Combined ivermectin and doxycycline treatment has microfilaricidal and adulticidal activity against *Dirofilaria immitis* in experimentally infected dogs


**Abstract**

There is still a pressing need for effective adulticide treatment for human and animal filarial infections. Like many filarial nematodes, *Dirofilaria immitis*, the causative agent of canine heartworm disease, harbours the bacterial endosymbiont *Wolbachia*, which has been shown to be essential for worm development, fecundity and survival. Here the authors report the effect of different treatment regimens in dogs experimentally infected with adult *D. immitis* on microfilariemia, antigenemia, worm recovery and *Wolbachia* content. Treatment with ivermectin (IVM; 6 l g/kg per os weekly) combined with doxycycline (DOXY; 10 mg/kg/day orally from Weeks 0–6, 10–12, 16–18, 22–26 and 28–34) resulted in a significantly faster decrease of circulating microfilariae and higher adulticidal activity compared with either IVM or DOXY alone. Quantitative PCR analysis of *ftsZ* (*Wolbachia* DNA) and 18S rDNA (nematode DNA) absolute copy numbers showed significant decreases in *Wolbachia* content compared with controls in worms recovered from DOXY-treated dogs that were not, however, associated with worm death. Worms from IVM/DOXY-treated dogs, on the other hand, had *Wolbachia*/nematode DNA ratios similar to those of control worms, suggesting a loss of both *Wolbachia* and nematode DNA as indicated by absolute copy number values. Histology and transmission electron microscopy of worms recovered from the IVM/DOXY combination group showed complete loss of uterine content in females and immunohistochemistry for *Wolbachia* was negative. Results indicate that the combination of these two drugs causes adult worm death. This could have important implications for control of human and animal filarial infections.

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**Keywords**: *Dirofilaria immitis*, Doxycycline; Ivermectin; *Wolbachia*; Q-PCR; Immunohistochemistry; TEM

1. Introduction

Infection with filarial nematodes causes severe disease in humans and animals. Current control strategies against infection in humans are based on the periodic administration of microfilaricidal drugs aimed at breaking the life-cycle of the parasite and reducing clinical disease (Addiss and Mackenzie, 2004). However, there is still a pressing need for new antifilarial drugs that are effective against adult parasites (Hoerauf et al., 2000). Recently, antibiotic therapy with doxycycline (DOXY) has been shown to be macrofilaricidal against both human (*Wuchereria bancrofti*, Debrah et al., 2007; *Onchocerca volvulus*, Hoerauf et al., 2007) and animal (*Onchocerca ochengi*; Gilbert et al., 2005) filarial worms, opening a promising new chapter in the control of infection and disease based on the hypothesis that the bacterial endosymbiont *Wolbachia* represents a suitable target. *Dirofilaria immitis* is a filarial nematode that causes canine heartworm disease in many countries...
of the world (Genchi et al., 2005). There is only one drug registered for adulticidal therapy against canine heartworm, melarsomine (Immiticide®), but treatment is often followed by severe pulmonary thrombosis. Long-term monthly administration of the macrocyclic lactone (ML) ivermectin (IVM) has been shown to be macrofilaricidal against *D. immitis*, but complete elimination of adult worms takes up to 36 months and the drug is not currently registered for this use (McCall et al., 2001). *Dirofilaria immitis* also harbours the bacterial endosymbiont *Wolbachia*. Bandi et al. (1999) have reported that tetracycline treatment of naturally infected dogs causes a block in embryogenesis and loss of *Wolbachia* from female worms.

The aim of the present study was to compare the effects of IVM, DOXY and IVM/DOXY combination treatment of experimentally infected dogs on microfilariae, antigenemia, worm recovery and *Wolbachia* content in *D. immitis*.

