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A combination of doxycycline and ivermectin is adulticidal in dogs with naturally acquired heartworm disease (*Dirofilaria immitis*)

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ABSTRACT

Canine heartworm disease is caused by infection with *Dirofilaria immitis*, a filarial nematode that resides in the pulmonary arteries and occasionally in the right heart chambers of infected dogs. Here the authors evaluated the effect of a combination of doxycycline (10 mg/kg/sid for 30 days) and ivermectin–pyrantel (6 μg/kg to 14 mg/kg every 15 days for 180 days) on microfilariemia, antigenemia and parasite load at echocardiography in naturally infected dogs from an endemic region of Italy. Dogs were examined monthly for 6 months and followed-up 4 months later. One hundred percent of dogs became negative for circulating microfilariae by day 90, while 8/11 (72.7%) of dogs became antigen-negative by day 300. Of the 7 dogs that were positive for visualization of parasites at echocardiography, 6 (85.7%) became negative by day 300. Treatment was well-tolerated by all dogs. These results suggest that a combination of doxycycline and ivermectin is adulticide in dogs with *D. immitis*.

1. Introduction

Dirofilaria immitis is a filarial nematode that causes canine heartworm disease (HWD) in many countries of the world (Genchi et al., 2005). There is currently only one drug registered for adulticide therapy against canine heartworm, melarsomine dihydrochloride (Immiticide®, Merial), but treatment is often followed by severe pulmonary thrombosis (Kramer et al., 2008). Long-term monthly administration of the macrocyclic lactone (ML) ivermectin has been shown to be macrofilaricidal against *D. immitis*. McCall et al. (2001) have reported that monthly administration of prophylactic doses of ivermectin resulted in complete elimination of adult worms in experimentally infected dogs in 36 months. More recently, Venco et al. (2004) evaluated the adulticide activity of monthly ivermectin in naturally infected dogs from an endemic area. After 24 months, approximately 71% of infected dogs were negative for the presence of circulating antigens. *D. immitis*, like many other filarial worms, harbours the bacterial endosymbiont *Wolbachia* (Kramer et al., 2003). Recent studies have shown that elimination of *Wolbachia* through antibiotic treatment of the filarial-infected host is macrofilaricidal against both human (*Wuchereria bancrofti*, Debrah et al., 2007; *Onchocerca volvulus*, Hoerauf et al., 2008) and animal (*Onchocerca ochengi*, Gilbert et al., 2005) filarial worms. We have recently reported that a combination of long-term ivermectin and doxycycline treatment had significant micro- and macrofilaricidal effects against *D. immitis* when compared to either drug administered alone to experimentally infected dogs (Bazzocchi et al., 2008).

The aim of the present study was to evaluate the effect of a combination of doxycyline and ivermectin on microfilariemia, antigenemia and parasite load evaluated by echocardiography in dogs with naturally acquired HWD.

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2. Materials and methods

2.1. Animals and treatment

Dogs were recruited during routine clinical examination at the University of Parma Veterinary Teaching Hospital. Dogs that were positive for circulating microfilariae of *D. immitis* and/or circulating parasite antigens and that had not received any preventives in the previous 3 months were included in the study, following owner's consent. A total of 11 dogs participated in the study. Six were female and five were male. Eight of the dogs were from the local shelter and it was not possible to reliably determine the exact age. However, all were adult dogs over 2 years old. The 3 privately owned dogs ranged in age from 3 to 10 years. Enrolled dogs were treated with doxycycline (Ronaxan®, Merial) at 10 mg/kg daily for 30 days and with ivermectin–pyrantel pamoate (Cardotek Plus®, Merial) at a minimum dose of 6 μg/kg to 14 mg/kg once every 15 days for 6 months. Dogs were submitted to complete physical examination, thoracic radiography, echocardiography and blood sampling for the evaluation of parasitological parameters at enrollment and once a month during the entire study period (days 0, 30, 60, 90, 120, 150, 180). A last complete examination was conducted 4 months after the last monthly visit (day 300 after onset of therapy) to assess definitive parasitological cure.

