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**Prevalence of *Dirofilaria* spp. in Hungary
and veterinary importance, the
experience of treatment**

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1. Introduction

Dirofilaria-species are frequent endoparasites of pets and wild animals, mainly carnivores in Mediterranean region of Europe, America, Australia, Asia, and a part of Africa. Prevalence of mosquito-borne *Dirofilaria* (*Nochtiella*) *repens* /Railliet and Henry, 2011/ (Filarioidea, Onchocercidae) is increasing in dogs and cats, and even in humans. The biological vectors of *D. repens* are mosquitoes of the genera *Aedes*, *Culex* and *Anopheles*. In these intermediate hosts the microfilariae develop into third-stage larvae (L3). These infective forms are transmitted to the final hosts via a mosquito bite, and in 8-9 months L3 develop to adults. Worms present in the subcutaneous tissue can give rise to nodules or dermatitis in dogs, but severe clinical signs are rarely observed in connection with *D. repens* infection. The veterinary importance of *Dirofilaria immitis* (heartworm) is definitely greater as this parasite lives in the right cardiac chambers and pulmonary vessels of dog and cats and can cause fatal disease. Both *Dirofilaria*-species have real zoonotic significance. In Hungary first autochthonous case of *D. repens* was diagnosed at the end of 1990, but heartworm infections were only imported cases until our study.

The aim of our work was to get more information about the prevalence, the significance, the clinical manifestation and the therapeutics of cutaneous dirofilariosis. Moreover we tried to observe the presence of

D. immitis in Hungary. On the basis of these, our research had three different strands:

1. In order to estimate the prevalence of dirofilariosis in dogs and cats, we made a country-wide survey between 2005 and 2009 to detect the circulating microfilariae in blood samples. We also used PCR and serological tests in case of a few samples. To get information about the circumstances and clinical status of examined dogs, the owners and veterinarians filled out a data sheet. Mosquito trapping was carried out during our survey in three different places. After species identification the collected mosquitoes were ordered in pool samples, and *D. repens* species specific PCR was loaded.

2. With the aim of collecting more data on the clinical aspects of cutaneous dirofilariosis, we analysed haematological and biochemical parameters of infected and not infected dogs. Moreover, we made pathological examinations on microfilaraemic dogs with severe kidney failure.

3. Intent on getting information on therapy of *D. repens* infection, we carried out two different field efficacy survey using macrocyclic lactones (moxidectin and selamectin).

2. Materials and Methods

2.1. Investigations for the occurrence of dirofilarioses in Hungary

2.1.1. Country-wide survey

Between 2005-2009 a country-wide survey was carried out to determine the prevalence of *Dirofilaria*-species in dogs and cats. 3104 blood samples of dogs and 78 blood samples of cats were examined by modified Knott's method. 2260 data sheets were filled out by local veterinarians and dog owners. 143 blood samples were checked by molecular techniques all of them by *D. repens* species-specific PCR, 24 samples by *D. immitis* species-specific PCR, and 7 samples by *Acanthocheilonema reconditum* species-specific PCR. Moreover the DNA of 10 samples were sequenced. We also made serological examinations from 125 blood samples by immunochromatographic Speed® Diro test (BVT, France). Fisher one-sided test and binomial one-sided test were used for statistical analysis of the data sheets. The common influence of the covariates was tested by probit regression.

2.1.2. Comparison of laboratory techniques

During the country-wide survey, the blood samples were tested by different laboratory methods to check their sensitivity. Modified Knott's method was compared to *D. repens* species-specific PCR (143 samples) and haematology blood smear examination (58 samples).

2.1.3. Investigation of pregnant bitches and their puppies

In order to realise if transplacental infection is possible in case of *D. repens*, 22 bitches were examined during the gestation period by modified Knott's method. The blood samples from puppies of microfilaraemic mothers were tested to find circulating microfilariae before they reached six-month-old age.

2.1.4. Collection and identification of mosquitoes, DNA examination

Between June and September 2008 Culicidae mosquitoes were collected in Budapest and Miskolc. Trapping was carried out with the 1012 model of New Standard Miniature Light Trap. Location of trapping: Rákosliget, Csepel-island, Miskolc.

For molecular examinations the collected mosquitoes were arranged in pool samples according to their species and the locality of collection. We divided the mosquito body into two parts, as cephalothorax and abdomen. DNA of mosquitoes was extracted by DNeasy Blood & Tissue kit (Qiagen). All of the pool samples were tested by *D. repens* species-specific PCR based on amplifications 12S rDNA.

2.2. Clinical aspects of dirofilariosis

2.2.1. Analysis of laboratory parameters

Between October 2007 and August 2008 457 blood samples were collected from dogs which visited Internal Medicine Clinic and had laboratory findings made by Internal Medicine Laboratory. To diagnose *D. repens* infection blood samples were tested for circulating microfilariae by modified Knott's method. Mann-Whitney-Wilcoxon test and Fisher test were used for statistical analysis of laboratory results.

