The Use of Doxycycline in Microfilaremic *Dirofilaria immitis* (Leidy, 1856) Naturally Infected Dogs

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ABSTRACT

Heartworm is a mosquito-borne disease that affects different mammalian species with worldwide distribution. The agent, *Dirofilaria immitis* (Leidy, 1856), infects dogs from all continents and, in the Americas, the parasite has been detected in 5 of the 13 countries. The species *D immitis* harbors an endosymbiont intracellular bacteria of the genus *Wolbachia*, known to be vital for the worms. These bacteria are sensitive to tetracycline-like drugs, therefore the use of antibiotics can affect the worms. The effect of the use of doxycycline on microfilaremia of naturally infected microfilaremic dogs was studied by treating dogs with 3 cycles of doxycycline (10 mg/kg SID for 21 days) at 6-month intervals. Blood samples were collected on Days 0, 7, 21, and 111 of each cycle and microfilariae detected and counted. Thirteen dogs completed 1 cycle, 10 dogs completed 2 cycles, and 6 dogs completed all 3 cycles. Five dogs could be sampled at 7 months after the end of the third cycle. All 13 dogs were still microfilaremic while they were given doxycycline treatment. On Day 111 of the first cycle, 40% became amicrofilaremic and from then on, amicrofilaremic dogs were always detected. Overall, treatment lowered the microfilariae mean counts, which can be observed when the 3 cycles are compared. The microfilariae mean counts tended to be lower at Day 111 of each cycle, suggesting that the effect of the antibiotic on *Wolbachia* organisms and the consequent impact on the fitness of the worms is a slow process. The lowered microfilariae stock provided by doxycycline-treated dogs (amicrofilaremic dogs and lowered microfilariae concentrations) may have impaired heartworm transmission once the chances for the vectors to become infected were reduced.

INTRODUCTION

Heartworm is a worldwide-distributed, mosquito-borne disease that can affect many different mammal species.[1] The adult worms live preferentially in the pulmonary arteries where they release the first-stage larvae (microfilariae) into the bloodstream.[2] The microfilariae circulate in the blood of the dog and are capable of living up to 2.5 years,[3] although some dogs never develop microfilaremia.[4]

Dirofilaria immitis, like other nematodes of the Onchocercidae family harbor an intracellular bacteria (Rickettsia) of the genus *Wolbachia*.[5,6] This bacteria was first identified infecting Onchocercidae individuals in the 70s[7] and is now known to be vital for the worms development, reproduction, and survival[5,8]; therefore, the bacteria is said to be an endosymbiont of *D immitis*. Because the *Wolbachia* endosimbyont is important to vital functions of *D immitis*, because individuals of the genus *Wolbachia* are sensitive to tetracycline and tetracycline-like treatments,[9] and because the use of those antibiotics has been suggested to reduce transmission of heartworm,[10] the effect of doxycycline administered to naturally infected microfilaremic dogs was studied.

MATERIALS AND METHODS

After the owners' consent and agreement not to administer any drug or vaccine to their dogs without a formal consent provided by the veterinarians of the study group, a total of 13 *D immitis* naturally infected microfilaremic and antigenemic (ELISA-SNAP-3DX®, IDEXX) dogs of the same area (22.92417° S; 42.22431° W) were treated with 3 cycles of doxycycline (10 mg/kg SID for 21 days/ PO – Doxifin®, Ouro Fino) at 6-month intervals. All dogs were bled on Days 0, 7, 21, and 111 of each treatment cycle and at 7 months after the end of the third cycle, for microfilariae detection and count. Blood samplings were always done according to the same protocol, starting at the same time and following the same order to avoid individual daily variations of microfilariae counts. All dogs were uniquely identified and data registered, and all the medication was administered by the veterinarians. Dogs were excluded from the protocol when any of the procedures of a treatment cycle was missed or if the dogs were treated with another drug besides doxycycline.

Blood samplings were kept at 4°C until processing and all samples were examined by the third day after collection. Microfilariae detections were performed using modified Knott's test.[11] Smears of 20 μL (25 × 15 mm) of hemolised blood, were fixed and stained with Giemsa for microfilariae counts.[12] Two slides of each sample were prepared and always examined (40× magnification) by the same 2 veterinarians. The final microfilariae count was given by the mean of the means of the 2 counts of each examiner and corrected to 1 mL.

