is still ongoing and other data from the study is being published.

2. Materials and methods

2.1. Study design

An overview of the study design for, 30-dog experiment and is presented in Table 1.

Table 1
Timing of treatments and evaluations in dogs with induced infections with adult *Dirofilaria immitis*.

Treatment	Timing of treatments and evaluations
Ivermectin	Weeks 1–36
Doxycycline	Weeks 1–6, 10–11, 16–17, 22–25, 28–33
Ivermectin+	Weeks 1–36
Doxycycline+	Weeks 1–6, 10–11, 16–17, 22–25, 28–33
Melarsomine	Week 24, 28a/b[a]
Melarsomine	Weeks 24, 28a/b[a]
Ivermectin+	Weeks 1–36
Doxycycline	Weeks 1–6, 10–11, 16–17, 22–25, 28–33
Control	No treatment
Evaluations	
Microfilarial counts/Ag	Weeks –6, 0, 6, 10, 12, 16, 18, 24, 26, 28, 32, 36
Radiographs	Weeks –6, 0, 12, 24, 28, 32, 36
Hematology/clinical pathology /physical exam/urinalysis	Weeks 0, 12, 24, 28, 32, 36

[a] Two injections of melarsomine were administered 24 h apart at Week 28.

2.2. Animals

A total of 30 purpose-bred beagles, including 23 males and 7 females, 12–16.5 months of age, weighing 15.1–34.2 kg, were used in the study. The dogs were housed in mosquito-proof indoor pens in a purpose-built building, with controlled temperature and ventilation systems. The dogs were fed at least once daily an appropriate quantity of commercially available maintenance diet and water was supplied *ad libitum*. Treated dogs were housed individually in runs, and nontreated dogs were housed 1–3 of the same sex to a run. The animals were maintained with due regard for their welfare and in accordance with applicable laws, regulations and guidelines. The protocol was approved by the Institutional Animal Care and Use Committee prior to initiation of the study.

An additional 11 heartworm-naive beagles were obtained from the commercial supplier for the mosquito studies (M-1, M-2, and M-3). Two of these dogs were experimentally infected with heartworms, became microfilaremic, and were used as donors in study M-3. The remaining 9 dogs were used as recipients in the mosquito studies.

All personnel making observations, performing tests and procedures, and collecting data were blinded in regard to which were treated and control animals. Groups were color-coded for identification by laboratory personnel throughout the study.

2.3. Parasite

Each of the 30 dogs was infected with nine adult female and seven adult male heartworms by intravenous transplantation via a jugular vein (Dzimianski et al., 1989). The heartworms ranged in age from 10 to 21 months and represented three different laboratory strains of the parasite (Butch, Lady, and Missouri strains).

2.4. Dog treatment study

Approximately 6 weeks after infection, the 30 dogs were ranked by microfilariae count within gender, and then randomly allocated to a nontreated control group of five dogs and five test groups of five dogs each. Each group contained at least one dog of each sex.

2.5. Study drugs

Commercially available chewables (Heartgard® Plus, Merial Limited, Duluth, GA) containing IVM and pyrantel pamoate were administered orally according to instructions on the label, with IVM given at a minimum dosage of 6 mcg/kg of body weight and pyrantel given at a minimum dosage of 5 mg/kg of body weight.

Doxycycline hyclate (West-ward Pharmaceuticals, Inc., Eatontown, NJ) was given orally for varying periods throughout the study at a dosage equivalent to 10 mg doxycycline/(kg day). Melarsomine dihydrochloride (Immiticide®, Merial Limited, Duluth, GA) was administered as intramuscular injections in the epaxial muscles at 2.5 mg/kg of body weight per injection. The three-injection protocol (alternate schedule) was used: one injection followed 1 month later by two injections 24 h apart.

All treatments with IVM and DOXY were started on Day 0 (Table 1). Group 1 dogs were given IVM weekly for 36 weeks. Group 2 dogs were given DOXY daily during Weeks 1–6, 10–11, 16–17, 22–25, and 28–33. Group 3 dogs were given both IVM plus DOXY as for Groups 1 and 2 and MEL (IVM + DOXY + MEL) as for Group 4. Group 4 dogs were given one injection of melarsomine (MEL) at 24 weeks, followed 1 month later by 2 injections 24 h apart. Group 5 dogs were given IVM plus DOXY as for Groups 1 and 2. Group 6 served as the nontreated control.