2.2. Parasitological evaluation

2.2.1. Microfilariemia

Detection and enumeration of circulating microfilariae (mf) of *D. immitis* were carried out at each visit by the modified Knott test, according to Bazzocchi et al. (2008). Briefly, 1 ml venous blood was mixed with 10 ml of 2% buffered formalin and centrifuged for 5 min at 200 × *g*. One hundred microlitres of sediment was mixed with equal parts of a 1:1000 methylene blue stain. An aliquot of 20 ml 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.

2.2.2. Antigenemia

Serology for circulating *D. immitis* antigens was carried out monthly for 6 months and once again at 10 months from the onset of therapy using a commercial ELISA kit (Canine Heartworm Antigen Test Kit – PetChek HTWM PF – Idexx Laboratories, Milan, Italy). Optical densities (O.D.) were measured at 650 nm in an Easy Reader BioRad.

2.2.3. Echocardiography

Parasite load was determined with echocardiograms and was scored as follows—0: no adult worms evident in the main pulmonary artery (MPA) and in the right (rPA) and left (lPA) pulmonary arteries; 1: low parasitic load (one to two adult worms evident in the rPA and/or lPA); 2: moderate parasitic load (more than two adult worms evident in the rPA and/or lPA); 3: high parasitic load (several worms evident in the MPA and in the rPA and/or lPA).

3. Results

Results from parasitological analyses are reported in Tables 1–3 and Fig. 1. Eight of the eleven dogs (72.7%) presented circulating mf at the beginning of the study with counts ranging from 80 to 29,910 mf/ml. Treatment caused a rapid decrease in circulating microfilariae: 37.5% (3/8) of dogs were negative by day 30, 87.5% (7/8) by day 60 and 100% were negative by day 90 (Table 1).

All dogs were positive for circulating antigens at the beginning of the study except for dog 5, which resulted positive at day 30. Approximately 18.1% of the dogs (2/11) were negative at day 90 after the beginning of treatment, 36.2% were negative (4/11) by day 120, (5/11) 45.4% were negative by day 180, and 72.7% were negative by the 4-month follow-up at day 300 (8/11) (Table 2).

A gradual decline in parasite load scores was observed in all those dogs in which adult parasites were seen at echocardiography (63.6%; 7/11) on day 0. Worms were no longer visible in 1/7 (14.3%) dogs on day 30, in 2/7 (28.5%) on day 150, in 4/7 (57.1%) on day 180 and in 6/7 (85.7%) dogs on day 300 (Table 3 and Fig. 1). Dogs scored as 0 at enrollment (36.4%; 4/11) remained negative for adult worms throughout the study.

Treatment was well-tolerated by all dogs. Three dogs presented episodes of coughing at approximately day 30–60. These were treated with prednisone at 0.5 mg/kg daily and placed under exercise restriction until remission of symptoms.

4. Discussion

The only drug currently registered for adulticide therapy against canine heartworm is the arsenic derivative melarsomine dihydrochloride (Immiticide®, Merial), but treatment is often followed by severe pulmonary thrombosis following the death of the entire worm population in a short period of time (Kramer et al., 2008). For this reason, the American Heartworm Society guidelines now advise, regardless of stage of disease, a slower and more gradual elimination of adult parasites and recommend a three-injection protocol of one dose initially, followed in 4–6 weeks with a two-dose treatment. In this way, worms are

Table 1
Results of microfilariemia[a] in *D. immitis* naturally infected dogs treated with a combination of doxycycline and ivermectin.

Dog	Day							
	0	30	60	90	120	150	180	300
1	10,350	460	20	0	0	0	0	0
2	29,910	340	0	0	0	0	0	0
3	710	30	0	0	0	0	0	0
4	2400	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
7	10,640	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0
9	420	20	0	0	0	0	0	0
10	80	80	0	0	0	0	0	0
11	1740	2010	0	0	0	0	0	0

[a] Number of mf/ml.

