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Short Communication

Doxycycline levels and anti-*Wolbachia* antibodies in sera from dogs experimentally infected with *Dirofilaria immitis* and treated with a combination of ivermectin/doxycycline

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ABSTRACT

Sera from *Dirofilaria immitis*-experimentally infected dogs treated with a combination of ivermectin/doxycycline were analysed for doxycycline levels by HPLC and anti-*Wolbachia* Surface Protein (rWSP) antibodies by ELISA and compared with sera from dogs treated with doxycycline alone. Results show that doxycycline levels were not statistically different between the two groups. Circulating anti-WSP antibody titres were markedly lower in both treatment groups when compared to control *D. immitis* infected dogs, indicating that doxycycline is able to reduce *Wolbachia* and prevent the immune response against the bacteria. The combination treatment protocol has been shown to be highly adulticidal and further studies are needed to better understand the interaction between doxycycline and ivermectin in *D. immitis* infected dogs.

1. Introduction

Heartworm infection (HW; *Dirofilaria immitis*) in dogs causes chronic pulmonary disease that, if left untreated, can lead to right-side congestive heart failure. Currently, the only registered drug for adulticide therapy in dogs with heartworm disease (HWD) is melarsomine dihydrochloride (Immiticide®, Merial). Due to concerns of severe, post-treatment thromboembolism in some dogs (Kramer et al., 2008) and recent problems with availability of melarsomine on several international markets, there is increasing interest in alternative adulticide treatments (Colby et al., 2011).

The recent targeting of the bacterial endosymbiont *Wolbachia*, through antibiotic therapy of the infected host, has offered an interesting alternative for the treatment of HWD. Indeed, *Wolbachia* is necessary for the reproductive capacity and long-term survival of those filarial parasites that harbour the endosymbiont. The adulticide effects of doxycycline (DOXY) have been studied in *D. immitis*-experimentally infected dogs (Bazzocchi et al., 2008). No significant adulticide effects at 8 months post infection following several cycles of DOXY was observed, even though treatment was able to reduce *Wolbachia* populations. The same study reported that when DOXY was combined with the macrocyclic lactone ivermectin (IVM), adulticide efficacy was approximately 80% vs. 9% when dogs were treated with DOXY alone. The adulticide effect of this combination has also been confirmed in naturally infected dogs (Grandi et al., 2010). It is not clear why the two drugs work better together in eliminating a large population of heartworms in a relatively short period of time (8–10 months). It is not yet known if this is due to a simple summation effect or if there exists a certain synergism between the two

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Table 1
Treatment protocols in *D. immitis*-experimentally infected dogs (Bazzocchi et al., 2008).

Group	Treatment (weeks post-infection)	
	Doxycycline (10 mg/kg)	Ivermectin (6 μg/kg)
DOXY	Weeks 0–6, 10–12, 16–18, 22–26, 28–34	–
IVM + DOXY	Weeks 0–6, 10–12, 16–18, 22–26, 28–34	Weekly for 34 weeks
CONTROL	–	–

drugs, including in pharmacokinetics. The present study was aimed at evaluating DOXY levels and circulating antibodies against *Wolbachia* Surface Protein (WSP) in serum from dogs treated with DOXY alone or in combination with IVM, according to Bazzocchi et al. (2008).

2. Material and methods

2.1. Animals and sera

Briefly, serum samples conserved at −20 °C from a previous study of *D. immitis*-experimentally infected dogs were used (Bazzocchi et al., 2008). Treatment protocols are reported in Table 1. Each group consisted of five dogs experimentally infected with adult heartworms (7 males and 9 females) by intravenous transplantation. Drugs were given with food in the morning and samples were taken at approximately 6 h later. Serum samples from 2 drug administration days, corresponding to the weekly IVM treatment, were analysed for drug concentrations: T1 (6 weeks post-infection, p.i.) and T4 (34 weeks p.i.). For anti-WSP ELISA, serum samples from T0 (6 weeks before infection to determine cut-off values), T1, T2 (10 weeks p.i.) and T4 were analysed.

