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Evaluation of the Therapeutic and Preventive Efficacy of 2.5% Moxidectin/10% Imidacloprid (Advocate®, Bayer Animal Health) in Dogs Naturally Infected or at Risk of Natural Infection by *Dirofilaria repens*

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Abstract

The objective of this GCP-compliant clinical field study was to evaluate the efficacy of the combination of moxidectin (minimum dose of 2.5 mg/kg body weight) and imidacloprid (minimum dose of 10.0 mg/kg body weight) spot-on (Advocate®) as a preventive and therapeutic treatment of natural infection by *Dirofilaria repens* in dogs in the Czech Republic.

There were two arms of the study, both negatively controlled. 34 animals were randomly allocated to two groups of the treatment arm; 90 negative animals were randomly allocated to the prevention arm groups. All enrolled dogs were observed physically and blood was sampled monthly for *Dirofilaria repens* microfilaria counts for 18 months by modified Knott test and PCR.

34 dogs were positive for microfilaria and enrolled in the treatment arm of this study (treated: 18, untreated: 16). The reduction of the log-transformed microfilaria counts was significantly higher in the treatment group on day 28 ($p = 0.007$), 56, 84 and 112 ($p < 0.001$). All animals treated were negative after a single treatment. In the untreated control group 93.75% remained positive ($p < 0.001$). 87 dogs were negative for microfilaria prior to allocation to the "preventive" arm (treated: 49; untreated: 38; 3 excluded). One dog in the untreated control group became positive for *Dirofilaria repens* microfilaria, while none of the treated dogs became positive.

Advocate® was effective in the treatment of dogs infected with microfilaria of *Dirofilaria repens*. Due to the low rate of natural infections the preventive efficacy could not be proven, but no dog treated became positive.

Introduction

The occurrence of cutaneous dirofilariosis (Figs. 1 and 2) or identification of microfilaria of *Dirofilaria repens* (Fig. 3) as a parasite associated with or without clinical signs has been reported more frequently in recent years throughout Europe. While the occurrence in southern Europe is well known (Marconcini et al. 1993; Genchi et al. 2002), reports of identifying the pathogen in various countries in eastern and Central Europe occurred only lately: Austria (Duscher et al. 2009), Czech Republic (Svobodova et al. 2006), Germany (Hermosilla et al. 2006, Kershaw et al. 2009, Sassnau et al. 2009), Hungary (Fok et al. 1998), Slovak Republic (Svobodova et al. 2005, 2007) and Ukraine (Vasylyk 2004). Trotz-Williams and Trees (2003) report in their systematic review that the prevalence of *Dirofilaria repens* ranges between 0 and 40% in dogs in Italy and Greece. It is not clear if this increase is due to the parasite and/or its vectors being able to adapt to the climate in such more northern areas or if it is purely due to the increased travel and associated risks of importing diseases or if it is just increased awareness of diseases associated with microfilariosis (Genchi et al. 2010). In any case, it is desirable to be able to recommend an appropriate prevention to dog owners to protect their animals from being infected, playing a potential role as carrier or transporter of the pathogen which also has a zoonotic potential. In addition, it would be desirable to have a treatment option available to eliminate an existing infection and thus reduce further spread of the disease in an endemic area or when moving the animals.

Imidacloprid 10% and moxidectin 2.5% spot-on (Advocate®, Bayer Animal Health) is indicated for monthly treatments for various parasites including *Dirofilaria immitis* in Europe. The efficacy in the treatment and prevention of *Dirofilaria repens* is relatively unknown, although various authors have reported the efficacy of macrocyclic lactones like ivermectin (Marconcini et al. 1993; McCall et al. 2005), doramectin (Baneth et al. 2002), selamectin (Genchi et al. 2002) and moxidectin (Rossi et al. 2002, 2004; Genchi et al. 2010). Fok et al. (2010) reported on a study indicating the therapeutic efficacy of the combination of imidacloprid 10%/moxidectin 2.5% spot-on formulation (Advocate®) to be present after 3-monthly treatments. Successful prophylactic use of moxidectin against *Dirofilaria repens* infection in dogs has been demonstrated in several investigations indicating an efficacy on development stages L3 and L4 of the parasite (Rossi et al. 2002, 2004). Moxidectin has long been known to be nematocidal and is licensed for the treatment of various adult nematodes. Based on its high bioavailability after spot-on treatment, it is likely that moxidectin has got adulticidal activity against *Dirofilaria repens*. The objective of this study was to prove the therapeutic and preventive efficacy of Advocate® in dogs infected or at risk of an infection with *Dirofilaria repens* by treating animals living in an endemic area in the Czech Republic.

