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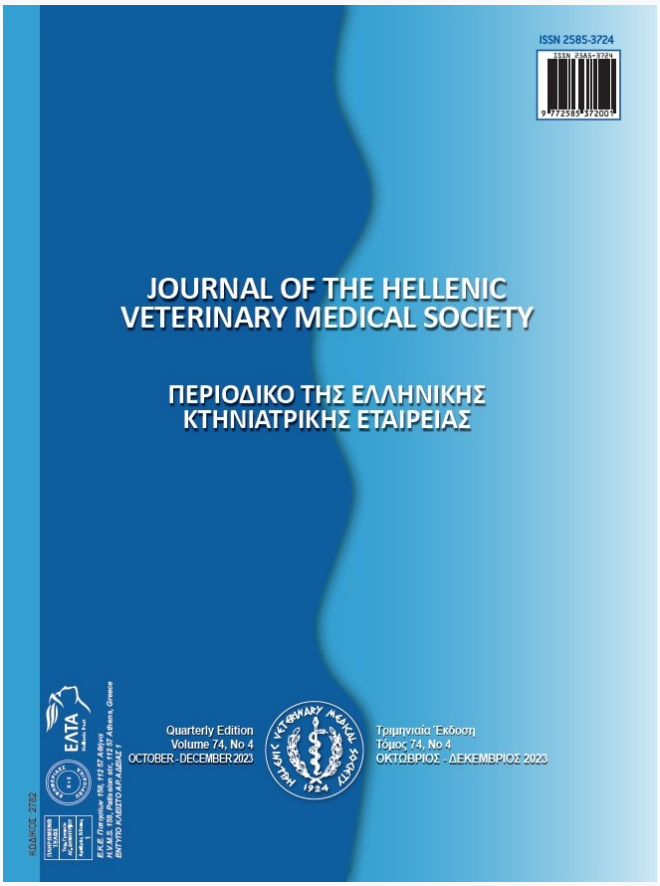

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Research article
Ερευνητικό άρθρο

Effectiveness of doxycycline/ivermectin therapy for heartworm disease in regard to *Wolbachia* status in dogs

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ABSTRACT: Heartworm disease (HWD), a highly important canine disease in veterinary medicine, is caused by *Dirofilaria immitis* (*D. immitis*). A therapeutic approach (the standard treatment or an alternative one, or even surgical intervention) in dogs naturally infected with *D. immitis* should be individually adjusted. The goal of this study was to test the effectiveness of an alternative therapeutic protocol for *D.immitis* and *Wolbachia* in 30 naturally infected dogs. Clinical and parasitological examinations (modified Knott test and SNAP Test IDEXX) were used for diagnosing *Dirofilaria* spp. Blood and urine laboratory, and radiographic examinations were used for the determination of HWD classes. An alternative therapy consisted of oral doxycycline and ivermectin administration until microfilaremic and antigenic negativization, and was not longer than 9 months. Polymerase chain reaction (PCR) for *wsp* and 16S rRNA genes at the beginning and at the end of the therapy in 15 dogs was used for *Wolbachia* detection. The effectiveness of the alternative therapy for *D. immitis* and *Wolbachia* was 83% and 75%, respectively. Neither the number of cured dogs nor the duration of the alternative therapy depended on the presence of *Wolbachia*. Thus, the alternative therapy with doxycycline and ivermectin is not based solely on targeting *Wolbachia.*

***Keywords*:** Heartworm disease; Wolbachia; alternative therapy

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INTRODUCTION

Dog heartworm disease (HWD) is a clinically important parasitic disease caused by *Dirofilaria immitis (D. immitis)*. The most serious complications are pulmonary thromboembolism (PTE), right heart failure and *caval syndrome* (CS). However, as a chronic infection, it can cause changes in immune system with allergic pneumonitis, glomerulonephritis and reactive arthritis as possible clinical manifestations (Dunn, 2000).

Wolbachia is intracellular, endosymbiontic bacteria, which plays the essential role in pathogenesis of HWD, intensifying the severity of clinical signs (Maia et al., 2016). *Wolbachia* is located in subdermal lateral cords of adult nematode and microfilariae, and in female reproductive system from where it is transmitted vertically into the eggs (Martin and Gavotte, 2010).