Table 2
Results of antigenemia[a] in *D. immitis* naturally infected dogs treated with a combination of doxycycline and ivermectin.

Dog	Day							
	0	30	60	90	120	150	180	300
1	0.297	1.186	0.258	0	0	0	0	0
2	1.862	1.649	2.194	1.798	0.189	0	0	0
3	1.564	1.468	1.919	1.389	1.910	1.787	0	0
4	2.248	2.102	2.190	1.298	1.565	1.798	0.387	0
5	0	0.957	0	0	0	0	0	0
6	2.084	1.981	1.856	1.952	1.769	1.588	1.943	0
7	0.138	0	0	0	0	0	0	0
8	1.889	1.600	1.504	1.812	1.684	1.871	1.666	0
9	2.154	2.012	1.868	1.953	1.873	1.694	2.211	2.484
10	1.813	1.731	1.763	1.752	1.235	2.009	0.290	2.261
11	1.638	1.998	2.074	1.931	1.906	2.034	2.272	1.341

[a] O.D. values: cut-off O.D. 0.135. Values = 0 were below the cut-off.

completely eliminated by approximately 2–3 months from the first treatment (Nelson et al., 2005).

The effects of long-term monthly administration of the macrocyclic lactone ivermectin (IVM) have been widely studied in experimentally infected dogs. IVM has potent activity against the third and fourth stage larvae of the parasite, interrupting development into the adult stage. However, monthly administration of IVM at the standard preventive dose over 12–31 months to experimentally infected dogs has also shown a high level of efficacy against many other stages of the parasite, including immature worms (4–5 months of age) and young adults (6–7 months of age). Efficacy reported against these stages ranges from 95 to 98% (McCall et al., 1995, 1996, 2001). The continuous administration of IVM also has been found to be efficacious against adult parasites 8 months of age that had been transplanted intravenously (McCall et al., 1998) 1 month earlier. Results of the latter study demonstrated marginal efficacy (56%) against 8-month-old adult worms after 16 monthly doses. Venco et al. (2004) evaluated the microfilaricide and adulticide effects of monthly IVM in 14 naturally infected dogs. The authors reported that 100% of dogs were negative for circulating mf after 4 months of treatment, while 21, 21, 43, and 71% of dogs were negative for circulating antigens after 10, 14, 19, and 24 monthly doses, respectively.

Recently, Bazzocchi et al. (2008) reported the significant adulticide effect of a combination of doxycycline and ivermectin in experimentally infected dogs. In that study, dogs were treated with weekly prophylactic doses of IVM (6 μg/kg) orally for 34 weeks and with doxycycline (10 mg/kg/day) orally from weeks 0 to 6, 10 to 12, 16 to 18, 22 to 26, and 28 to 34. Necropsy at week 36 showed that this treatment regimen resulted in an adulticide effect on 78% of the worm population. The exact mechanism of action for the synergistic effect of the two drugs has not been determined, but is likely due to a combination of *Wolbachia* depletion by doxycycline and somatic degeneration due to the effects of ivermectin. In the present study, we chose to modify the treatment protocol in order to evaluate if a more applicable approach (1 month of daily doxycycline and ivermectin at bi-weekly intervals) could lead to parasitological cure in naturally infected dogs.

Table 3
Results of parasite load scoring with echocardiograms in *D. immitis* naturally infected dogs treated with a combination of doxycycline and ivermectin.