Materials and methods

Therapeutic arm:

Animals enrolled in this arm of the study had to be positive in the modified Knott test for microfilaria prior to the first treatment. Advocate® spot-on for dogs was applied monthly for 4 consecutive months to the Investigational Veterinary Product (IVP) group (T01a) using the approved dosage instructions of a minimum dose of 2.5 mg moxidectin/kg body weight (b.w.) and 10.0 mg imidacloprid/kg b.w. A second group of animals (T03) served as negative control group, while appropriate exit criteria had been defined in case of clinical signs of dirofilariosis in order not to risk the health of the animals. For animals allocated to the treated group in the therapeutic arm, the study ended as soon as the result of the blood sample had become negative by therapeutic treatment (latest on day 112 ± 2). Afterwards, 16 of these dogs continued as patients in the prevention arm T01b.

Preventive arm:

In the second arm of the study, the ability of the combination product containing imidacloprid and moxidectin to prevent dogs from being infected with *Dirofilaria repens* was tested. Animals had been tested negative for the presence of microfilaria prior to enrolment. Animals of the investigational group T01b received monthly treatments with the IVP at the recommended minimum dose of 2.5 mg moxidectin/kg b.w. and 10.0 mg imidacloprid/kg b.w. for the duration of the study (18 months), while another group (T02) received no treatment. Appropriate exit criteria had been defined for animals being identified to be positive for microfilaria during the study assuring immediate treatment according to best practice. Any animal in the negative control group that proved positive for microfilaria within the first 4 months of the study was withdrawn from this arm and received appropriate rescue treatment. Based on the life cycle of the parasite, such animals had been infected prior to enrolment.

Design

This study was conducted after obtaining regulatory approval in the Czech Republic and following the guideline for Good Veterinary Practice (VICH GL9). Both arms of this study were negative controlled, randomised and blinded. Animals were enrolled from various households, but all investigations were done by the team of the Institute of Parasitology of the University of Veterinary and Pharmaceutical Sciences in Brno. The experimental unit was the animal.

Procedure

Prior to any observations and samplings, the informed owner consent was obtained. Between day –7 and day 0, dogs living in the target region of high prevalence of *Dirofilaria repens* were tested for the presence of microfilaria by observing blood samples with the modified Knott test.

Depending on the result and the household they were living in, they were allocated to treatment groups based on the following criteria:

a. the infection status for microfilaria of *Dirofilaria repens* of the individual dog and of each other dog to be enrolled and living in the same household,
b. dogs living in the same household had to be allocated to the same treatment group in order to avoid contamination of dogs in the untreated control group by the spot-on medication of the treated group.

Households with positive and negative dogs to be enrolled (mixed households) were randomised as follows:

- Negative dogs were allocated to the prevention arm of the study, treatment group T02.
- Positive dogs were allocated to the treatment arm of the study, treatment group T03, using the provided allocation list.
- When the target number of patients in treatment group T03 was enrolled, only positive dogs from mixed households were enrolled in the study, allocated to treatment group T01a using the provided random allocation list. The negative dogs of these households were not enrolled.

Households with either positive or negative dogs to be enrolled were randomised as follows:

- In case all dogs of one household were negative, they were allocated to the prevention arm of the study, either treatment group T02 or T01b using the provided random allocation list.
- In case all dogs of one household were positive, the animals were allocated to treatment group T01a using the provided allocation list.