2. Materials and methods

2.1. Animals and treatment groups

A total of 20 young adult beagles (male and female) were used. The animals were born and raised in a mosquito-proof environment and did not have any prior exposure to natural infection with *D. immitis*. Seven male and nine female adult heartworms harvested from dogs approximately 8 months p.i. were introduced by i.v. transplantation into each dog (McCall et al., 1998). Approximately 6 weeks later (Day 0), the dogs were ranked within gender by microfilarial count and randomly allocated to four groups of five dogs each. Beginning on Day 0, the dogs in one group were given weekly prophylactic doses of IVM (6 µg/kg) orally for 34 weeks. Weekly treatment was chosen to evaluate if previously reported adulticidal effects following monthly treatment could be achieved in a shorter period of time. The dogs in another group were given DOXY (10 mg/kg/day) orally from Weeks 0–6, 10–12, 16–18, 22–26 and 28–34. This protocol was chosen based on recent reports of adulticidal activity against *O. ochengi*. Gilbert et al. (2005) showed that i.m. administration of a long-acting oxytetracycline to *O. ochengi*-infected cattle at monthly intervals for 6 months eliminated more than 80% of viable adult worms, whereas short-term treatment was ineffective. The dogs in the third group were given a combination of IVM and DOXY at the same dosages and treatment schedules as used for the first and second groups, respectively. The remaining group served as the non-treated (or infection) control. Approximately 36 weeks after i.v. transplantation, the dogs were humanely euthanized by barbiturate injection and necropsied for recovery of heartworms. The study protocol was approved by the Institutional Animal Care and Use Committee prior to initiation of the study.

2.2. Microfilariaemia, antigen testing and worm recovery

Blood was collected from each dog for examination by the modified Knott test for detection and enumeration of microfilariae (mf) of *D. immitis* before i.v. transplantation of adult heartworms, just prior to initiation of IVM and DOXY treatment at Day 0, and thereafter at Weeks 6, 10, 12, 16, 18, 24, 26, 28, 32 and 36 (i.e. just prior to necropsy). Samples were collected at approximately the same time each day throughout the study. Detection and enumeration of mf of *D. immitis* were carried out with the modified Knott test. Briefly, 1 ml venous blood was mixed with 10 ml of 2% buffered formalin and centrifuged for 5 min at 200 g. One hundred microlitre of sediment was mixed with equal parts of a 1:1,000 methylene blue stain. Twenty microlitre of stained sediment was placed on a slide, covered with a coverslip and examined under a microscope. The number of mf was multiplied by 10 and expressed as mf/ml.

Serology for circulating *D. immitis* antigens was carried out using a commercial ELISA kit (Canine Heartworm Antigen Test Kit – PetChek HTWM PF – Idexx Laboratories, Milan, Italy) at Weeks 0, 6, 10, 12, 24 and 36. O.D.s were measured at 650 nm in an Easy Reader Bio-Rad.

At Week 36, necropsies were performed. The heart and pulmonary vasculature were examined thoroughly for heartworms. All recovered worms were counted and sexed. Worms were then placed in 10% buffered formalin for histology and immunohistochemistry, in Karnovsky’s fixative for transmission electron microscopy (TEM) and frozen at −80 °C for PCR.

2.3. Quantitative PCR (Q-PCR) for Wolbachia

Worms kept at −80 °C were thawed, weighed and placed separately in 300 µl (female worms) or 200 µl (male worms) of Tris–HCl pH 8. Each sample was heated at 90 °C for 10 min, mechanically disrupted, treated with proteinase-K (final concentration of 200 µg/ml) at 55 °C for 3 h and reheated at 90 °C for 10 min. After centrifugation at maximum speed for 10 min, DNA was extracted from 100 µl of the supernatant with a commercial kit (DNeasy Tissue kit; Qiagen, GmbH, Hilden, Germany) and re-suspended in 100 µl of water. The purity and concentration of DNA samples were verified using a microspectrophotometer. After DNA extraction, samples were normalised to 150 µg of initial worm weight for 1 µl of purified DNA.

