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Wolbachia, doxycycline and macrocyclic lactones: New prospects in the treatment of canine heartworm disease

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ABSTRACT

Melarsomine dihydrochloride (Immiticide®, Merial) is the only approved adulticidal drug for the treatment of canine heartworm disease (HWD). However, in cases where arsenical therapy is not possible or is contraindicated, a monthly heartworm preventive along with doxycycline for a 4-week period, which targets the bacterial endosymbiont *Wolbachia*, might be considered. There are published reports on the efficacy of ivermectin and doxycycline in both experimentally and naturally infected dogs, but no data on the use of other macrocyclic lactones (MLs) with a similar treatment regime. Preliminary results of studies in dogs show that a topical formulation of moxidectin, the only ML currently registered as a microfilaricide, is also adulticidal when combined with doxycycline. It is not yet known if the efficacy of these combination therapies is due to pharmacokinetic synergism. A recent study showed that serum levels of doxycycline in dogs treated with the combination protocol were not statistically different compared to dogs treated with doxycycline alone. However, lungs from dogs treated with the combination therapy showed a marked reduction in T regulatory cells, indicating that treatment efficacy may be due to a heightened immune response against the parasite. Further studies are necessary to evaluate the long-term clinical outcome of combination protocols and to establish the most efficient treatment for HWD in dogs.

1. Introduction

Canine heartworm disease (HWD) is caused by the filarial nematode *Dirofilaria immitis*, a vector-borne parasite transmitted by several mosquito species. The presence of adult worms in the pulmonary arteries of infected dogs causes changes in arterial structure and function that can lead to pulmonary hypertension and, eventually, to right-sided congestive heart failure (Bowman and Atkins, 2009).

The disease is endemic in many parts of the world and currently affected areas in Europe include Spain (including the Canary Islands), Portugal, the south of France, parts of southern Switzerland, Italy, Greece, Turkey, the Czech Republic, Slovenia, Romania and Bulgaria (Genchi et al., 2014). There are several factors that may explain the spread of the parasite from endemic areas to previously unaffected areas, including climate change, density of the vector population and the movement of microfilaraemic dogs that travel throughout Europe for holidays or as “rescue” adoptions.

Melarsomine dihydrochloride (Immiticide®, Merial) is the only approved adulticidal drug for treatment of HWD. However, treatment can often be followed by severe pulmonary thrombosis (Kramer et al., 2011) and the drug is not registered for use in many countries. In cases where treatment with melarsomine is not possible or is contraindicated, a monthly heartworm preventive along with doxycycline for a 4-week period, which targets the bacterial endosymbiont *Wolbachia*, might be considered. The following review will discuss the principles behind the combination protocols and the current application of this alternative adulticide strategy in dogs with HWD.

2. *Wolbachia* is an anti-filarial target

The endosymbiont *Wolbachia* was first described in *D. immitis* by Sironi et al. (1995). Since then, *Wolbachia* has been identified in a number of filarial worm species, including several that cause human diseases like elephantiasis (*Wuchereria bancrofti*) and river blindness (*Onchocerca volvulus*) Taylor et al. (2013). Endosymbiosis has been defined as the intimate association between two different organisms maintained through generations because host and symbiont equally benefit from the association. Studies have shown that *Wolbachia* is required for normal parasite development, fertility and long-term survival of different filarial species (Taylor et al., 2013; McCall et al., 2014).

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Genetic and molecular studies in the last several years have been aimed at defining exactly what these bacteria do for heartworms and other filarial nematodes (Darby et al., 2012, Godel et al., 2012). Comparison of *Wolbachia* genomes with filarial genomes have revealed that these nematodes have become dependent on their endosymbionts for a wide range of biological processes, including the production of important metabolites like heme, nucleotides and riboflavin (Darby et al., 2012; Scott et al., 2012). This was confirmed by the recent sequencing of the *D. immitis*-derived *Wolbachia* genome. Indeed, heartworm *Wolbachia* encode enzymes for anabolic pathways that are missing in the worm such as biosynthesis of heme, purine, and pyrimidines (Godel et al., 2012). Thus, current research is aimed at the removal of *Wolbachia* through antibiotic treatment of infected hosts as an adulticide strategy.