The goals of heartworm treatment are to improve the clinical condition of the animal and to eliminate all life stages of heartworm with minimal post-treatment complications. Melarsomine dihydrochloride is the only effective drug available for treating adult heartworm infections (ESCCAP, 2019; AHS, 2020). According to AHS recommendations, standard treatment with melarsomine includes pretreatment with prednisone, doxycycline and macrocyclic lactones. Alternatively, monthly oral ivermectin or topical moxidectin heartworm preventive with doxycycline might be considered as a salvage procedure (AHS, 2020). Surgical intervention is advised when multiple worms have been displaced into the right cardiac chambers, producing sudden onset of CS (ESCCAP, 2019; AHS, 2020). Being the part of both standard and alternative therapy, the role of tetracycline is worth attention. Tetracycline treatment targets *Wolbachia* (Bandi et al., 1999), and the protocols which include doxycycline administration result in less arterial lesions, thrombi formation, respiratory complication and mortality rates in dogs, both experimentally (Kramer et al. 2011) and naturally infected (Nelson et al., 2017). However, chemically modified tetracyclines without antimicrobial activity are also able to cause detrimental effects on filarial worms (Rajan, 2004), and ivermectin, which causes intestinal alterations of *D. immitis,* enables an accumulation of higher concentrations of doxycycline within the nematode (Bazzochi et al., 2008).

Knowing the fact that no therapy is ideal for every patient, and that it should be individually adjusted to each patient, we wanted to assess the effectiveness of an alternative therapy for HWD based on doxycycline and ivermectin in dogs naturally infected with *D. immitis* in northern part of Serbia, which is endemic for HWD (Potkonjak et al., 2020). In addition, since it is not clear whether the use of an antibiotic in alternative therapeutic protocols against *D. immitis* targets *Wolbachia*, we have determined the effectiveness of the alternative therapy from the aspect of *Wolbachia* infection.

MATERIALS AND METHODS

Study design

A total of 30 dogs naturally infected with *D. immitis* were submitted to the same protocol of an alternative therapy for HWD. The purpose was to analyze a clinical follow up, the outcome of the alternative therapy for 12 months and to estimate its effectiveness. Afterwards, we randomly chose 15 out of these dogs in which we retrospectively analyzed a presence of *Wolbachia* at the beginning and at the end of the therapy. The aim of this part of the study was to estimate the effectiveness of the alternative therapy for *Dirofilaria/Wolbachia* coinfection.

Dogs

This study was designed as the field study of 30 client-owned dogs naturally infected with *D. immitis* in the endemic region of Serbia. The dogs included in the study were of various breeds, sex, age and body weight. Since this research was designed as clinical case study, according to the national law, an ethical approval for this research was not needed. However, a written consent for participation in the study was signed by each dog's owner who were informed about specific conditions of this study: at the moment of examination a dog should have been at least 7 months old, exposed minimally to one mosquito season and without a history of treatment with macrocyclic lactones. The owners were informed that the duration of the alternative therapy could vary but would not be longer than 9 months. The owners were obliged to bring their dogs for clinical and parasitological examinations once per month during the therapy, and then again 12 months after the establishment of HWD diagnosis.

Parasitological examinations

Blood samples were tested by modified Knott test for the identification of microfilaria (mf) and their number (microfilariemia) was estimated by mf/ml (Genchi et al., 2007; Bazzocchi et al., 2008). Mod-

ified Knott test was performed by mixing 1ml of venous blood with 10ml of 2% buffered formalin, followed by centrifugation for 5 min at 200g. Equal parts of the sediment and 1:1,000 methylene blue stain were mixed in the quantity of 200µl. A total of 20µl of stained sediment was examined microscopically. Detected number of mf was multiplied by 10 and expressed as mf/ml. Whole blood or blood serum samples were tested for *D. immitis* antigen (Ag) using commercially available assays (Canine Heartworm Antigen Test Kit-Snapp HTWM, IDEXX, USA). The infection was established in case of detection of either Ag or mf of *D. immitis* or both.