Whole blood and serum samples were collected for microfilariae and *D. immitis* antigen (Ag) testing, immunologic evaluation and molecular biology procedures before IVM and DOXY dosing was initiated and again at Weeks 6, 10, 12, 16 (microfilariae/Ag only), 18 (microfilaria/Ag only), 24, 26 (microfilariae/Ag only), 28, 32, and 36. Radiography, physical examinations, hematology/clinical chemistry testing and urinalysis were performed before infection (radiography only), before Day 0, and again at Weeks 12, 24, 28 (Groups 3 and 4), 32 (Groups 3 and 4) and 36 (Table 2). All of the dogs were humanely euthanized and necropsied at 36 weeks.

2.6. Microfilarial counting and antigen testing

At each blood collection, a 20-μL sample was removed to make a Giemsa-stained preparation (Schlotthauer et al., 1986), and 1 mL was retained for later examination by the modified Knott method. For the modified Knott method, the 1-mL samples were mixed with 2% formalin and kept until after the 20-μL slide preparations were examined. If microfilariae were not seen on this preparation, the entire 1-mL sample was examined.

Table 2
Efficacy of ivermectin and doxycycline, given alone or together with or without melarsomine, beginning 6 weeks after infection at necropsy 36 weeks after intravenous transplantation of live adult *Dirofilaria immitis*.

Treatment	Mean no. live adult heartworms				Percent efficacy
	Male	Female	Total	Range	
Weekly ivermectin (6 μg/kg)	4.2[a]	6.8[a]	11.0[a]	5–14	20.3
Intermittent doxycycline (10 mg/(kg day))	4.6[a]	8.0[a]	12.6[a]	9–15	8.7
Weekly ivermectin + intermittent doxycycline + melarsomine (three injections)	0.8[b]	0.2[c]	1.0[b,c]	0–4	92.8
Melarsomine (three injections)	0[b]	0[c]	0[c]	–	100
Weekly ivermectin + intermittent doxycycline	0.4[b]	2.6[b]	3.0[b]	0–6	78.3
Nontreated control	5.4[a]	8.4[a]	13.4[a]	12–16	NA

Different superscripts in a column indicate a significant difference between groups ($p \leq 0.01$), NA = not applicable.

At each sampling, whole blood samples were collected in EDTA for later use, and serum was collected and stored at −70 °C until needed. Serum was examined for adult *D. immitis* Ag using a commercially available kit (DiroCHEK®, Symbiotics Corporation, San Diego, CA). A subjective scoring system, based on the intensity of the color reaction, was used to express the results of each test as negative (−, 0); very weak positive (vwk+, 1), weak positive (wk+, 2); positive (+, 3); strong positive (++, 4); or very strong positive (+++, 5) (McCall et al., 1996).

2.7. Necropsy

Just prior to necropsy, each dog was given approximately 2 mL of heparin (1000 USP units/mL) intravenously and the appropriate dose of a barbiturate euthanasia solution. The heart and lungs were examined grossly for pathological changes. The pleural and peritoneal cavities were examined for *D. immitis* and the anterior and posterior venae cavae were clamped before removal of the heart and lungs. The right atrium, right ventricle, and pulmonary arteries (including those coursing through the lungs) were dissected and examined for worms. The worms from each dog were recorded as either dead or alive; live worms were further classified by motility and appearance compared with those from control dogs. Worms were then sexed and counted. For later studies, some female worms from each dog were dissected and the removed intestines and reproductive tracts were placed in EM fixative, some entire worms were placed in EM fixative, some worms were frozen, some were placed in RNA later, and some were placed in 10% formalin. Also for later studies, lung, liver, kidney, and spleen samples were taken from each dog for examination by immunohistochemistry, conventional microscopy, transmission electron microscopy and molecular biology techniques.