To quantify *Wolbachia*, a sensitive quantitative PCR method (Q-PCR) was devised. Genes from the *Wolbachia* genome (*ftsZ*, a bacterial tubulin homologue gene) and the *D. immitis* genome (18S rDNA, a ribosomal gene) were amplified. PCR products were quantified in real time through incorporation of SYBR green dye using a Bio-Rad i-Cycler. The number of worms analysed for each treatment group is indicated in Table 2.

Based on the gene sequence of the *Wolbachia* of *D. immitis* present in GenBank (Accession No. AJ495000) a pri-
mer pair was designed (FTSZQ-F: 5′ ACGGGTAGT GG GGACATGA 3′; FTSZQ-R: 5′ TCCATCGCTTG TCGA AAGT 3′) to amplify a fragment of 116 bp. Specific primers for the amplification of 18S rDNA (18SQ-F: 5′ GGCAAAGCGGTGTTTAGC 3′; 18SQ-R: 5′ GCAC GCTGATTCCTCAGT 3′) were designed based on the available gene sequence (Accession No. AF036638) to amplify a fragment of 102 bp.

Plasmids containing inserts of the amplified ftsZ and 18S rDNA gene sequences were prepared as standards in the Q-PCR. Briefly, a conventional PCR was performed using primers previously described (annealing temperature of 60 °C; final primer concentration 1 μM) and PCR products were ligated into the pGEM T-easy vector (Promega corporation, Madison, WI) and cloned into DH5α cells. Positive colonies were screened by PCR and one plasmid for each target was purified and sequenced (ABI Prism 310; Applied Biosystem, Foster City, CA, USA) to verify that the correct sequence had been amplified. Plasmid DNA was quantified by a spectrophotometer and 10-fold dilutions were prepared between 102 and 109 copies/μl.

Q-PCR mixtures were performed in duplicate in 25 μl with target DNA (1 μl of normalised DNA for ftsZ amplification and 1 μl of a 1:10 dilution of the same DNA for 18S rDNA amplification), 12.5 μl of SYBR green mix (Bio-Rad, Hercules, CA) and 350 nM of FTSZQ-F and FTSZQ-R primers or 450 nM of 18SQ-F and 18SQ-R primers. The thermal profile used (95 °C for 90 s; 50 cycles of 95 °C for 15 s and 58 °C for 60 s; for melting curve construction, 55 °C for 60 s and 80 cycles starting to 55 °C and increasing 0.5 °C each 10 s) was the same for each target gene. Melting curves were performed to confirm the presence of specific products and the absence of non-specific products.

Thermocycler software generated linear standard curves from plots of plasmid logarithmic copy numbers against the cycle numbers at which the fluorescence released by SYBR Green molecules increased above background levels. Copy numbers of ftsZ and 18S rDNA genes in 1 μl of the normalised DNA were calculated by reference to the standard curves and multiplied, in the case of the 18S rDNA gene, by the dilution factor. Results were expressed both as ftsZ/18S (Wolbachia/nematode) ratios and as absolute values of copy numbers for ftsZ and 18S genes.

2.4. Histology and Wolbachia immunohistochemistry

Worms (at least one male and two females from each group, except Group IVM/DOXY where only one female was available for examination) were immediately fixed in 10% buffered formalin and then processed for routine histology. Each worm was sectioned into five equal parts and each section was embedded separately to ensure reliable comparison among groups. Worms were stained with H & E. Six micron-thick sections were also placed on polystyrene pre-treated slides and immunohistochemistry was performed with a polyclonal antibody against the Wolbachia Surface Protein (WSP), according to Kramer et al. (2003).