Clinical examinations

Upon diagnosing HW infection in dogs, thorough clinical examination with blood and urine analysis, and thoracic radiography were performed in order to define the class of HWD in each dog (AHS 2020; Jones 2016; Ware, 2011). Class 1: asymptomatic to mild HWD (no clinical signs or occasional cough; normal laboratory parameters; radiographic signs absent). Class 2: moderate HWD (occasional cough, exercise intolerance, abnormal lung sounds, mild loss of body condition; mild anemia with or without mild proteinuria; radiographic signs: RV enlargement, mild PA enlargement, circumscribed perivascular infiltrates and/or mixed alveolar/interstitial infiltrates). Class 3: severe HWD (persistent cough, constant fatigue, dyspnea, abnormal heart and lung sounds, hepatomegaly, syncope, ascites, jugular distension and pulse, death; anemia, other hematological abnormalities or proteinuria; radiographic signs: RV and RA enlargement, severe PA enlargement, circumscribed to diffuse mixed patterns of pulmonary infiltrates, signs of pulmonary embolism. Class 4: caval syndrome (sudden onset of severe lethargy, weakness, dark red to black coffee colored urine; hemoglobinemia, hemoglobinuria, proteinuria, bilirubinuria and mf in urine sediment; numerous short, white, parallel lines within the right atrium, ventricle and tricuspid orifice detected by echocardiography) (AHS 2020; Jones 2016; Ware, 2011).

Therapeutic protocol

Specific therapy for HW was an alternative therapy based on doxycycline and ivermectin (Table 1). All dogs were treated with the alternative protocol - doxycycline 10 mg/kg b.w. perorally, once a day for 6 weeks, then alternately 4 weeks without and 2 weeks with the medication, and ivermectin 6-14 µg/kg b.w. perorally, every 2 weeks, until negative results were reported for antigenemia, but not longer than 9 months (Bazzocchi et al., 2008). In dogs that were in heart failure or in higher risk for pulmonary thromboembolism (PTE), the therapy was individually adjusted with appropriate drugs (prednisone, nadroparine calcium, enalapril maleate, furosemide, aminophylline). Also, the owners were advised to subject the dogs only to light physical activity (on a short leash) and to implement a cardiac diet.

Molecular detection of Wolbachia

Genomic DNA was extracted from whole blood samples, taken at the beginning and at the end of the therapy, using commercial Quick Blood DNA Purification Kit (EUR_X Ltd. Gdansk, Poland), according to the manufacturer's protocol. Total DNA samples of 15 dogs were retrospectively analyzed for the presence of *Wolbachia* by conventional PCR, using a primer pair specific for *wsp* gene, previously described by Simsek and Turkan Ciftci (2016). Furthermore, additional conventional PCR was done for the same samples targeting 16S rRNA gene, as previously described by Foster et al. (2008). PCR products for *wsp* and 16S rRNA were separated on agarose gel and stained by ethidium bromide and visualized under ultraviolet light.

Table 1. Timetable of examinations and the alternative therapy for HWD

Days	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	360
Par.ex	◊		◊		◊		◊		◊		◊		◊		◊		◊		◊	◊
Cl.ex	●		●		●		●		●		●		●		●		●		●	●
Lab.	○																			
Rg.	□																			
Ivr	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Doxy	*	←	→	*		*	←→	*		*	←→	*		*	←→	*		*	←→	*

◊ Par.ex. - parasitological examination, ● Cl.ex - clinical examination, ○ Lab. - laboratory examination, □ Rg. - radiographic examination, + Ivr - ivermectin administration, * Doxy - doxycycline administration

The effectiveness of the alternative therapy

The dog was considered cured of HWD if clinical signs have disappeared and if no *D.immitis* antigenemia and microfilaremia have been detected during the alternative therapy and/or 12 months after the beginning of the therapy. In the second part of the study, in addition to clinical and parasitological criteria, negative PCR tests for *wsp* and 16S rRNA genes at the end of the alternative therapy were posed as the goal.

Statistical analysis

A commercial software package Statistica 64 is used for statistical analyses (basic statistics, the Fisher probability test, the t-test for independent samples). A probability value of $P<0.05$ was considered as significant.