2.8. Statistical analysis

The numbers of live male worms, live female worms, and total live worms were determined for each dog. The numbers of worms recovered for each dog were transformed to the natural logarithm (count + 1) for calculation of geometric means and analysis, using one-way analysis of variance for complete randomized design. Each group was compared with the control group, using a single degree of freedom contrast. Groups were compared with each other using Duncan's multiple range test. Efficacy against male, female, and total worms in each test group was calculated as the percent reductions, using geometric means, compared with the control group.

2.9. Mosquito studies

For M-1 and M-2 experiments, pooled blood samples from several dogs in each of Groups 1 (IVM only), 2 (DOXY only), and 6 (nontreated control) were membrane-fed to adult female *A. aegypti* (Liverpool Black-eyed strain) mosquitoes (McCall, 1981). Sixteen days later, L_3 from each of the three sets of mosquitoes were collected in approximately 1 mL of Hanks' balanced salt solution (pH 7.0) containing penicillin (0.4 units/mL) and streptomycin (0.4 μg/mL) and injected subcutaneously into the inguinal area of each of three dogs using a tuberculin syringe with a 20-gauge needle.

For the M-3 experiment, blood from a donor dog treated only with DOXY (Lady strain) as for M-1 and blood from a donor control dog (Lady strain) were membrane-fed to two groups of mosquitoes. Infective-stage larvae from the DOXY mosquitoes were injected subcutaneously into two recipient dogs, and one recipient dog was given L_3 from the mosquitoes fed on the blood from the donor control dog.

The mosquitoes for the M-1 experiment were infected with microfilaremic blood on Day 66 (Week 9, 3 days after the last dose of IVM and 25 days after the last dose of DOXY). Sixteen days later, the total of six L_3 collected from the IVM mosquitoes were injected subcutaneously into Recipient dog 1, 40 of the L_3 collected from the DOXY mosquitoes were injected subcutaneously into Recipient dog 2, and 40 of the L_3 collected from the control mosquitoes were injected into Recipient dog 3. Microfilariae and Ag testing was performed 5.8, 6.7, 7.1, 8.0, 8.8, 9.4, and 10.4 months after infection and the three dogs were necropsied for recovery of adult worms at 10.8 months.

The mosquitoes for the M-2 experiment were infected on Day 129 (Week 18). At this point in the study (i.e., 3 days after the last dose of IVM and 4 days after the last dose of DOXY), microfilariae counts for the IVM- and DOXY-treated groups had already decreased substantially. The total (3) L_3 collected from the IVM mosquitoes were injected into Recipient dog 4; the total (5) L_3 collected from the DOXY mosquitoes were injected into Recipient dog 5; and 40 of those from the control mosquitoes were injected into Recipient dog 6. Microfilaria and Ag testing was done at 5.0, 5.9, 6.7, 7.3 and 8.3 months after infection and the dogs were necropsied 8.7 months after infection.

For the M-3 experiment, the mosquitoes were infected with microfilaremic blood on Day 54 (Week 8, 14 days after the last dose of DOXY). Sixteen days later, Recipient dogs 7 and 8 were each injected with 40 L_3 larvae from the DOXY mosquitoes and Recipient dog 9 received 40 control larvae. Recipient dog 7 was necropsied 4.2 months after infection, which was too early for microfilariae and Ag testing. Microfilariae and Ag tests were performed for the two remaining Recipient dogs (8 and 9) just prior to necropsy at 6 months after infection.

All dogs infected by either intravenous transplantation or subcutaneous injection of L_3 were negative for heartworm microfilariae and Ag prior to infection.

3. Results

3.1. Dog treatment study

Weekly administration of IVM plus intermittent DOXY (IVM + DOXY) for 36 weeks was 78.3% effective in reducing the adult worm burden (Table 2). One of the five dogs in this group was completely cleared of worms, and most of the remaining worms in the other four dogs were abnormal in appearance, with intermittent translucent areas in the body and dark anterior ends. In comparison, treatment with only IVM was only 20.3% effective. All dogs in this group had adult worms, and most of the worms were abnormal in appearance, with intermittent translucent areas in the body and dark anterior ends. Treatment with only DOXY was only 8.7% effective. Treatment with IVM + DOXY + MEL was 92.8% effective against adult worms. Three of the five dogs in this group were cleared of worms, and the two remaining dogs had 1 male and 4 (3 males, 1 female) live worms, respectively. All of these worms were abnormal in appearance. MEL alone was 100% effective in reducing the worm burden; one dead female worm was recovered from one dog and four of the five dogs had fragments of worms. In comparison, all nontreated control dogs had live worms, with an average of 13.4 per dog (range = 12–16). All except two of the worms from these nontreated control dogs were normal in appearance.