3. Doxycycline and canine HWD

Elimination of *Wolbachia* is detrimental for the filarial worm and studies on different filarial species including *O. volvulus, W. bancrofti* and *Brugia pahangi* have shown that doxycycline treatment of infected hosts causes degeneration of oocytes and early embryonic stages, is microfilaricidal and has varying adulticide effects. Death of adult worms usually takes from 18 to 24 months (Taylor et al., 2013).

Several studies have also described the effects of doxycycline in dogs with experimentally acquired HWD. Bazzocchi et al. (2008) reported that following 2–6 weeks, intermittent cycles of doxycycline treatment (10 mg/kg/day) resulted in only 8.6% efficacy against adult worms in experimentally infected dogs. However, this study evaluated worm survival at 8 months post-treatment and, as mentioned above, it is likely that death of adult worms takes longer. In a more recent study (McCall et al., 2014), dogs with experimentally-acquired patent infection were treated with doxycycline for 1 month at 20 mg/kg/day. Worm survival was evaluated at 12 and 13 months post-infection (p.i.) and results suggested that treatment had a slow-kill, adulticidal effect, which was more evident at 13, than at 12 months after doxycycline treatment started. The most interesting result of the study, however, was the inability of microfilariae from doxycycline-treated dogs that developed into infective larvae in mosquitos to successfully develop into adults after subsequent infection in new dogs. This would suggest that *Wolbachia* plays an essential role in parasite development in the mammalian host.

Indeed, doxycycline treatment has also been evaluated for its effect on developing worms. McCall et al (2011) treated experimentally infected dogs with doxycycline for 1 month at 20 mg/kg/day at different time points p.i. with third stage larvae (L3) and then verified the presence of adult worms at 8 months p.i. When doxycycline was administered during the first month p.i., no live worms were observed at necropsy, indicating 100% efficacy against infective larvae. When antibiotic treatment was given starting at 40 days p.i., efficacy against developing worms was 98.4%. Finally, when treatment was initiated 65 days p.i., efficacy against juvenile worms was 69.6%. Interestingly, none of the dogs harboring live worms were microfilariemic.

4. Macrocyclic lactone (ML)/doxycycline combination protocols

As mentioned above, doxycycline has various detrimental effects on developing and adult heartworms, but adulticide efficacy is low and slow. Macrocyclic lactones (MLs) are highly efficacious against L3 and young fourth stage larvae (L4) and prevention of disease is based on this activity. However, preventive doses of MLs have been reported as also having a so-called “slow kill” effect against adult *D. immitis* (for review see McCall, 2005). The effects of MLs on adult worms include neuromuscular dysfunction and degeneration of late-stage embryos, thus also reducing the production of microfilariae. Venco et al. (2004) evaluated the use of monthly preventive doses of ivermectin in naturally infected dogs with HWD and showed that 100% of dogs were negative for circulating microfilariae by 4 months, while 71% became negative for circulating antigens after 24 monthly doses. The study also reported a significant decrease in parasite burden as evaluated by echocardiography. However, signs of pulmonary hypertension and interstitial inflammation increased in approximately 20% of dogs and the authors concluded that this adulticide protocol should be used with caution. Furthermore, the long-term use of ML preventives in dogs with HW infection has been implicated in the development of resistant *D. immitis* strains in the southern United States (Wolstenholme et al., 2015). Thus, any method utilizing only macrocyclic lactones as a slow-kill adulticide is not currently recommended.

The hypothesis that the combination of doxycycline and MLs may have greater activity against adult heartworms than either drug alone was first tested in experimentally-infected dogs (Bazzocchi et al., 2008). Treatment with 2–6 week, intermittent cycles of doxycycline (10 mg/kg/day) combined with weekly preventive doses of ivermectin resulted in 80% adulticide activity. The adulticide effect of ivermectin/doxycycline combination protocols has also been confirmed in naturally infected dogs (Grandi et al., 2010). Importantly, the combination of ivermectin and doxycycline was well tolerated and post-adulticide complications were minimal. Indeed, it has been reported that the elimination of *Wolbachia* results in a marked decrease in pro-inflammatory responses to dead and dying worms (Kramer et al., 2008; Kramer et al., 2011; Mavropoulou et al., 2014).