2.5. Transmission electron microscopy (TEM)

Adult females of D. immitis (two worms from each group) were collected and dissected in saline solution. The uteri and ovaries were harvested and prefixed in Karnovsky’s fixative in cacodylate buffer (pH 7.2) at 4 °C. The samples were then washed in the same buffer and post-fixed in 1% OsO4 for 1.5 h at 4 °C. The samples were then dehydrated in graded ethanol solutions, transferred to propylene oxide and embedded in Epon 812. The semithin section (1 μm) for light microscopy were stained with 0.5% toluidine blue; thin sections (80 nm) were stained with uranil acetate and lead citrate and examined under a Zeiss EM900 transmission electron microscope.

2.6. Statistical analysis

The non-parametric Kruskal–Wallis H-test was used to identify differences among treatment groups compared with controls. P values < 0.05 were considered to be significant. The software program SPSS (version 12.0) was used to analyse data (SPSS Inc., USA).

3. Results

3.1. Combination IVM/DOXY has significant micro- and macrofilaricidal activity against D. immitis

The treatment protocol was well tolerated by the dogs and no adverse effects were observed. The results of mean microfilarial counts obtained during the study are shown in Fig. 1. There was a significant (P < 0.05) fall in circulating mf in the combination IVM/DOXY treatment group beginning at Week 6 of treatment and all dogs from this group were amicrofilaremic at Week 12 and thereafter.

![Fig. 1. Mean microfilarial counts (± SDs) obtained during the study. The decrease in microfilariae/ml is significant (P < 0.05) in the combination ivermectin/doxycycline (IVM/DOXY) treatment group compared with the other groups from Week 6 onward. The decrease in microfilariae/ml is significant (P < 0.05) compared with controls in the dogs treated with IVM or DOXY alone from Week 12 onward.](image-url)
Microfilariaemia also significantly decreased ($P < 0.05$) in dogs treated with IVM or DOXY alone from 12 weeks onward, but most still had some circulating mf at necropsy. Mean O.D. values for antigenemia (Fig. 2) also gradually decreased with IVM/DOXY treatment: two of the five treated dogs had O.D. values close to zero at the end of the study, causing the mean value to decrease by approximately 50% (differences however in mean O.D. values were not significant). O.D. values for IVM or DOXY alone were similar to controls throughout the study. The results of worm recovery during necropsy are shown in Table 1.

When compared with the control group, the IVM/DOXY combination resulted in an average of 92.6% mortality for male worms and 69% mortality for female worms, for a total adulticidal effect of 78.26% ($P < 0.001$). In this group, one of five dogs was worm-free at the end of the study. Weekly IVM treatment caused death of 20.3% of the adult worms, while intermittent DOXY only eliminated 8.7% of the adult worms.

### Table 1

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Mean number alive adult worms</th>
<th>SD (±)</th>
<th>95% Confidence limits</th>
<th>Percent efficacy</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Total</td>
<td>Range</td>
</tr>
<tr>
<td>IVM</td>
<td>4.2</td>
<td>6.8</td>
<td>11</td>
<td>(5–14)</td>
</tr>
<tr>
<td>DOXY</td>
<td>4.6</td>
<td>8.0</td>
<td>12.6</td>
<td>(9–15)</td>
</tr>
<tr>
<td>IVM/DOXY</td>
<td>0.4</td>
<td>2.6</td>
<td>3.0</td>
<td>(0–6)</td>
</tr>
<tr>
<td>Control</td>
<td>5.4</td>
<td>8.4</td>
<td>13.8</td>
<td>(12–16)</td>
</tr>
</tbody>
</table>

$^a P < 0.001$.