3.1.1. Microfilarial counts

Group mean microfilarial counts are presented in Fig. 1. All dogs were negative for circulating microfilariae prior to infection. All dogs also were positive for microfilariae just prior to Day 0, except one dog, which became microfilaremic by Week 6. For both groups that were given IVM plus DOXY (with or without MEL), mean microfilariae counts dropped relatively soon after treatment started, and no microfilariae were seen in any of these 10 dogs after Week 9. Beginning approximately Week 10, mean microfilariae counts for dogs that received IVM + -DOXY dropped gradually to negative or near-negative values (generally $\leq$ 10 microfilariae/mL) just prior to necropsy. At necropsy, all five DOXY dogs had microfilariae. Two of the five dogs treated with IVM were amicrofilaremic, and three of them had 2, 4, and 100 microfilariae/mL, respectively, just prior to

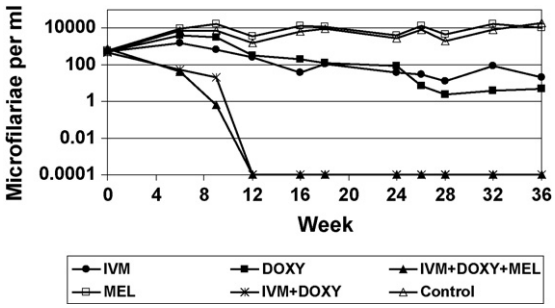

Fig. 1. Mean microfilarial counts for dogs given weekly ivermectin (IVM) or intermittent doxycycline (DOXY), alone or together (with or without melarsomine [MEL] later), beginning 6 weeks after intravenous transplantation of adult heartworms.

necropsy at 36 weeks. Mean microfilariae counts in the MEL and nontreated control groups remained relatively high throughout the study.

3.1.2. Antigen scores

Group mean Ag scores for the nontreated control and treated groups are presented in Fig. 2. Although the score for the IVM + DOXY + MEL group spiked at Week 32, the scores for both groups that received IVM plus DOXY (IVM + DOXY and IVM + DOXY + -MEL) gradually dropped after Week 26, and the score for both groups was 2.2 just prior to necropsy. The Ag score for the MEL-treated group did not drop until just prior to necropsy (score = 1.8). The mean scores for the groups that received only IVM or DOXY were similar to those for nontreated controls throughout the study (range 3.8–4.2).

3.2. Mosquito studies

3.2.1. M-1 study

Recipient dog 3 that was given 40 infective larvae from mosquitoes that had fed on blood from nontreated dogs was positive for microfilariae and Ag 6.7 months post-infection and remained positive on both tests through to the time of necropsy at 10.8 months PI. At necropsy, this dog had a total of 27 adult worms. Recipient dog 1 that was given 6 infective larvae from the mosquitoes that had fed on blood from the dogs treated with IVM was negative for microfilariae and Ag throughout the study and had two live adult male worms at necropsy. Recipient dog 2 that received 40 infective larvae from mosquitoes that had fed on blood from dogs treated with DOXY was negative for microfilariae and Ag throughout the study and had no live worms at necropsy.

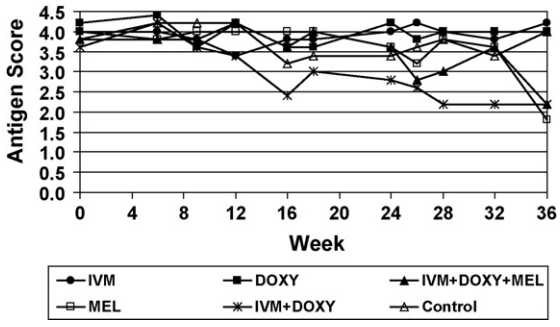

Fig. 2. Mean heartworm antigen test (DiroCHEK®) scores for dogs administered weekly ivermectin (IVM) or intermittent doxycycline (DOXY), alone or together (with or without melarsomine [MEL] later), beginning 6 weeks after intravenous transplantation of adult heartworms.