Since the distribution of the microfilariae counts means was shown to be not-normal, statistical analyses of the means were done with the Kruskall-Wallis test. The mean of log(x+1) was used for the graphic presentation to guarantee the proper visualization of data.[13] The numbers of amicrofilaremic dogs were compared among the cycles by contingency table analysis, with comparisons by Chi square test (χ^2).

RESULTS

The number of dogs included in each of the 3 treatment cycles varied due to migrations, deaths, or owner's noncompliance. Thirteen dogs completed 1 cycle, 10 completed 2 cycles, and 6 completed all 3 cycles. Five dogs could be sampled at 7 months after the end of the third cycle.

At the beginning of the study (Day 0 of the first cycle), the microfilaremia varied between 150 and 29,800 mf/mL with a mean of 10,066 mf/mL. During the first cycle of treatment, the microfilaremia mean of Days 0 through 21 showed no difference ($H = 0.5302$; $P = 0.7671$; $df = 2$); however, the mean observed on Day 111 dropped to 28 mf/mL ($H = 28.246$; $P = 0.000$; $df = 3$) (Table 1). It is interesting to note that all 13 dogs were still microfilaremic while they were given doxycycline and that on Day 111, 6 became amicrofilaremic (46%).

Table 1. Arithmetic Means of the Microfilaria Count on Canine Blood Samples of Dogs Receiving Doxycycline Treatment During 21 Days for 3 Cycles at 6-Month Intervals.

Day	Cycle 1 (mf/mL)	Cycle 2 (mf/mL)	Cycle 3 (mf/mL)
0	10,006[a]	415[b]	794[b]
7	8,186[a]	662[b]	735[b]
21	10,002[a]	1,160[b]	1,035[b]
111	28[b]	59[b]	154[b]

Values within columns or lines having different superscript letters are significantly different ($P < 0.05$).

When the second treatment cycle started, 3 of the 10 dogs showed no microfilaremia and the mean microfilariae count was 415 mf/mL. There was no difference among microfilaremia means within the second treatment cycle, including at Day 111 (H = 1.8026; $P = 0.6144$; df = 3) (Table 1). On Day 111, 40% (4/10) of the dogs were amicrofilaremic.

The third cycle started with 2 amicrofilaremic dogs and the mean microfilariae count was 794 mf/mL. There was no difference among microfilaremia means within the third treatment cycle, including at Day 111 (H = 0.9921; $P = 0.8032$; df = 3) (Table 1). On Day 111, 33% (2/6) of the dogs were amicrofilaremic.

At 7 months after the end of the third cycle, 1 of the 5 examined dogs was amicrofilaremic (20%) and the mean microfilariae count was 4,258 mf/mL.

Overall, treatment lowered the microfilariae mean counts, which can be observed when the 3 cycles are compared (Figure 1). The first cycle microfilariae count means were higher than the means of Cycles 2 (H = 53.0289; $P = 0.000$; df = 7) and 3 (H = 43.1697; $P = 0.000$; df = 7) (Table 1). Although no difference was observed among microfilaremia levels on Days 111 of the different cycles, a sharp drop in microfilariae count was observed from the last doxycycline administration day (Day 21) to Day 111 of Cycle 1 (H = 18.8099; $P = 0.0000$) (Figure 1 & Table 1); such a decrease was not observed in Cycles 2 (H = 0.5714; $P = 0.4497$) and 3 (H = 1.2564; $P = 0.2623$). When the microfilaremia at 7 months after the end of the third cycle was compared to Days 111 of the different cycles, microfilariae counts were shown to be higher than on Day 111 of Cycles 1 (H = 4.9445; $P = 0.0262$) and 2 (H = 4.3350; $P = 0.0373$), but no difference was observed in comparison to Day 111 of cycle 3 (H = 2.7; $P = 0.1003$).