Dog	Day							
	0	30	60	90	120	150	180	300
1	1	1	1	1	1	1	1	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	2	2	2	1	1	1	0	0
5	2	1	1	1	1	1	0	0
6	2	2	2	2	1	0	0	0
7	1	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0
9	3	3	3	3	3	3	3	2
10	0	0	0	0	0	0	0	0
11	2	2	2	1	1	1	1	0

Here, 37.5% of dogs were negative for circulating mf by day 30, 87.5% by day 60 and 100% were negative by day 90. Even though the rapidity with which reservoirs of infection in the dog are eliminated is less important than eventually achieving the goal, the rapid clearance of mf observed in this study could contribute to controlling the spread of heartworms in hyper-endemic areas.

The combined treatment also caused a decrease in antigen levels from day 30 onward. Heartworm antigen testing is the most reliable method of confirming the efficacy of adulticide therapy and a negative test is considered definitive for parasitological cure. Eight out of eleven dogs were cleared of circulating antigens by 10 months from the onset of therapy, suggesting early death of adult worms. Following melarsomine dihydrochloride treatment, heartworm antigen usually becomes undetectable by 6 months post-treatment. However, when considering the reported adulticide effect of ivermectin, which generally requires more than a year of continuous monthly administrations and may take more than 2 years before dogs are antigen-negative and heartworms are eliminated completely (McCall et al., 1998; Venco et al., 2004), the present protocol would appear to eliminate adults worms much faster.

Echocardiograms carried out on those dogs in which adult parasites were visible showed a consistent reduction in parasite load, starting at day 30 and continuing throughout the study period, further suggesting precocious death of *D. immitis*. Cardiac ultrasound is not a reliable diagnostic tool for *D. immitis* infection, but is useful