3.2.2. M-2 study

Recipient dog 6 that was given 40 larvae from mosquitoes that had fed on blood from the nontreated dogs was positive for microfilariae and Ag at 5.9 months after infection and remained positive on both tests through to the time of necropsy at 8.7 months after infection. At necropsy, this dog had a total of 26 adult worms. Recipient dog 5 that received five infective larvae from mosquitoes that had fed on blood from dogs treated with DOXY was negative for microfilariae and Ag throughout the study and had no live worms at necropsy. Recipient dog 4 that was given six infective larvae from mosquitoes that had fed on blood from dogs treated with IVM was negative for both microfilariae and Ag throughout the study but had one live adult female worm at necropsy.

3.2.3. M-3 study

No live worms were recovered from Recipient dog 7 necropsied 4.2 months after infection with 40 larvae from mosquitoes that had fed on a dog treated with DOXY. Just prior to necropsy at 6 months after infection, Recipient dog 8, which was also infected with larvae from mosquitoes that fed on a dog treated with DOXY, was negative for microfilariae and Ag and had no worms at necropsy. Control dog 9 was positive for both microfilariae and Ag and had a total of 21 live adult worms at necropsy.

4. Discussion

Weekly administration of prophylactic doses (6 mcg/kg) of IVM resulted in a gradual reduction in microfilarial count to negative (for two dogs) or near-negative (2–100 microfilariae/mL) values by the end of the study. These results are similar to those obtained in other studies where IVM was given monthly at the same dosage (McCall et al., 1998, 2001; Bowman et al., 2001). Intermittent administration of daily doses of DOXY caused a gradual reduction in microfilariae count, with a pattern similar to that of the IVM-only group, but all of the five dogs had a few microfilariae ($\leq$10 microfilariae/mL) just prior to necropsy at 36 weeks. Starting treatment with DOXY and IVM treatments approximately 6 months before the first treatment with MEL was to clear the *Wolbachia* from the worms as much as possible before initiating treatment with MEL. In our previous laboratory studies, we have seen a rebound in *Wolbachia* at varying times after treatment. Giving intermittent treatments over the course of the study was expected to preclude that rebound.

These gradual reductions in microfilariae counts during administration of IVM or DOXY only strongly suggest that inhibition of embryogenesis (DOXY) and death of developing microfilariae stages *in utero* (IVM) led to microfilariae reduction due to attrition rather than to a direct microfilaricidal effect of the drugs (Lok et al., 1989; McCall et al., 2001; Taylor et al., 2005a). The complete disappearance of circulating microfilariae after Week 10 in all 10 dogs treated with DOXY + IVM suggests that this combination of the drugs had a direct microfilaricidal effect as well.

The lower mean Ag scores in the dogs that were given MEL only, IVM + DOXY or IVM + DOXY + -MEL generally reflected their lower worm burdens (along with the presence or absence of worm fragments as a source of Ag), compared with the other groups. The gradual reduction in average Ag scores for the groups of dogs that received IVM + DOXY (with or without MEL) indicates that some of the worms died relatively early during the treatment period and only a few worms were alive at necropsy, as evidenced by the 78.3% efficacy in the IVM + DOXY group and the 92.8% efficacy for IVM + DOXY + MEL. The spike in Ag score for the IVM + DOXY + MEL group at 32 weeks was probably due to the release of Ag from worms killed by MEL, and the dramatic reduction in Ag score in the MEL group between Weeks 32 and 36 was probably due to the death and clearance of Ag, since efficacy was 100% for this group.