RESULTS

The most common clinical signs in dogs with natural HWD were cough, fatigue and dyspnea (Table 2). Nine of 30 dogs (30%) enrolled were asymptomatic.

Table 2. Clinical manifestations of the dogs naturally infected with *D. immitis* at the beginning of the alternative therapy

Clinical signs*	Number (%) of the dogs
Cough	16 (53)
Fatigue	8 (27)
Dyspnea	5 (17)
Skin lesions	2 (7)
Ascites	2 (7)
Panting	1 (3)
Peripheral edema	1 (3)
Lameness	1 (3)
No signs	9 (30)

*In nine dogs (30%) more than 2 clinical signs were present

At the moment of HW infection diagnosis, mf were detected in 21 dogs (70%), while 9 dogs (30%) had occult HW infection. In 4 dogs (13%) mf of both *D. immitis* and *D. repens* were detected, while in 2 dogs (7%) there was only mf of *D. repens* detected by Knott test. Although registered during study, at the moment of diagnosis and/or in control examinations, *D. repens* was not in the focus of our study (Table 3).

According to their clinical signs, radiographic findings and laboratory parameters, 8 dogs (27%) were classified in class 1 (asymptomatic to mild HWD), 18 dogs (60%) were in class 2 (moderate HWD) and 4 dogs (13%) were in class 3 (severe HWD). None of the dogs were in class 4 (CS).

Upon beginning of the alternative therapy, clinical improvement (improvement of clinical signs based on anamnesis and general clinical examination) was registered during the first month of the therapy in the majority of dog patients. In two dogs with heart failure, ascites resolved in the third month of the therapy. Two dogs died during the studying period. One died due to complication of HWD, i.e. acute PTE. The other dog was successfully cured from HWD after 6 months of therapy, but died due to babesiosis twelve months after the beginning of the therapy.

After three months of the alternative therapy there were no dogs with mf. After 6 months antigen was not detected in 9 out of 26 dogs (35%). But, in three dogs Ag was detected after 12 months. With 25 out of 30 dogs reaching the goal of the therapy, the effectiveness of 83% was estimated (Table 3).

The average duration of the alternative therapy

Table 3. Number of dogs with antigenemia (Ag) and microfilariaemia (mf) at the moment of HW infestation diagnosis and during therapy

Checkups	Ag ++ *D. immitis*	Ag + *D. immitis*	Ag ND *D.immitis*	mf *D. immitis*	mf *D. immitis* *D. repens*	mf *D. repens*
Day 0	13	13	4	15	4	2
Day 30	11	15	4	7	1	1
Day 60	10	15	5	1	0	0
Day 90	8	17	4	1	0	0
Day 120	4	21	4	0	0	0
Day 150	5	18	6	0	0	0
Day 180	2	12	15	0	0	0
Day 210	1	9	19	0	0	0
Day 240	2	8	19	0	0	0
Day 270	2	5	22	0	0	0
Day 360	1	2	25	0	0	0

Ag++ high level of antigen detected; Ag+ low level of antigen; ND antigen not detected; mf microfilariae

varied in dogs with different class of HWD (class 1: 4.71±2.36 months, class 2: 8.00±2.74 months, class 3: 7.33±5.03 months), with statistical difference between the dogs in class 1 and 2 (P=0.01).

In the second part of this study, when blood samples were retrospectively analyzed for *Wolbachia* presence, the prevalence was 53% (8/15 dogs). *Wolbachia* was detected either with both (3 dogs) or just with one gene (16S rRNA gene of *Wolbachia* was detected in 5 dogs) at the beginning of the alternative therapy. At the end of the alternative therapy, when no Ag and no mf of *D. immitis* were detected (14 dogs or 93%), the majority of these dogs had neither *D. immitis* nor *Wolbachia* detected (13 dogs or 87% D-W-), and in one dog *wsp* gene of *Wolbachia* was detected (1 dog or 7% D-W+). This dog initially did not have *Wolbachia* genes detected but at the end of the therapy *wsp* gene of *Wolbachia* was detected. The effectiveness of the alternative therapy for *D. immitis* was 87% (13/15 dogs) and 75% (6/8 dogs) for *D.immitis*/*Wolbachia* coinfection. No significant difference was detected between the duration of therapy in D+W+ and D+W- dogs (6.75 ± 3.84 months and 7.50 ± 4.55 months; P=0.74).