It is not clear why the two drugs work better together in eliminating a large population of heart-worms 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 drugs. Menozzi et al. (2015) evaluated doxycycline levels by high-performance liquid chromatography (HPLC) in sera from *D. immitis*-experimentally infected dogs treated with a combination of ivermectin/doxycycline and compared them with sera from dogs treated with doxycycline alone. Results showed that doxycycline levels were not statistically different between the two groups, suggesting that the adulticide effect is not due to a higher drug concentration of doxycycline in the combination protocol. Passeri et al. (2014) reported there was a significantly lower number of T regulatory cells in the lungs of dogs treated with a combination of both drugs when compared either to the control group or to the other groups treated with either drug alone or with melarsomine. T regulatory cells play an important role in inducing hypo-responsiveness to helminth infections (Maizels et al., 2004; D’Elia et al., 2009), so these results would suggest that successful adulticide effects of doxycycline and ivermectin are associated with a decrease in immune tolerance towards the parasite.

5. The application of ML/doxycycline combination protocols in practice

5.1. Preparation for melarsomine treatment

There have been several reports on the clinical benefits of ML/doxycycline treatment before administration of melarsomine in HW-infected dogs. Indeed, the greatest risk of adulticide therapy is the subsequent rise in pulmonary pressure due to the formation of thrombi around dead and dying worms, together with an intense inflammatory reaction against the parasite. Pre-treatment with MLs/doxy can reduce worm mass and eliminate pro-inflammatory antigens (including *Wolbachia*), thus reducing post-adulticide complications. Kramer et al. (2011) reported that experimentally-infected dogs that received a combined doxycycline/ ivermectin protocol before treatment with melarsomine showed less severe arterial lesions and the virtual absence of thrombi when compared to dogs treated with melarsomine alone. Once HWD has been diagnosed, it is currently recommended to treat dogs with 1 month of doxycycline at 10 mg/kg/BID and with 3 months of monthly preventives; at days 60, 90 and 91, treat with melarsomine with a three-step protocol. Dogs are considered cleared of infection when antigen testing is negative at 6 months post-treatment.

5.2. As an alternative adulticide

As illustrated above, ML/doxycycline combinations have been shown to be adulticidal in both experimentally and naturally-infected dogs. All published studies have concentrated on preventive doses of ivermectin, either weekly or bi-weekly for 6 months, combined with doxycycline at 10 mg/kg either SID or BID (Bazzocchi et al., 2008; Grandi et al., 2010;Mavropoulou et al., 2014). Infected dogs usually begin to be negative for circulating antigens at about 12 months from the beginning of therapy. Treatment is well-tolerated with minimal radiological and clinical signs.

A recent report has evaluated the effects of another ML in combination with doxycycline. Topical moxidectin (Advantage Multi®, Bayer) is active against infective L3-L4 larvae and against circulating microfilariae. Savadelis et al. (2016) reported in *D. immitis*-experimentally infected dogs that 9 monthly doses of topical moxidectin combined with 30 days of 10 mg/kg doxycycline BID resulted in the elimination of microfilariae by 21 days post-treatment, thus breaking the transmission cycle of the parasite very quickly. Furthermore, heartworm antigen began declining 3 months post-treatment and treatment resulted in a 95.9% efficacy in eliminating mature adult heartworms by 10 months, indicating a more marked adulticidal effect compared to that of combinations with ivermectin. It is well documented that orally administered macrocyclic lactones have a typical specific pharmacokinetic (PK) pattern showing a high peak and a very short half live and thus a very short exposure to the worm. This is dose dependent of course, but even with higher doses the general PK pattern does not change (Daurio et al., 1992). In contrast, the moxidectin PK for the spot on formulation is much different with a peak establishing several days following application, a half-life of about 28 days, and the development of a steady state level after about 4 months, ensuring a constant and high exposure of the parasite to the drug (Bowman et al., 2016).

6. Conclusions

Doxycycline and ML combinations are adulticidal for *D. immitis*. Research should, however, continue for the identification of other anti-*Wolbachia* molecules and targets. It is also necessary to further study the interaction between MLs and doxycycline/others in order to determine the best combination protocol in terms of clinical and parasitological response. Finally, as is true for any treatment of any sick dog, it is always necessary to periodically monitor the patient's response to therapy.

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