2.2. HPLC for doxycycline serum levels

The concentrations of DOXY in serum were measured by means of HPLC method, following the technique by Nielsen and Gyrd-Hansen (1996), slightly modified. The HPLC system consisted of a Prostar LC Workstation (Varian Co., Walnut Creek, CA, USA), with a Prostar 325 UV-Vis detector and a 10 μL loop. Chromatographic separations were obtained using a Syncronis C18 analytical column (Thermo, Milan, Italy) (5 μm particle size, 150 mm × 4.6 mm), maintained at room temperature (20 °C). The analytical wavelength was set at 350 nm. The mobile phase consisted of acetonitrile and 0.01 mol/L trifluoroacetic acid (30:70, v/v), with a flow rate of 1.0 mL/min. All used solvents and reagents were of HPLC grade purity and were purchased from Sigma–Aldrich (Milan, Italy).

Samples were prepared by adding 400 μL buffer EDTA (0.1 mol/L sodium phosphate, containing 0.1 mol/L disodium EDTA; pH of the buffer mixture was adjusted to 5.0 by adding 0.1 mol/L phosphoric acid) and 100 μL perchloric acid 20% to 500 μL of serum and the mixture was placed in vortex mixer for 2 min and then centrifuged at 7500 × *g* for 20 min. The supernatant was collected, filtered through a 0.22-μm syringe filter, put in sample vial and injected into the HPLC system. A serum sample from a *D. immitis* infected dog receiving no treatment was used as negative control.

2.3. ELISA for anti-WSP antibodies

The recombinant protein WSP of the *Wolbachia* of *D. immitis* (rWSP) was produced in *Escherichia coli* and purified as described in Bazzocchi et al. (2000). Wells of ELISA flat-bottom plates were coated with 0.1 μg/well of rWSP. Sera were analysed in duplicate at a dilution of 1:100 and the anti-dog IgG HRP-conjugated antibody (Sigma–Aldrich) was diluted at 1:5000. The optical density (O.D.) was measured at 492 nm. The cut-off was established at an O.D. of 0.65, which is the mean O.D. of the control sera (sera from each dog at the moment of infection) plus three times their standard deviation. Samples with O.D. less than of 0.65 were classified as negative and samples with O.D. greater than or equal to 0.65 were classified as positive.

2.4. Statistical analysis

Differences in DOXY serum levels (mg/L) at each time point were analysed by comparing median values by Mann–Whitney *U* test (Genstat, 7th edition) and $p < 0.05$ was considered to be a significant difference.

3. Results and discussion

Serum levels of antibiotic in dogs treated with the combination IVM/DOXY protocol were not statistically different compared to dogs treated with DOXY alone at any time points considered (Fig. 1). Therefore it is unlikely that the adulticide effect of the combination treatment shown in the previous study was due to a difference in tissue/worm distribution of DOXY. There was, however, a wide range of variability in serum concentrations among dogs and among time points, making interpretation of results difficult. Interestingly, dogs from both the combination group and the DOXY group showed markedly lower values for anti-WSP antibodies when compared to untreated HW-infected

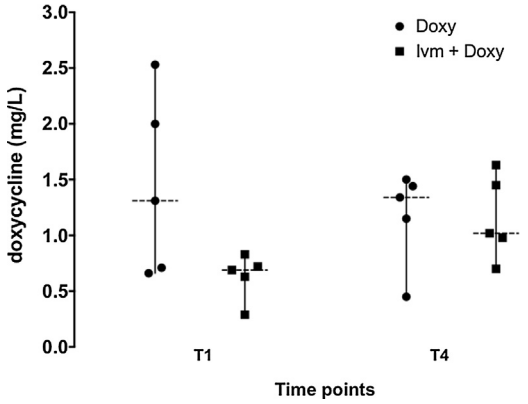

Fig. 1. Serum concentrations of DOXY (mg/L) in HW-infected dogs (5 per group) treated with DOXY alone or with the combination IVM + DOXY, measured at time points T1 (6 weeks p.i.) and T4 (34 weeks p.i.). The graph shows the distribution of individual DOXY levels around the median value for the two time points.