### Table 2

<table>
<thead>
<tr>
<th>Worms examined</th>
<th>$ftsZ$/18S genes ratio</th>
<th>18S copy number</th>
<th>$ftsZ$ copy number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Range</td>
<td>Median</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVM (13)</td>
<td>0.49$^a$</td>
<td>0.114–1.29</td>
<td>7.2 $\times$ 10$^{4a}$</td>
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<tr>
<td>DOXY (14)</td>
<td>4 $\times$ 10$^{-3a}$</td>
<td>1.3 $\times$ 10$^{-3}$–4 $\times$ 10$^{-4}$</td>
<td>4.4 $\times$ 10$^{3}$</td>
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<tr>
<td>IVM/DOXY (4)</td>
<td>7.1 $\times$ 10$^{-3}$</td>
<td>10$^{-3}$–1.4 $\times$ 10$^{-2}$</td>
<td>2.5 $\times$ 10$^{4}$</td>
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<tr>
<td>Control (18)</td>
<td>3.3 $\times$ 10$^{-3}$</td>
<td>5.2 $\times$ 10$^{-4}$–1.6 $\times$ 10$^{-2}$</td>
<td>3.8 $\times$ 10$^{6}$</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVM (5)</td>
<td>2.2 $\times$ 10$^{-2}$</td>
<td>1.5 $\times$ 10$^{-2}$–7.1 $\times$ 10$^{-2}$</td>
<td>1.3 $\times$ 10$^{3}$</td>
</tr>
<tr>
<td>DOXY (5)</td>
<td>1.8 $\times$ 10$^{-4a}$</td>
<td>1.3 $\times$ 10$^{-4}$–9 $\times$ 10$^{-4}$</td>
<td>1.4 $\times$ 10$^{3}$</td>
</tr>
<tr>
<td>Control (4)</td>
<td>5.1 $\times$ 10$^{-2}$</td>
<td>3 $\times$ 10$^{-2}$–7.5 $\times$ 10$^{-2}$</td>
<td>1.2 $\times$ 10$^{3}$</td>
</tr>
</tbody>
</table>

$^a P < 0.05$.
ment. This phenomenon is explained by a decrease of median values of 18 S copy numbers for control versus IVM (from $3.8 \times 10^6$ to $7.2 \times 10^5$) as shown in Table 2. Interestingly, ftsZ/18 S rDNA ratios did not differ significantly between male worms from the IVM group (median value: $2.2 \times 10^{-2}$) and controls (median value of $5.1 \times 10^{-2}$). Worms recovered from the IVM/DOXY treatment group had Wolbachia/nematode ratios for female worms (median value: $7.1 \times 10^{-3}$) similar to controls since there was a contemporary decrease both in Wolbachia and filarial DNA as shown by absolute values in Table 2. No male worms were analysed for this group.

3.3. IVM/DOXY combination treatment causes complete loss of immature and mature embryonic stages in D. immitis and results in depletion of Wolbachia

Histology and anti-WSP immunohistochemistry of worms from the different treatment groups are shown in Fig. 3A–F. Male and female worms from control dogs showed normal morphology and strong anti-WSP staining in lateral chords and embryos (Fig. 3A). Worms from animals treated with IVM showed degeneration of stretched mf in the cranial part of the uterus and dilation of the oesophagus which appeared full of blood (Fig. 3B), while anti-WSP immunohistochemistry showed many Wolbachia in the hypodermis (Fig. 3C). Histological examination of females from DOXY-treated dogs showed degenerating oocytes and morulae that were negative for anti-WSP immunohistochemistry. However, DOXY treatment alone was unable to efficiently clear Wolbachia from D. immitis and bacteria were still present within lateral chords of females (Fig. 3D). Males from DOXY-treated dogs, however, appeared necrotic on histology and showed only residual “halo” staining for WSP (Fig. 3E). The only worm available for examination from the IVM/DOXY group was the female shown in Fig. 3F. Histology revealed the lack of any discernible structures within the uterus or only shadows of what were likely stretched mf, and anti-WSP staining was absent.