2.2.2. Kidney failure and dirofilariosis

13 blood samples were collected from young dogs suffering from kidney problems, which means high karbamid and creatinin levels according to Internal Medicine Laboratory. These samples were checked by modified Knott's method.

2.3. Therapeutic examinations

2.3.1. Moxidectin (Advocate® spot on, Bayer)

Two field trials were conducted in Budapest (Trial 1) and in Pécs (Trial 2). Trial 1 was performed as controlled field efficacy study with 28 dogs. Trial 2 was a blinded, randomised controlled field study with 36 dogs. A total of 64 dogs have been confirmed positive for infection with *D. repens* following detection of circulating microfilariae. The animals were treated with Advocate® spot on according to different treatment protocols or left untreated. Microfilaria counts were performed before treatment, after 2 weeks, 4

weeks, and thereafter every 4 weeks up to 6 months after the last treatment. The statistical analysis was performed with the validated programme Testimate Version 6.4 from IDV Gauting.

2.3.2. Selamectin (Stronghold® spot on, Pfizer)

The trial was carried out from April 2007 to March 2008 on a beagle breeding farm. 23 of these animals (3 males and 20 females; age range: 6 months to 5 years) were randomly selected for inclusion into the study. Four groups were formed according to the treatment schedule. Blood samples were collected by venipuncture from each dog on Days 0, 16, 28, 56, 84, 112, 140, 168, 196, 224, 252, 280, 308 and 336. Microfilaria counts were carried out in 0.5 ml of blood. Data were analysed by Sign test and Mann-Whitney-Wilcoxon test.

3. Results

3.1. Investigations for the occurrence of dirofilarioses in Hungary

3.1.1. Country-wide survey

563 of 3104 blood samples of dogs were microfilaria positive by modified Knott's method, which means 18.1% prevalence. 3 of 78 cats were microfilaraemic (3.8%). According to morphological criterions 561 were identified as *D. repens* infection, while 2 samples contained smaller microfilariae, suspected *A. reconditum*. *D. immitis* serological tests were negatives in case of 124 samples, and in one occasion it resulted doubtful (mild) positive line. *D. repens* species-specific PCR was positive in 87 of 143 cases. From the 56 PCR-negative samples 32 were microfilaraemic by modified Knott's method. *A. reconditum* species-specific PCR was negative in all 7 cases. Using *D. immitis* specific primer 1 sample proved to be positive. The same sample contained *D. repens* DNA too. This coinfection was originated in Hungary. During this period a nematode removed from a ferret was sent to our lab that we identified by morphometric and molecular methods as *D. immitis*.

3.1.2. Comparison of laboratory techniques

The sensitivity of PCR compared to modified Knott's method was 73.1%, while that of blood smear examination was only 2.5%.

3.1.3. Investigation of pregnant bitches and their puppies

2 of 22 bitches were microfilaraemic, but no samples of their puppies contained microfilariae.

3.1.4. Analysis of data sheets

The statistical analysis of the results showed, that *D. repens* infection is more frequent in dogs which are older, male, living in extensive circumstances, partaking no antiparasitic treatment.

3.1.5. Collection and identification of mosquitoes, DNA examination

217 collected female mosquitoes were identified on species level, as 150 *Culex pipiens*, 45 *Aedes vexans*, 22 *Anopheles maculipennis*, while 18 individuals only on genus level, as 14 *Ochlerotatus* genus, 4 *Culiseta* genus. 217 mosquitoes were arranged in 42 pool samples. *D. repens* species-specific PCR resulted 33,3% positivity (14 pool samples were positive). All of the 6 pool samples that contained *A. maculipennis* proved to be positive, both abdomen and cephalotoracal parts. 6 of 10 *Ae. vexans* samples were positive (60%), but two of them contained only abdomen of mosquitoes. In case of *C. pipiens*, only 2 of 26 samples were positive (7.69%), which included the head and the cephalothorax of the same mosquitoes.

3.2. Clinical aspects of dirofilariosis

3.2.1. Analysis of laboratory parameters

The comparative examination of the hematological and serum biochemical profiles of the dogs revealed leukocytosis, eosinophilia, higher ALT, ALKP, urea, lipase, CK values, and lower thrombocyte number and LDH value in the infected group compared to those not infected. Only urea level was significantly higher according to statistical analysis, and decrease of thrombocyte number was almost significant.

3.2.2. Kidney failure and dirofilariosis

2 of 13 samples contained *D. repens* microfilariae. After euthanasia of both dogs dissection was carried out, while severe kidney failure was diagnosed without any other causative agent, but microfilaraemia.

3.3. Therapeutic examinations

3.3.1. Moxidectin (Advocate® spot on, Bayer)

Two weeks after the first treatment, 38 of 44 dogs were found to be negative for microfilariae. Four weeks after the initial treatment, one dog still showed a low total microfilaria count in Trial 1. Following the second treatment, all treated dogs were negative in all treated groups in both trials.