The person doing the modified Knott test was blinded to treatment.

All dogs enrolled in the study were monthly observed for clinical signs and an EDTA blood sample was collected for microfilaria testing. The trial design is described in Tab. 1. The treatment regimen is detailed in Tab. 2. Treatments were administered according to the instructions of the product leaflet.

Tab. 1 Study design and allocation to treatment

Treatment code	Investigational veterinary product/control	Status of infection with microfilaria of *Dirofilaria repens*		Study arm	No. of dogs treated	Observation days
T01	Advocate®	T01a	Positive	Treatment arm	18	0*, 28 ± 2*, 56 ± 2*, 84 ± 2*, 112 ± 2*
	Advocate®	T01b	Negative at enrolment OR Negative after treatment (T01a) latest at day 84±2	Prevention arm	49**	0*, 28 ± 2*, 56 ± 2*, 84 ± 2*, 112 ± 2*, 140 ± 2, 168 ± 2, 196 ± 2, 224 ± 2, 252 ± 2, 280 ± 2, 308 ± 2, 336 ± 2, 364 ± 2, 392 ± 2, 420 ± 2, 448 ± 2, 476 ± 2
T02	N/A	Negative		Prevention arm	41 (38)***	0, 28 ± 2, 56 ± 2, 84 ± 2, 112 ± 2, 140 ± 2, 168 ± 2, 196 ± 2, 224 ± 2, 252 ± 2, 280 ± 2, 308 ± 2, 336 ± 2, 364 ± 2, 392 ± 2, 420 ± 2, 448 ± 2, 476 ± 2
T03	N/A	Positive		Treatment arm	16	0, 28 ± 2, 56 ± 2, 84 ± 2, 112 ± 2

* laboratory results from day 0 to 112 ± 2 were evaluated both for the treatment arm and the prevention arm of T01
** this number included the 16 animals coming from T01a
*** the per-protocol population used for the analysis of efficacy included 38 dogs, as 3 dogs had to be excluded due to identified microfilariae within the first 4 months of the study

Parasitological test

EDTA blood samples were observed using the modified Knott test after been submitted to the laboratory.

1. Take 1 ml of non-coagulated EDTA blood,
2. mix with 4–5 drops of saponin solution and pour into 1 ml purified water in a centrifuge tube,
3. centrifuge for 3–5 minutes at approximately 1,500 rpm,
4. stain the content of the tube with some drops of methylen blue,
5. examination of the whole compound under a microscope without using a cover slip. Counting of each single microfilaria in this 1 ml sample under a stereomicroscope,
6. record the number of microfilaria counted per ml (mf/ml).

Samples analysed by Knott test were additionally examined for *Dirofilaria* species using the multiplex-PCR (Rishniw et al. 2006) at the Department of Comparative Tropical Veterinary Medicine, LMU Munich, Germany.

Data handling and analysis

As planned in the protocol, statistical analysis was conducted at the end of the study for both arms of the study comparing each treatment group with the relevant control group for superiority using Fisher's exact test and Wilcoxon rank sum statistic after the data had been entered into a data base using double data entry technique and downloading the data into SAS version 8.2.

Results

Animals enrolled and general health

One hundred and eight different animals were enrolled in the study. Thirty-four animals were allocated to the treatment arm (18 in T01a, 16 in T03). Seventy-four dogs (33 in T01b, 41 in T02) were allocated on day 0 to the prevention arm. Sixteen animals completing the treatment arm in group T01a with a negative result for *Dirofilaria repens* continued the study in the prevention arm