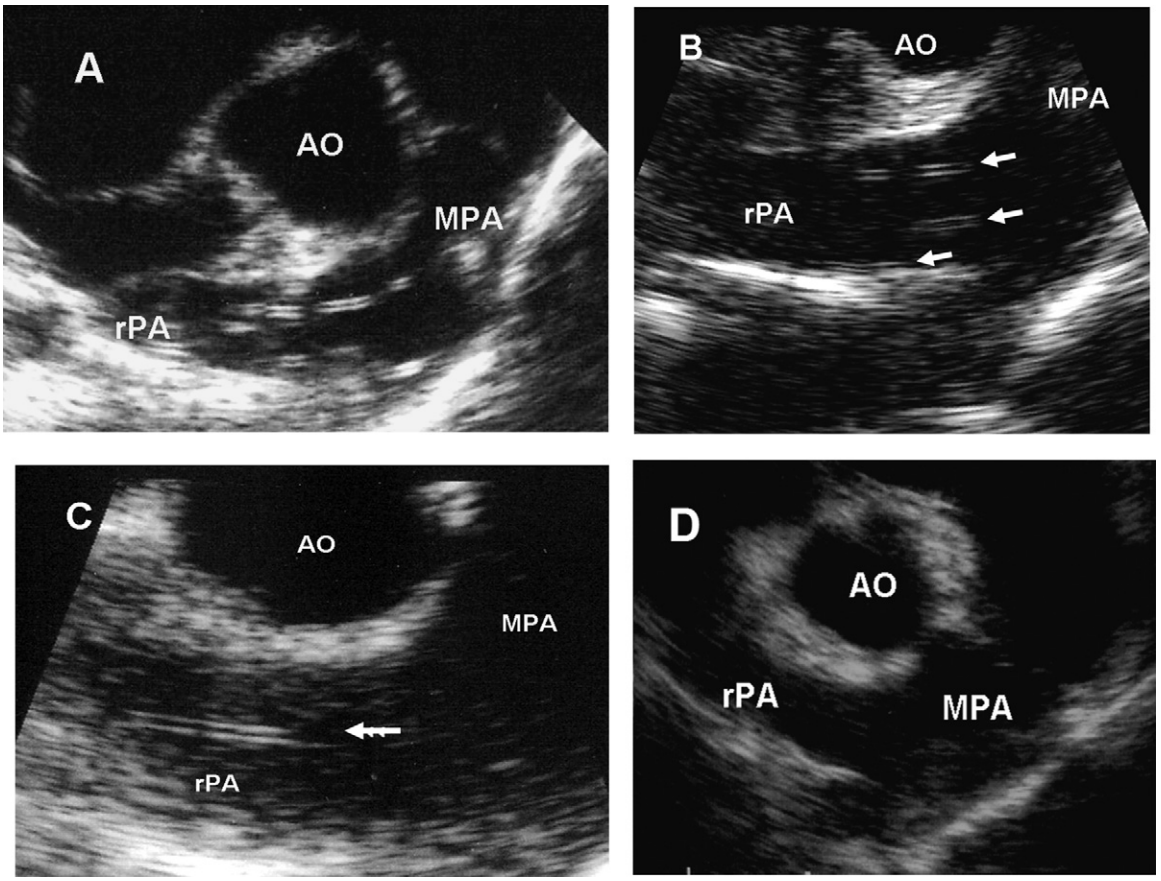

Fig. 1. Parasite load by echocardiography at four different time-points in the same dog. (A) Day 0. At day 0 numerous adult worms were evident in the rPA and lPA (score 2); (B) at day 60 more than 2 adult worms are still evident (score 2), but the number is clearly lower than at the first examination; (C) at Day 180 only one adult worm was evident in the rPA (score 1); (D) adult worms were no longer visible at day 300 (score 0).

for establishing parasite load (Venco et al., 2004) and the decrease in/disappearance of visible parasites in echocardiography-positive dogs could be interpreted as a decrease in parasite load and thus treatment efficacy. Interestingly, worms were visible in dogs 1 and 5 even when these had become negative for circulating antigens. If we consider, however, that circulating antigens are metabolites from fecund females, and that elimination of *Wolbachia* by doxycycline has been shown to dramatically reduce female fecundity (Bazzocchi et al., 2008), the authors' hypothesis is that these visible worms in antigen-negative dogs are either males or females that are no longer reproductively active.

According to the American Heartworm Society guidelines, exercise should be restricted in dogs treated with prophylactic doses of ivermectin as the adulticide and they should be examined by a veterinarian at least once every 4–6 months until all of the worms are dead (Nelson et al., 2005). While it is feasible to monitor dogs periodically with thoracic X-rays, as we have also done here it would be more difficult to control physical activity for such a long period of time, thus increasing the risk of thromboembolism. The authors feel that the protocol used in the present study could allow for closer monitoring in a clinical setting and easier management of treated dogs. Finally, as reported by Bazzocchi et al. (2008), a combination of doxycycline and ivermectin results in the depletion of *Wolbachia* from *D. immitis* in experimentally infected dogs. Interestingly, the death of these worms resulted in less pulmonary pathology than that seen in untreated dogs, following melarsomine dihydrochloride therapy (Kramer et al., 2008). The therapeutic protocol evaluated here may also reduce the severity of harmful effects due to adulticide therapy when compared to currently available options.

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References

Bazzocchi, C., Mortasino, M., Grandi, G., Kramer, L., Genchi, C., Bandi, C., Genchi, M., Sacchi, L., McCall, J.W., 2008. Combined ivermectin and doxycycline treatment has microfilaricidal and adulticidal activity

against *Dirofilaria immitis* in experimentally infected dogs. Int. J. Parasitol. 38 (12), 1401–1410.

Debrah, A.Y., Mand, S., Marfo-Debrekyei, Y., Batsa, L., Pfarr, K., Buttner, M., Adjei, O., Buttner, D., Hoerauf, A., 2007. Macrofilaricidal effect of 4 weeks of treatment with doxycycline on *Wuchereria bancrofti*. Trop. Med. Int. Health 12, 1433–1441.

Genchi, C., Rinaldi, L., Cascone, C., Mortarino, M., Cringoli, G., 2005. Is heartworm disease really spreading in Europe? Vet. Parasitol. 133, 137–148.