3.3.2. Selamectin (Stronghold® spot on, Pfizer)

Microfilaria counts carried out on Day 0 showed wide differences in counts (range 4–2279/0.5 ml of blood).

On Day 16, the number of microfilariae decreased in some blood samples while in others microfilaraemia was increased. From Day 84, a general reduction of the microfilaria count was observed, and subsequently an increasing number of samples became negative.

4. Discussion

The country-wide survey showed 18.1% prevalence of *D. repens* in dogs. This value is much higher than it has been found earlier in Tolna county, and also the prevalence measured in countries to the north and to the west are lower. No data has been available about the prevalence of *D. repens* in cats in Hungary, and our result (3.8%) is similar to those found in Italy. These data show that the rate of infection in Hungary is close to that of the Mediterranean countries. In our survey *A. reconditum* was not found, but we diagnosed the first autochthonous *D. immitis* case in dogs, and *D. immitis* was identified in a ferret too.

In the Hungarian fauna of mosquitoes *A. maculipennis* seems to be the most potent vector of *D. repens*, followed by *Ae. vexans*, while the less significant role has *C. pipiens*.

The comparative results of haematological and biochemical parameters in infected and not infected dogs are according to our expectations. The alterations of laboratory findings are similar of those in heartworm infected dogs, which may mean that these deviations are caused by microfilariae and not adults. Histopathological disorders of the kidneys were connected with *D. immitis* infection in publications. If microfilaraemia affects kidneys, in case of *D. repens* infection kidney failure could also evolve.

Moxidectin (Advocate® spot on) applied monthly during long treatment period successfully eliminated

microfilariae of *D. repens* in dogs. A marked reduction of the microfilaria count was recorded in dogs following chronic selamectin (Stronghold® spot on) treatment. In our opinion such a treatment regime would help to decrease the level of microfilaraemia and even eliminate adult worms too. So long term applications can avoid the spread of *D. repens* and this way the number of microfilaraemic dogs may be reduced.

5. New scientific results

By five-year-long country-wide survey:

1. We ascertained that the prevalence of *D. repens* infection is 18.1% among dogs and 3.8% among cats in Hungary.
2. We diagnosed the first autochthonous *D. immitis* infection in dogs and ferrets.
3. The statistical analysis of data sheets showed, that *D. repens* infection is more frequent in dogs which are older, male, living in extensive circumstances, partaking no antiparasitic treatment.
4. This was the first time to investigate *Dirofilaria*-DNA in mosquitoes in Hungary, which showed that *A. maculipennis*, *A. vexans* and *C. pipiens* have significant role in transmission and development of *D. repens*.

By the examination of the clinical aspects of cutaneous dirofilariosis:

1. Leukocytosis, eosinophilia, higher ALT, ALKP, urea, lipase, CK values, and lower thrombocyte number and LDH value was measured in the infected group compared to those not infected.
2. The necropsy of two microfilaraemic dogs revealed connection between kidney failure and cutan-dirofilariosis.

By field efficacy survey:

1. Advocate® spot on applied monthly during long treatment period successfully eliminated microfilariae of *D. repens* in dogs and it was effective against adults too.
2. A marked reduction of the microfilaria count was recorded in dogs following chronic selamectin spot-on treatment, while there was no side effect.

6. Publications

6.1. In peer reviewed journals, in the topic of the doctoral thesis

Jacsó O., Fok É.: **Detection of *Dirofilaria repens* infection in dogs and cats by laboratory methods. Literature review,** [In Hungarian] Magyar Állatorv. Lapja, 128. 683-690., 2006.

Jacsó O., Mándoki M., Majoros G., Pétsch M., Mortarino, M., Genchi, C., Fok É.: **First autochthonous *Dirofilaria immitis* (Leidy, 1856) infection in a dog in Hungary,** Helminthologia, 46. (3) 159-161., 2009.

Jacsó O., Fok É., Kiss G., Kökény G., Lang Zs.: **Preliminary findings on the efficacy of selamectin in the treatment of dogs naturally infected with *Dirofilaria repens*,** Acta Vet. Hung., 58. (4) 405-412., 2010.

Fok É., **Jacsó O.,** Szebeni Zs., Györffy A., Sükösd L., Lukács Z., Schaper, R.: **Long lasting elimination of *Dirofilaria repens* microfilariae in dogs with monthly treatments of moxidectin 2.5 % /imidacloprid 10% (Advocate®, Bayer) spot on.** Parasitol. Res., 106. 1141–1149., 2010.

Molnár V., Pazár P., Rigó D., Máthé D., Fok É., Glávits R., Vajdovich P., **Jacsó O.,** Balogh L., Sós E.: **Autochthonous *Dirofilaria immitis* infection in a ferret with aberrant larval migration in Europe,** J. Small Anim. Pract., 51. 393-396., 2010.