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

3

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21 **Abstract**

22

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

32

33 Key words: *Dirofilaria immitis*; doxycycline; ivermectin; *Wolbachia*

34

35 **Introduction**

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

42 The recent targeting of the bacterial endosymbiont *Wolbachia*, through antibiotic therapy of the
43 infected host, has offered an interesting alternative for the treatment of HWD. Indeed, *Wolbachia* is
44 necessary for the reproductive capacity and long-term survival of those filarial parasites that
45 harbour the endosymbiont. The adulticide effects of doxycycline (DOXY) have been studied in *D.*
46 *immitis* – experimentally infected dogs (Bazzocchi et al., 2008). No significant adulticide effects at
47 8 months post infection following several cycles of DOXY was observed, even though treatment
48 was able to reduce *Wolbachia* populations. The same study reported that when DOXY was
49 combined with the macrocyclic lactone ivermectin (IVM), adulticide efficacy was approximately
50 80% vs. 8% when dogs were treated with DOXY alone. The adulticide effect of this combination
51 has also been confirmed in naturally-infected dogs (Grandi et al., 2010). It is not clear why the two
52 drugs work better together in eliminating a large population of heartworms in a relatively short
53 period of time (8-10 months). It is not yet known if this is due to a simple summation effect or if
54 there exists a certain synergism between the two drugs, including in pharmacokinetics. The present
55 study was aimed at evaluating DOXY levels and circulating antibodies against *Wolbachia* Surface
56 Protein (WSP) in serum from dogs treated with DOXY alone or in combination with IVM,
57 according to Bazzocchi et al. (2008).

58

59

60

61 **Material and methods**

62 *Animals and sera*

63 Briefly, serum samples conserved at -20°C from a previous study of *D. immitis* – experimentally
64 infected dogs were used (Bazzocchi et al., 2008). Treatment protocols are reported in Table 1. Each
65 group consisted of five dogs **experimentally infected with adult heartworms by intravenous**
66 **transplantation**. Drugs were given with food in the morning and samples were taken at
67 approximately 6 hours later. Serum samples from 2 drug administration days, corresponding to the
68 weekly IVM treatment, were analyzed for drug concentrations: T1 (41 days **post-infection, p.i.**) and
69 T4 (250 days p.i.). For anti-WSP ELISA, serum samples from T0 (day 46 **pre-infection** to
70 determine cut-off values), T1, T2 (**??? days p.i.**) and T4 were analysed.

71

72 *HPLC for doxycycline serum levels*

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

82 Samples were prepared by adding 400 µl buffer EDTA (0.1 mol/L sodium phosphate, containing
83 0.1 mol/L disodium EDTA; pH of the buffer mixture was adjusted to 5.0 by adding 0.1 mol/L
84 phosphoric acid) and 100 µL perchloric acid 20% to 500 µl of serum and the mixture was placed in
85 vortex mixer for 2 min and then centrifuged at 7500 x g for 20 min. The supernatant was collected,

86 filtered through a 0.22-µm syringe filter, put in sample vial and injected into the HPLC system. A
87 serum sample from a *D. immitis* infected dog receiving no treatment was used as negative control.

88

89 *ELISA for anti-WSP antibodies*

90 The recombinant protein WSP of the *Wolbachia* of *D. immitis* (rWSP) was produced in *Escherichia*
91 *coli* and purified as described in Bazzocchi et al. (2000). Wells of ELISA flat-bottom plates were
92 coated with 0.1 µg/well of rWSP. Sera were analysed in duplicate at a dilution of 1:100 and the anti
93 IgG HRP-conjugated antibody was diluted at 1:5000. The optical density (O.D.) was measured at
94 492 nm. The cut-off was established at an O.D. of 0.65, which is the mean O.D. of the control sera
95 (sera from each dog at the moment of infection) plus three times their standard deviation. Samples
96 with O.D. less than of 0.65 were classified as negative and samples with O.D. greater than or equal
97 to 0.65 were classified as positive.