The 20.3% reduction in worm burden following weekly administration of prophylactic doses of IVM for about 8.5–9 months was not surprising. In an earlier study, 16 monthly doses of the drug were only 56% effective against adult heartworms (McCall et al., 1998), which suggests that weekly dosing only with IVM at the prophylactic dosage is no more effective in killing adult heartworms than monthly dosing. The lack of early adulticidal efficacy of DOXY alone against heartworms is consistent with findings for other filariae, which usually require at least 1.5–2 years for adulticidal effects to be evident (Langworthy et al., 2000; Gilbert et al., 2005; Taylor et al., 2005a, b). IVM + DOXY had substantial (78.3%) adulticidal efficacy, and it seems reasonable to assume that the remaining worms would have died if the dogs had been held a few more months before necropsy. This high level of adulticidal activity of IVM + DOXY strongly suggests a synergistic effect of these two drugs and warrants further attention. In the current study, treatment with IVM and DOXY was purposefully excessive in an attempt to obtain near maximal effect. Follow-up studies will focus on increasing the daily dosage of DOXY and shortening the initial treatment period to 4 weeks, followed by treatment 4–6 months later and administering IVM no more frequently than every 2 weeks. Limiting DOXY treatment to one period of 4–6 weeks has been effective in treatment patients with human filariasis (Taylor et al., 2005b).

The three-injection protocol for MEL was 100% effective against adult heartworms, whereas IVM + -DOXY + MEL was 92.8% effective. This might suggest drug interference, however, MEL is not always 100% effective (Dzimianski et al., 1989; Di Sacco and Vezzoni, 1992; Keister et al., 1992; Vezzoni et al., 1992). Confirmation of drug interference would require a study with a larger dog population.

Population dynamics in relation to the role of *Wolbachia* in various life-cycle stages has been studied in filariae, particularly *Brugia malayi* (McGarry et al., 2004; Fenn and Blaxter, 2004; Kozek, 2005; Taylor et al., 2005a). Individual worms appear to vary widely in their bacterial load, which may reflect a dynamic change of population size over time or if constant, the potential for a selective advantage in terms of longevity or fecundity in worms carrying more bacteria. However, at the population level, the numbers of bacteria remain static in microfilariae and the mosquito-borne stages (L_2 and L_3), with the lowest ratios of *Wolbachia*/nematode DNA. Within the first week of infection of the mammalian host, bacteria numbers increase dramatically and the bacteria/worm ratio is the highest of all life-cycle stages. The rapid multiplication continues throughout L_4 development, so that the major period of bacterial growth occurs within the first month of infection. There are few bacteria in mosquito-derived L_3 but many, in large groups, in L_4 collected 1–3 weeks after infection. It appears that the large clusters of bacteria observed throughout the hypodermal cord of adult worms originate from this rapid period of division and thereafter are maintained at that level, as seen in adult male worms. In females, bacterial numbers increase further as the worms mature and as the ovary and embryonic larval stages become infected. Thus, population dynamics is consistent with (1) the two processes compromised most by ‘‘tetracycline’’ treatment (i.e., molts from L_3–L_4 and L_4–juvenile; microfilariae production) and (2) evasion of mammalian immunity (i.e., L_3–L_4 and L_4–juvenile molts and adult worm survival) allowing for long-term survival of adult filariae. In the present study, the survival of microfilariae after DOXY treatment of HW-microfilaremic dogs, followed by ‘‘normal’’ development of these microfilariae to L_3 and the inability of these L_3 to develop to adults in recipient dogs strongly suggest that

relatively few (if any) *Wolbachia* are required for microfilariae to survive and develop into L_3, but substantial numbers are needed for development of L_3 into adults in the mammalian host.

Arsenical drugs have been the mainstay for heartworm adulticidal therapy for the past four to five decades. However, treatment strategies have changed, and indeed, continue to evolve since MEL replaced thiacetsamide sodium in the early 1990s. The manufacturer provides two treatment protocols for MEL: the ''standard'' two-injection protocol for Class I (mild clinical signs) and Class II (moderate clinical signs) dogs and the ''alternative'' three-injection protocol for Class III (severe clinical signs) disease. Generally, this was followed 3–4 weeks later by administration of a microfilaricide, which often had to be repeated once or twice to clear the dog of circulating microfilariae. During the past several years, this microfilaricidal treatment generally has been reduced to a by-product of macrocyclic lactone chemoprophylaxis (Nelson et al., 2005).