Tab. 2 Treatment regimen

Body weight	Advocate® Size of pipettes	Volume of Advocate® in 1 pipette (ml)	Moxidectin (mg/kg)	Imidacloprid (mg/kg)
1.0–≤ 4.0 kg	Advocate® for small dogs ≤ 4 kg (S)	0.4	2.5–10.0	10–40
> 4.0–≤ 10.0 kg	Advocate® for medium dogs > 4–10 kg (M)	1.0	2.5–6.25	10–25
> 10.0–≤ 25.0 kg	Advocate® for large dogs > 10–25 kg (L)	2.5	2.5–6.25	10–25
> 25.0–≤ 40.0 kg	Advocate® for extra-large dogs > 25–40 kg (XL)	4.0	2.5–4.0	10–16

(T01b). Thus in total 90 (49 in T01b, 41 in T02) animals formed the intent-to-treat population in the prevention arm, but 3 were excluded from T02 to have totally 87 dogs as per protocol population.

The treatment groups were compared descriptively to assess their comparability. Baseline comparability of treatment groups was assessed by means of descriptive tables on the following baseline information of day 0: animal characteristics (sex, age) and physical examinations. Age ($p > 0.066$) and sex ($p > 0.397$) were not significantly different between treatment groups. None of the animals showed any clinical sign of dirofilariosis in either treatment group. In 96 % of the samples the infection status determined by Knott test was confirmed by multiplex-PCR.

Efficacy in the treatment of *Dirofilaria repens*

Efficacy in the treatment arm was evaluated based on the reduction of microfilaria counts of *Dirofilaria repens* comparing treatment groups T01a and T03 at any observation day starting from 28 ± 2 days post start of treatment on day 0 and the microfilaria counts observed at monthly intervals until day 112 ± 2. The mean counts on day 0 were 1711.4 mf/ml (± 3858.2) in T01a and 1345.9 mf/ml (± 2313.5) in T03. The difference was statistically not significant $p = 0.385$).

At each observation day, microfilaria count data were not normally distributed, right-skewed and peaked. Therefore, counts were log-transformed. After the log-transformation, the distribution became normal. The reductions of the log-transformed counts were significantly higher in T01a compared to T03 starting from day 28 ($p = 0.007$) to day 56, 84 and 112 ($p < 0.001$ at each point in time, Wilcoxon rank sum statistic). All results are displayed in Tab. 3.

The percentage of animals not showing microfilaria of *Dirofilaria repens* at days 28 to 112 was analysed using Fisher's exact test. The difference between groups was presented with 95 % confidence intervals (Tab. 3).

Form day 56 onwards, all animals in T01a were negative for *Dirofilaria repens*, whereas in group T03 93.75 % of the dogs were positive on all observations days. Taking into account that six animals were transferred from group T03 to T01a, receiving their first treatment on day 28 of the study, and being tested negative for *Dirofilaria repens* 28 days later, all animals treated were negative after a single treatment. Thus the evaluation of the secondary efficacy criterion supports superiority shown for the primary efficacy criterion ($p < 0.001$, Fisher's exact test) (Tab. 4).

Efficacy in the prevention of *Dirofilaria repens*

The preventive efficacy evaluation was based on the condition that the infection rate with *Dirofilaria repens* in untreated animals would be at least 40 %. As only one dog in the control group T03 became

Tab. 3 Absolute counts (microfilaria/ml blood) from day 0 to day 112 and reduction of the count of microfilaria of *Dirofilaria repens* from day 28 to day 112 based on log-transformed counts

Day	Parameter	Advocate® T01a (T1)	Untreated control T03 (T2)	Statistic T1 vs. T2 p =
Day 0	N	18	16	
	Arithmetic mean of absolute counts (SD)	1,711.4 (3,858.2)	1,345.9 (2,313.5)	
Day 28*	N	18	16	
	Arithmetic mean of absolute counts (SD)	166.9 (419.6)	1,176.9 (1,585.4)	
	Mean***	–3.4328	–0.4363	0.007**
	SE***	0.76991	0.37035	
Day 56	N	17	16	
	Arithmetic mean of absolute counts (SD)	0.0 (0.0)	771.5 (988.5)	
	Mean***	–4.9747	–0.7725	<.001**
	SE***	0.63574	0.40729	
Day 84	N	17	14	
	Arithmetic mean of absolute counts (SD)	0.0 (0.0)	1,085.1 (1,610.3)	
	Mean***	–4.9747	–0.3443	<.001**
	SE***	0.63574	0.35918	
Day 112	N	16	14	
	Arithmetic mean of absolute counts (SD)	0.0 (0.0)	794.1 (1115.6)	
	Mean***	–4.9113	–0.4343	<.001**
	SE***	0.67342	0.40354	