Gilbert, J., Nfon, C.K., Makepeace, B.L., Njongmeta, L.M., Hastings, I.M., Pfarr, K.M., Renz, A., Tanya, V.N., Trees, A.J., 2005. Antibiotic chemotherapy of onchocerciasis: in a bovine model, killing of adult parasites requires a sustained depletion of endosymbiotic bacteria (*Wolbachia* species). J. Infect. Dis. 192, 1483–1493.

Hoerauf, A., Specht, S., Büttner, M., Pfarr, K., Mand, S., Fimmers, R., Marfo-Debrekyei, Y., Konadu, P., Debrah, A.Y., Bandi, C., Brattig, N., Albers, A., Larbi, J., Batsa, L., Adjei, O., Büttner, D.W., 2008. *Wolbachia* endobacteria depletion by doxycycline as antifilarial therapy has macrofilaricidal activity in onchocerciasis: a randomized placebo-controlled study. Med. Microbiol. Immunol. 197 (3), 295–311.

Kramer, L.H., Passeri, B., Corona, S., Simoncini, L., Casiraghi, M., 2003. Immunohistochemical/immunogold detection and distribution of the endosymbiont *Wolbachia* of *D. immitis* and *Brugia pahangi* using a polyclonal antiserum raised against WSP (*Wolbachia* surface protein). Parasitol. Res. 89, 381–386.

Kramer, L., Grandi, G., Leoni, M., Passeri, B., McCall, J., Genchi, C., Mortasino, M., Bazzocchi, C., 2008. Wolbachia and its influence on the pathology and immunology of *Dirofilaria immitis* infection. Vet. Parasitol. 158 (3), 191–195.

McCall, J.W., McTier, T.L., Supakorndej, N., Ricketts, R., 1995. Clinical prophylactic activity of macrolides on young adult heartworms. In: Soll, M.D., Knight, D.H. (Eds.), Proceedings of the Heartworm Symposium '95. American Heartworm Society, Batavia, IL, pp. 187–195.

McCall, J.W., McTier, T.L., Ryan, W.G., Gross, S.J., Soll, M.D., 1996. Evaluation of ivermectin and milbemycin oxime efficacy against *Dirofilaria immitis* infections of three and four months' duration in dogs. Am. J. Vet. Res. 57, 1189–1192.

McCall, J.W., Ryan, W.G., Roberts, R.E., Dzimianski, M.T., 1998. Heartworm adulticidal activity of prophylactic doses of ivermectin (6 μg/kg) plus pyrantel administered monthly to dogs. In: Seward, R.L. (Ed.), Recent Advances in Heartworm Disease: Symposium '98. American Heartworm Society, Batavia, IL, pp. 209–215.

McCall, J.W., Guerrero, J., Roberts, R.E., Supakorndej, N., Mansour, A.E., Dzimianski, M.T., McCall, S.D., 2001. Further evidence of clinical prophylactic, retro-active (reach-back) and adulticidal activity of monthly administrations of ivermectin (Heartgard Plus™) in dogs experimentally infected with heartworms. In: Seward, R.L. (Ed.), Recent Advances in Heartworm Disease: Symposium '01. American Heartworm Society, Batavia, IL, pp. 189–200.

Nelson, C.T., McCall, J.W., Rubin, S.B., Buzhardt, L.F., Dorion, D.W., Graham, W., Longhofer, S.L., Guerrero, J., Robertson-Plouch, C., Paul, A., Executive Board of the American Heartworm Society, 2005. Guidelines for the diagnosis, prevention and management of heartworm (*Dirofilaria immitis*) infection in dogs. Vet. Parasitol. 133 (2–3), 255–266.

Venco, L., McCall, J.W., Guerrero, J., Genchi, C., 2004. Efficacy of long-term monthly administration of ivermectin on the progress of naturally acquired heartworm infections in dogs. Vet. Parasitol. 124 (3–4), 259–268.