Starting in 1995, several studies have demonstrated that prolonged administration of monthly prophylactic doses of a macrocyclic lactone, particularly IVM, kills older larvae, immatures (juveniles), young adults and mature adults (see McCall, 2005). Moreover, a high percentage of the dogs become amicrofilaremic within 6–9 months after dosing is started. The rate of kill with this slow-kill treatment is dependent on the age of the heartworms when treatment is started, with 3-month-old larvae requiring up to 1 year and mature adults needing 2.5 years to provide efficacy of at least 95%. Although monthly IVM is not an approved alternative to MEL therapy and it is particularly risky in very active and symptomatic dogs (Venco et al., 2004), it clearly provides potent ''safety-net'' activity against older larvae, immatures (juveniles), and young adults in cases of owner compliance failure, even when the owner and veterinarian are not aware that the animal is infected.

The American Heartworm Society (Nelson et al., 2005) recognizes the safety-net and adulticidal properties of some of the macrocyclic lactones, particularly IVM. Their 2005 canine guidelines state that administration of a chemoprophylactic dose of a macrocyclic lactone should begin as soon as the dog is diagnosed with a heartworm infection. While controversial due to the theoretical risk of inducing resistance to macrocyclic lactones, it may be beneficial to administer a macrocyclic lactone for up to 6 months prior to administration of MEL when the presentation does not demand immediate intervention. The reasoning for this approach is to greatly reduce, if not completely eliminate, circulating microfilariae and kill migrating *D. immitis* larvae and (in the case of IVM) to stunt immature *D. immitis* ($\geq$20%) (McCall, 1993, unpublished data) and reduce female worm mass ($\geq$20%) (McCall, 1993, unpublished data) by inhibiting the reproductive system. Moreover, administration for 3 months also will allow immature worms to reach an age at which they are known to be susceptible to killing by MEL (Keister et al., 1992; Atkins and Miller, 2003) and administration for longer than 3 months should result in reduced antigenic mass, which in turn may reduce the risk of thromboembolism.

By eliminating the *Wolbachia* endosymbionts, tetracyclines have been shown in several species of filariae to prevent development of the larval stages, inhibit embryogenesis in adult worms, and eventually kill adult filariae, usually 1.5–2 years after treatment is started in laboratory animals, large animals, and humans (Taylor et al., 2005a; Kramer et al., 2007).

In the present study, IVM + DOXY had the synergistic effects of completely clearing dogs of circulating microfilariae by Week 10 and killing 78.3% of the adult worms by Week 36. Interestingly, *Wolbachia* were still detected at necropsy in worms from dogs treated only with DOXY but were virtually eliminated in those receiving both DOXY and IVM (Bazzocchi et al., 2008). Gross pathology and histopathologic observations of the lungs of the 30 dogs revealed the most severe pathology in the dogs receiving only MEL and the least evidence of pathology in those receiving IVM + DOXY + MEL (no grossly visible lesions in 3 of 5 dogs). These latter dogs also showed significantly fewer severe arterial lesions and a virtual absence of thrombi (Kramer et al., 2008). Clinical findings of significance included eosinophilia in most dogs treated with IVM or DOXY alone at 12 weeks following a precipitous decline in microfilariae; only dogs that received IVM did not have elevations in serum alanine transaminase; and MEL had no apparent effect on serum chemistries (Dzimianski et al., 2006).

In the present study, L_3 collected from mosquitoes fed on microfilaremic blood from dogs treated with DOXY were normal in appearance and motility but were not able to develop in dogs. Thus, treatment with DOXY prevented further transmission of the disease even when microfilariae were present.

These and other observations strongly suggest that administration of both IVM and DOXY for several months prior to MEL or possibly without MEL, will eliminate adult heartworms with less potential for severe thromboembolism than MEL alone and will block transmission. Therefore, it is likely that DOXY

eventually will be included in heartworm adulticide therapy for dogs. Furthermore, the potentially life-threatening infections and high risk associated with MEL treatment strongly encourage the testing of DOXY plus IVM as an alternative adulticidal therapy for heartworm infected cats and ferrets (McCall et al., 2008).

Conflict of interest

J.W. McCall, Genchi, L. Kramer, Guerrero, Dzimianski, P. Supakorndej, Mansour, S.D. McCall, N. Supakorndej, Grandi, and Carson have no personal or financial relationship with other persons or organizations that could inappropriately influence or bias this study.

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