DISCUSSION

In this study the effectiveness of the alternative therapy based on combined doxycycline and ivermectin in dogs naturally infected with *D.immitis* was 83%. In dogs retrospectively analyzed for the presence of *Wolbachia*, the prevalence was 53%, and the effectiveness of the alternative therapy was 87% for *D. immitis*, and 75% for *D. immitis*/*Wolbachia* coinfection. The number of cured dogs was not dependent on the positivity for *Wolbachia* at the beginning of the alternative therapy, and the duration of the therapy was not significantly different between D+W+ and D+W- dogs.

In this study, we used clinical, laboratory and radiographic findings in order to classify dogs with HWD. The majority of dogs (18 dogs or 60%) were in class 2 (moderate) HWD, followed by 8 dogs (27%) classified in class 1 (asymptomatic to mild) HWD and 4 dogs (13%) in class 3 (severe HWD). There were no dogs with *caval syndrome*, which demands surgical intervention, and cannot be cured by medications. Resolution of the clinical signs was registered in all dogs of our study, even in those in which Ag was detected 12 months after HWD diagnosis and the beginning of the therapy, except in two dogs. It is of interest to emphasize the outcome of the alternative therapy for dogs in class 3 of HWD. Two dogs had PTE as a complication of HWD. One developed acute PTE and died after two months, although no Ag and no mf was detected at that time on the last parasitological control. The other had chronic form, and this was the dog with persistent cough and whose level of Ag only decreased during the therapy, and it was not considered cured of HWD by the alternative therapy. However, two dogs in heart failure were successfully cured with the alternative therapy. These results can lead us to hypothesize that coagulopathy is more difficult to cope with than hemodynamic disturbances which lead to heart failure in HWD.

Different genes can be used in PCR detection of *Wolbachia* such as *wsp*, 16S rRNA, *dnaA, ftsZ, groE, hcpA, gltA, fbpA, gatB and coxA* (Sarwar et al., 2018). In this study, we have used *wsp* and 16S rRNA genes for the detection of *Wolbachia*. It was shown that *wsp*, a major surface protein, induces chemokinetic activity and IL-8 production, and thus activates neutrophils in dogs infected with *D. immitis* (Bazzocchi et al., 2003). When adult heartworms die naturally or as a result of drug administration, the bacteria are released. The *wsp* genes recruit neutrophils and other immune cells which form a partial blockage of blood vessels at microscopic level obstructing normal blood flow (McCall et al., 2008). On the other hand, animals infected by filarias develop an antibody response against *wsp* (Bazzocchi et al., 2000, Simón et al., 2003). However, the higher variability of *wsp* gene among different *Wolbachia* strains leads to a lesser sensitivity of PCR amplification (Ren et al., 2020). Other different molecular marker, 16S rRNA, is used as a genetic marker to study bacterial phylogeny and taxonomy (Janda and Abbott, 2007). Being the most conserved marker gene among different *Wolbachia* strains, 16S rRNA provides more consistent PCR amplification (Ren et al., 2020). By choosing these two genes we aimed at both immunological role and molecular detection of *Wolbachia* in dogs naturally infected with *D. immitis*. The prevalence of 53% was estimated for *Wolbachia* in our study, similar to the results of the detection of *Wolbachia* in *Dirofilaria* spp. infected dogs (Landum et al., 2014).

Although being the only drug registered as adulticide against *D.immitis*, melarsomine is connected to serious side effects (from irritation and swelling to sterile granuloma or abscess at the injection site, neurological complications with paraparesis and paraly-

sis, and PTE). That fact limits its application in certain dog patients, especially in dogs with concurrent disease, or with moderate to severe HWD. Also, this drug is not available throughout the world. Because melarsomine is not registered in Serbia, an alternative therapy consisting of doxycycline and ivermectin in different application regimes is widely used in our country (Spasojević Kosić et al., 2014; Spasojević Kosić et al., 2020; Milojković et al., 2016; Stepanović et al., 2015). Based on the results of the study in experimentally infected dogs (Bazzocchi et al., 2008), with adulticide effect of 78%, complete loss of uterine content in female worms documented histologically, and negative immunochemistry for *Wolbachia*, we decided to use the same therapeutic protocol in dogs naturally infected with *D.immitis*. This study also proved that combined doxycycline and ivermectin act on *D.immitis* better than any of these drugs alone (Bazzocchi et al., 2008).