98

99 *Statistical analysis*

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

103

104 **Results and Discussion**

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

112 untreated HW-infected controls (Fig. 2). This is strongly suggestive of elimination of *Wolbachia*
113 from *D. immitis*, as previously shown by PCR analysis of worms collected from treated dogs at
114 necropsy (Bazzocchi et al., 2008). So, DOXY, whether alone or in combination, is actively
115 eliminating *Wolbachia* from adult worms efficiently enough to prevent the antibody response to it.
116 Yet, this is not sufficient for an adulticide effect greater than 9% (prima nel testo c'è scritto 8%)
117 (Bazzocchi et al., 2008). Only the combination of DOXY with IVM is able to kill the parasite. If the
118 antibiotic is taken up and distributed in a uniform way in both protocols, and the effect on
119 *Wolbachia* is comparable, it may be that the interaction in the combination protocol is synergistic.
120 Indeed, it is possible that DOXY has a detrimental effect on *D. immitis* independent of its effect on
121 *Wolbachia*, as has been suggested previously (Smith and Rajan, 2000). IVM causes neuromuscular
122 dysfunction, pharyngeal paralysis, and thickening of the gut epithelium in treated worms.
123 Ultrastructural analysis of IVM-treated *D. immitis* show retained ingesta and increased gut
124 permeability (Steffen et al. 1998). These alterations may lead to an increase in the concentration of
125 DOXY within the worm. The two drugs may also be interacting on a molecular level: it has been
126 reported that IVM is able to reduce cellular efflux of antibiotics in farm animals, thus increasing the
127 intracellular concentration of the latter. It would appear that this is due to IVM's ability to inhibit
128 the activity of various cellular transport systems (Lespine et al., 2006; Real et al., 2011; Ballent et
129 al., 2012). On the other hand, it cannot be excluded that DOXY in some way potentiates the effects
130 of IVM, even though this seems less likely. However, several compounds, including antibiotics,
131 have been shown to increase intracellular concentrations of MLs (per esteso? macrolactones) such
132 as moxidectin (Dupuy et al., 2006).
133 Furthermore, since tetracycline was shown to inhibit oxidation of fatty acids in mitochondria of
134 mice and man (Fréneaux et al., 1988), it is possible that DOXY could exert a toxic effect also on the
135 nematode mitochondria. Finally, tetracyclines are known to bind to bivalent ions such as calcium
136 and magnesium. An intriguing hypothesis could be that DOXY may interfere with calcium uptake
137 into parasite neurons. Indeed, it was observed that minocycline is able to cause calcium-dependent

138 neuromuscular block in rabbits (Hashimoto et al., 1979). It is therefore possible that partial
139 neuromuscular block induced by doxycycline could add to the paralyzing effect by IVM in a
140 synergistic fashion, thus resulting in a lethal effect for the parasite.

141 In conclusion, the results of the present study suggest that the adulticide effect of the association of
142 IVM and DOXY is not due to a higher drug concentration of DOXY in the combination protocol,
143 nor to a lack of efficacy in the removal of *Wolbachia* from the worm tissue. Future studies should
144 concentrate on the parasite target, perhaps through in vitro treatment of microfilariae with one or
145 both drugs in order to evaluate drug concentration and expression of cell detoxification mechanisms.

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201
202

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	-	-

203

204

205 **Figure legends**

206

207 **Figure 1.**

208 Serum concentrations of DOXY (mg/L) in HW-infected dogs (5 per group) treated with DOXY
209 alone or with the combination IVM+DOXY, measured at time points T1 (41 days p.i.) and T4 (250
210 days p.i.). The graph shows the distribution of individual DOXY levels around the median value for
211 the two time points.

212

213 **Figure 2.**

214 ELISA results for rWSP in HW+ dog sera. T0: day 46 pre-infection; T1 (day 41 post infection;
215 p.i.); T2 (day 82 p.i.); and T4 (day 250 p.i.). Control: O.D. values obtained from the five dog of
216 control group (not treated); DOXY: O.D. values obtained from the five dogs of the group treated
217 with DOXY alone; IVM+DOXY: O.D. values obtained from the five dogs of the group treated with
218 the drug combination. Bars indicate the means±SD. Cut-off value: 0.65.

219