In TEM, ultra-thin sections of the apical region of the ovary from untreated female worms revealed the presence of a large number of Wolbachia in the cytoplasm of oocytes. The bacteria were contained within membrane-bound vacuoles (Fig. 4A). The microorganisms also appeared in mf in the cephalic uterine portion of females from the untreated group (Fig. 4B). In worms treated with IVM, the eggs in the middle part of the ovary (Fig. 5A) and in the caudal portion of the uterus appeared slightly modified. In the eggs and developing embryos from the worms of the IVM group bacteria are easily observable (Fig. 5A); there is thus evidence that IVM had some detrimental effect on nematode embryonic development, but not on Wolbachia. In the cephalic portion of the uterus of IVM-treated worms, only degenerating embryos/mf were observed: the mf showed signs of cuticular degeneration and structures interpreted as fragments of embryos/mf were observed (Fig. 5B). In the worms from the DOXY group, oocytes in the ovary and in the caudal portion of the uterus showed marked morphological alterations of all cytoplasm organelles and only chromatin, scattered into an amorphous cellular material, was observed (Fig. 5C). In the cephalic portion of the uterus, where nematodes from control animals contained developed mf, highly degenerated embryos were observed, with only a few developed embryos surrounded by amorphous material (Fig. 5D). In the group treated with IVM/DOXY embryogenesis appeared dramatically altered: the cell cytoplasm showed several residual bodies and it was almost impossible to identify any cellular element, both in the cephalic parts of the reproductive apparatus (Fig. 5E), and in the cephalic uterine portion (Fig. 5F). Treatment with IVM/DOXY thus lead to marked degeneration of eggs and embryos at all levels of the reproductive apparatus, indicating that the molecules possibly have a synergistic activity on the oogenesis/embryogenesis of filariae. Finally, in the worms treated with IVM, the gut showed a degenerative process of the wall characterised by the presence of several vacuoles (Fig. 6B). This was not observed either in the worms from the DOXY group or in those from the control group (Fig. 6A).

4. Discussion

Here we have shown that long-term treatment with a combination of IVM and DOXY has superior and significant micro- and macrofilaricidal effects against D. immitis as compared with either drug administered alone. Effects included elimination of circulating mf, a decrease in circulating adult antigens and a significant decrease in the number of worms recovered at necropsy. The dramatic drop in circulating mf could be due to the summation of the effects observed in TEM of worms from dogs treated with each drug individually; i.e. degeneration of late embryonic stages by IVM and a block of early embryogenesis by DOXY. The combined treatment also caused a decrease in antigen levels in several dogs from this group from Week 24 on, suggesting early death of adult worms (the ELISA used here has been reported to detect circulating antigens for up to 4 months following worm death; McCall et al., 1998). However, the cause of this adulticide effect is not clear. Evaluation of absolute ftsZ copy numbers showed that treatment with DOXY alone was able to reduce Wolbachia loads as well as DOXY/IVM, but did not cause worm death at the post-treatment time point considered here. In previous reports, death of other filarial species following antibiotic treatment targeting Wolbachia was reported to be significant approximately 12–24 months post-treatment (Gilbert et al., 2005; Debrah et al., 2007; Hoerauf et al., 2007) and in this current work it cannot be excluded that worms may have died later. Anti-WSP immunohistochemistry of female nematodes from the DOXY group still showed positive staining within the lateral chords (results not observed in the one worm examined
from the IVM/DOXY group) and persistence of at least some viable *Wolbachia* may have contributed to the observed lack of adulticide effects. Similarly, IVM treatment alone caused a significant reduction in 18S rDNA absolute copy numbers without significantly affecting worm counts at necropsy. The 100-fold decrease in nema-