In our study, most of the treated dogs had no mf after one month of the therapy, with no mf detected after 90 days. Similar results were shown in another study with doxycycline and ivermectin in different regime of administration (Grandi et al., 2010). Concerning antigenemia in our study, the dogs needed treatment for 2-12 months to become negative. More promising results come from a monthly dose of moxidectin in combination with 30 days of doxycycline. This type of alternative therapy eliminates circulating mf within one month, and antigenemia after four to nine months (Genchi et al., 2019). The efficiency of the therapy with only melarsomine depends on the severity of disease, and most of the dogs with mild to moderate HWD become Ag-negative by four to five months (Genchi et al., 2019). In our study, the duration of the alternative therapy depended on the class of HWD, and it was significantly shorter in dogs with mild compared to dogs with moderate HWD ($P<0.05$). Two dogs with high levels of Ag became Ag-negative after 2 months of the alternative therapy, but with completely different clinical outcomes (*exitus lethalis* in one dog with class 3 HWD and complete clinical resolve in one dog with class 1 HWD). Doxycycline is recommended as a part of the standard treatment before melarsomine by both ESCCAP and AHS (ESCCAP, 2019; AHS, 2020), and it is used in combination with different macrocyclic lactones (mainly moxidectin or ivermectin) in alternative therapy protocols. However, recommended doses and duration of therapy vary in studies, depending on the type of the study (experimental or natural infection), and on the therapeutic protocol for HWD (the standard treatment with melarsomine or an alternative one) (McCall et al., 2008; Bazzocchi et al., 2008; Grandi et al., 2010; Rossi et al., 2010; Kramer et al., 2011; Savadelis et al., 2018). Based on the evaluation of antibodies against *Wolbachia* in serum samples from dogs, administration of lower dose of doxycycline is sufficient to achieve a significant reduction of *Wolbachia* (Carretón et al., 2020). In our study, effectiveness of the alternative therapy for *Dirofilaria*/*Wolbachia* coinfection was 75%, but the presence of *Wolbachia* did not affect the effectiveness of therapy and its duration for *D.immitis*. From the clinical point of view, a therapy should be individually adjusted to each dog, according to its clinical follow up. In the study presented here, for example, in one dog *Wolbachia* was detected only after the treatment. This dog had class 3 HWD due to heart failure but he made a full clinical recovery, with no Ag and mf detected, and considered cured of HWD. The reason for this might be false negative PCR for *Wolbachia* or repopulation of *Wolbachia*, the disintegration of parasites or false negative antigenemia during persistent infection, or even new infection with *D.immitis*. However, the alternative protocol may not be so effective in dogs with severe heartworm disease.

Some limitations of this study should be addressed. Ideally a control group using standard treatment protocol in naturally infected dogs was needed. Given that the high effectiveness of standard protocol is well known, it was not necessary to include this group. The number of dogs naturally infected with *D. immitis* was insufficient to test effectiveness of the alternaltive protocol for dogs with severe HWD. Also, conventional PCR for two genes of *Wolbachia* was not sufficient to assess the dose and duration of antibiotic treatment as a part of the alternative therapy. Further studies are needed in order to overcome these limitations.

CONCLUSION

The alternative therapy which combines doxycycline and ivermectin has adulticidal and microfilaricidal activity in dogs naturally infected with *D. immitis*. The duration of the alternative therapy varies from four to eight months in dogs with different class of HWD. The alternative therapy is similarly effective for *D.immitis* and *Wolbachia* (83% and 75%, respectively). The effectiveness of the alternative therapy with doxycycline and ivermectin is not dependent solely on targeting *Wolbachia*, since neither the number of cured dogs nor the duration of the alternative

therapy is influenced by the presence of *Wolbachia*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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