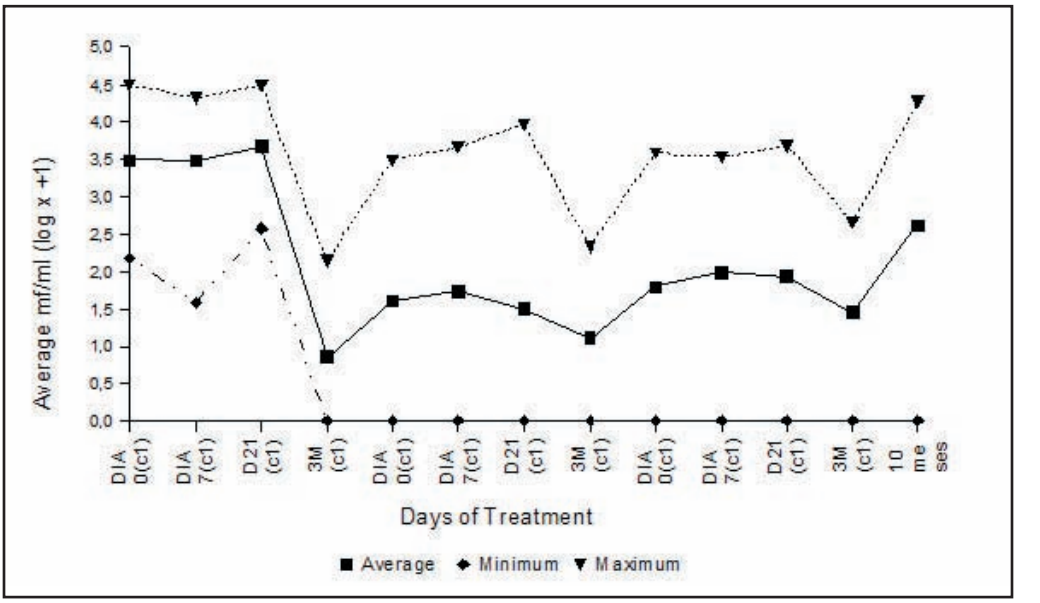
The number of amicrofilaremic dogs after treatment (Day 111) did not increase during the 3 treatment cycles, although it varied from 46% (6/13) to 33% (2/6) ($\chi^2 = 0.29$; $P = 0.865$; df = 2). Seven months after the end of the third cycle, the number of amicrofilaremic dogs (20% [1/5]) was shown to be similar to before the end of the third cycle ($\chi^2 = 1.12$; $P = 0.7714$; df = 3).

DISCUSSION

At the beginning of the study, the mean concentration of microfilariae was somewhat low in comparison to other undisturbed dog populations (10^3 to 10^5 mf/mL).[14] It was interesting, though, to register a large range in microfilaria concentration among the dogs, which could only be attributed to the host's individual reactions to the worms.

The fact that the microfilariae mean counts did not change during the 21 days of doxycycline treatment of the first cycle followed by a sharp drop on Day 111 (3 months after the last doxycycline dose) shows that *Wolbachia* reduction has no immediate effect on microfilariae concentration. It suggests that the reduction on the bacterial concentration in the tissues (longitudinal lateral cords and ovaries) of the worms needs time to play its role on microfilariae. *Wolbachia* organisms are known to be important to guarantee worms vital functions,[15,16] therefore the treatment with doxycycline probably reduced microfilariae lifespan drastically, causing premature death or destruction of microfilariae that would be expected to last for approximately 2 years.[3] Since *Wolbachia* organisms are essential for the reproductive fitness of the worms,[9,17-19] the treatment must have temporarily sterilized the adult worms (block in embryogen-

Figure 1. Average microfilariae (mf) concentration in canine blood during doxycycline treatment (3 cycles of 10 mg/kg during 21 days, every 6 months).



esis), halting new microfilariae releases to the bloodstream. Therefore, the reduction in lifespan associated to the lack of microfilariae release to the bloodstream probably determined the sharp drop on microfilariae concentration on Day 111.

Cycles 2 and 3 showed similar microfilariae concentrations that suggested that the doxycycline treatment pressure upon the worms halted the build up on microfilariae. Although no difference could be detected, Cycle 3 concentrations being somewhat higher than Cycle 2 concentrations may suggest a build up of *Wolbachia* resistance to the treatment as has been detected before.[17,20,21] This possibility gains strength by the fact that when treatment ceased (10 months after the last doxycycline dose of Cycle 3), microfilariae concentration increased when compared to Days 111 of Cycles 1 and 2, but no difference was shown when compared to Day 111 of Cycle 3. This possibility must be carefully considered once the number of dogs included in the cycles dropped from 13 in the first cycle to 6 in the third.

The lowered microfilariae stock (low microfilariae concentrations and amicrofilaremic dogs) provided by doxycycline-treated dogs to the mosquito vectors may have determined an important impact on heartworm transmission, once a heartworm's life cycle is mainly dependent upon microfilaremic dogs, despite abundance of vectors, environment conditions, and susceptible population being important as well.[22,23] Therefore, the use of tetracycline and tetracycline-like treatments, as suggested before,[10] seems to have the potential of impairing heartworm transmission.