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**Fig. 3.** Anti-*Wolbachia* surface protein (WSP) immunohistochemistry and histology of worms from the different treatment groups. (A) Female worm from a control dog. Note intense anti-WSP staining in lateral chords and embryos (ABC-Peroxidase, bar = 500 μm). (B) Female worm from a dog treated with ivermectin (IVM) shows dilation of the oesophagus (arrow, haematoxylin and eosin – H & E, bar = 500 μm) and the inset shows degeneration of stretched microfilariae (H & E, bar = 100 μm). (C) Anti-WSP immunohistochemistry of a female worm from an IVM-treated dog. Note the *Wolbachia* in the hypodermis (ABC-Peroxidase, bar = 200 μm). (D) Anti-WSP immunohistochemistry in a female worm recovered from a dog treated with doxycycline (DOXY). Note degenerating morulae, which are negative for WSP (arrows), and the persistence of a few *Wolbachia* within lateral chords (arrowheads, ABC-Peroxidase, bar = 100 μm). (E) Anti-WSP immunohistochemistry of a male worm from an IVM-treated dog. Note residual “halo” staining for WSP (arrows, ABC-Peroxidase, bar = 100 μm). (F) Anti-WSP immunohistochemistry of the female worm available for examination from the IVM/DOXY group. Note the lack of discernible structures within the uterus and of any anti-WSP staining (ABC-Peroxidase, bar = 200 μm).
tode DNA observed in the female worms recovered from this group is likely due to both the loss of mature mf from the uterus observed in histology and to the intestinal damage and tissue loss revealed by TEM.

Why then are worms dying when exposed to IVM/DOXY? One could hypothesise a simple “summation” effect of the two drugs. Absolute copy numbers for both DNA targets were significantly decreased in Q-PCR. However, while the mechanism of action of the macrocyclic lactones at therapeutic dosages is well known (i.e. inhibition of the inhibitory transmitters, causing impairment of neuromuscular function), it is still not known how antibiotics and/or Wolbachia depletion kill filarial worms. This obviously renders any feasible hypothesis on eventual synergism between the two drugs open to question.

Interestingly, both Debrah et al. (2007) and Hoerauf et al. (2007) administered IVM to patients infected with filarial worms (W. bancrofti and O. volvulus, respectively) approximately 6 months following 4- or 6-week DOXY treatment. These studies did not directly address possible synergism between the two drugs, but IVM may have potentiated the observed macrofilaricidal activity.

Our results suggest that depletion of Wolbachia is not the sole cause of worm death. Rajan (2004) reported that chemically modified tetracyclines without antimicrobial activity were able to cause detrimental effects on filarial worms similar to those observed with unmodified tetracyclines and suggested that other effects independent of Wolbachia depletion could be responsible. Could IVM in some way potentiate these effects? In our study, the observed intestinal alterations caused by IVM, also reported previously by Steffens and McCall (1998) following monthly IVM treatment, may have allowed for more rapid absorption of the antibiotic by worms. It is also possible that the accumulation of blood and ingesta, due to neuromuscular dysfunction within the altered intestine, allowed for better uptake of the antibiotic compared with the DOXY group. Furthermore, in-vitro experiments have shown that P-glycoprotein, an efflux pump for various drugs including tetracyclines (Kavallaris et al., 1993), is inhibited by IVM, reducing drug efflux (Pouliot et al., 1997). If IVM allowed accumulation of higher concentrations of DOXY within the nematode compared with DOXY alone, eventual lethal effects independent of Wolbachia depletion would have been observed sooner.

On the other hand, it cannot be excluded that DOXY in some way potentiates the effects of IVM, even though this seems less likely. Recently, however, several compounds,
including antibiotics, have been shown to increase intracellular concentrations of MLs such as moxidectin (Dupuy et al., 2006).

The protocol used here is unfeasible in a clinical setting for the treatment of canine heartworm disease. The therapy was well tolerated by the dogs, but the lengthy time course...
and the cost of treatment would likely discourage practitioners from considering it as an alternative to melarsomine. Further work is needed to determine if a shorter treatment regimen (6–8 weeks) has the same adulticidal effects observed in this study.

It has been recognised by the scientific community that a more efficient regime of adulticidal treatment would have important implications for control of infection and disease caused by human filariae and the search for a safe and effective adulticide is considered a priority (Addiss and Mackenzie, 2004). The significant adulticidal effect of a combination IVM/DOXY treatment reported here may contribute to this goal.

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