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REFERENCES

1. Guerrero J, Ducos de lahite J, Genchi C, et al: Update on the distribution of *Dirofilaria immitis* in dogs from southern Europe and Latin America. In: Proceedings of the Heartworm Symposium '92. Austin, TX; 1992:31-37.
2. Simón F, Kramer LH, Román A, et al: Immunopathology of *Dirofilaria immitis* infection. *Vet Res Comm* 2007;31:161-171.
3. Underwood PC, Harwood PD: Survival and location of the microfilariae of *Drofilaria immitis* in the dog. *J Parasitol* 1939;25:23-33.

4. Rawlings CA: *Heartworm Disease in Dogs and Cats*. Philadelphia, PA: WB Saunders Co.; 1986.
5. Bandi C, Anderson JC, Genchi C, Blaxter ML: Phylogeny of *Wolbachia* in filarial nematodes. *Proc Roy Soc London B* 1998;265:2407-2413.
6. Hise AG, Fergusson IG, Pearlman E: The role of endosymbiontic *Wolbachia* bacteria in filarial disease. *Cell Microbiol* 2004;6:97-104.
7. McLaren DJ, Worms MJ, Laurence BR, Simpson MG: Micro-organisms in filarial larvae (*Nematoda*). *Transact Royal Soc Trop Med Hyg* 1975;69:509-514.
8. Genchi C, Sacchi L, Bandi C, Venco L: Preliminary results on the effect of tetracycline on the embryogenesis and symbiotic bacteria (*Wolbachia*) of *Dirofilaria immitis*. *Parassitologia* 1998;40(Suppl 1):72.
9. Bandi C, McCall JW, Genchi C, Corona S, Venco L, Sacchi L: Effects of tetracycline on the filarial worms *Brugia pahangi* and *Dirofilaria immitis* and their bacterial endosymbionts *Wolbachia*. *Int J Parasitol* 1999;29:357-364.
10. Labarthe N, Guerrero J: The epidemiology of heartworm: what is happening in South America and Mexico? *Vet Parasitol* 2005;133:149-156.
11. Newton WL, Wright WH: The occurrence of a dog filariid other than *Dirofilaria immitis* in the United States. *J Parasitol* 1956;42:246-258
12. McCall JW, Jun JJ, Bandi C: *Wolbachia* and the antifilarial properties of tetracycline. An untold story. *Ital J Zool* 1999;66:7-10.
13. Williams CB: The use of logarithms in the interpretation of certain entomological problems. *Ann App Biol* 1937;24:404-414.
14. Lok JB: *Dirofilaria* sp.: taxonomy and distribution. In: Boreham PFL, Atwell RB, eds. *Dirofilariases*. Florida: CRC Press Florida; 1988.
15. Werren JH, O'Neill SL: The evolution of heritable symbionts. In: O'Neill SL, Werren JH, Hoffmann AA, eds. *Influencial Passengers*. New York: Oxford University Press; 1997:1-41.
16. Bandi C, Trees AJ, Bratting NW: *Wolbachia* in filarial nematodes: evolutionary aspects and implications for the pathogenesis and treatment of filarial diseases. *Vet Parasitol* 2001;98:215-238.
17. Casiraghi M, McCall JW, Simoncini L, et al: Tetracycline treatment and sex-ratio distortion: a role for *Wolbachia* in the moulting of filarial nematodes. *Intern J Parasitol* 2002;32:1457-1468.
18. Hoerauf A, Ohene A, Büttner DW: Antibiotics for the treatment of onchocerciasis and other filarial infections. *Curr Opinion Invest Drugs* 2002;3:533-537.
19. Kosek WJ: What is new in the *Wolbachia/Dirofilaria* interaction? *Vet Parasitol* 2005;133:127-132.
20. Bosshardt SC, McCall JW, Sharon UC, Karen LJ, Petit TA, Klei TR: Prophylactic activity of tetracycline against *Brugia pahangi* infections in jirds (Meriones unguiculatus). *J Parasitol* 1993;79:775-777.
21. Taylor MJ, Hoerauf A: *Wolbachia* bacteria of filarial nematodes. *Parasitol Today* 1999;15:437-442.
22. Guerrero J, McCall JW, Dzimianski MT, McTier TL, Holmer RA, Newcomb KM: Prevalence of *Dirofilaria immitis* infection in cats from the south-eastern United States. In: Proceedings of the Heartworm Symposium '92. Austin, TX; 1992:91-95.
23. Knight DH, Lok JB: Seasonal timing of heartworm chemoprophylaxis in the United States. In: Proceedings of the Heartworm Symposium '95. Auburn, AL; 1995:37-42.