* animals in T01a positive for microfilaria on day 28 received their first treatment on day 28
** p values are based on the Wilcoxon rank sum statistic
*** based on log-transformed counts
SD: standard deviation
SE: standard error

positive on day 168 or later, the primary efficacy criterion, comparison of numbers of dogs which are microfilaria-negative at day 392, was only summarised descriptively. None of the dogs treated with Advocate® (T01b) became positive during the study period. One dog in T03 was positive for *Dirofilaria repens* microfilaria on day 392.

Safety of Advocate®

In total, there were eight adverse events reported (treated: 7, untreated: 1). One event was categorised as potentially related to treatment. This animal showed one day after the first treatment apathy, lack of appetite, tiredness and fever. No treatment was necessary for this condition. The investigator found the animal without abnormal finding on the following observation day and no abnormal condition was reported after further treatments with the same product.

Discussion

This study was conducted according to VICH GCP, which assures the accurateness, integrity and correctness of the observations. The study was controlled by a negative group, animals were randomised to treatment and the laboratory scientist conducting the microfilaria counts was blinded. Some animals, highly microfilaria-positive in the negative control group of the treatment arm, were allocated to the treated group in order to assure treatment and avoid any potential damage. This was justified based on animal welfare rationale and done based

Tab. 4 Therapeutic efficacy based on prevalence of microfilaria of *Dirofilaria repens* from day 28 until day 112

Variable	Statistic	T01a (T1)	T03 (T2)	p values comparing T1 vs. T2
Day 28*	N	18	16	
	% Positive	27.78**	93.75	<.001
	% Negative	72.22**	6.25	
Day 56*	N	17	16	
	% Positive	0.00	93.75	<.001
	% Negative	100.00	6.25	
Day 84*	N	17	14	
	% Positive	0.00	92.86	<.001
	% Negative	100.00	7.14	
Day 112*	N	16	14	
	% Positive	0.00	85.71	<.001
	% Negative	100.00	14.29	
Over all days*	N	17	16	
	% Positive	23.53	93.75	<.001
	% Negative	76.47	6.25	

* p values are based on the Fisher's exact test
** 5 (27.78 %) animals in T01a positive on day 28 received their first treatment on day 28

on the infection and did not influence the study results. Although randomisation was not completely followed, the allocation to treatment was based on objective criteria rather than by any external bias.

The results confirm the results of Fok et al. (2010) and Traversa et al. (2011): one treatment with the licensed dose of Advocate® is able to remove almost all microfilaria of *Dirofilaria repens* from infected dogs. Traversa et al. (2011) achieved elimination of microfilaria after just one treatment; however 2 of 17 dogs showed low microfilaria counts again after some months. Sasaki and Kitagawa (1993) reported that milbemycin D administered orally to dogs with microfilaraemia caused by *Dirofilaria immitis* led to the disappearance of circulating microfilariae after one to five treatments. In spite of this fact, the study made it clear that milbemycin D does not kill intrauterine microfilariae but inhibits the development of heartworm embryos. Moreover, its prophylactic use in microfilaraemic dogs will induce cases of occult infection. Fok et al. (2010) treated either monthly or every fortnight for 3 to 6 months dogs with microfilaria of *Dirofilaria repens* and achieved long-lasting clearance of microfilaria in blood in all dogs and assumed an adulticidal efficacy. In our study, all animals received monthly treatments for 4 months. Thus this study confirms that monthly treatment and a therapy over several months is sufficient to achieve complete and long-lasting elimination of microfilaria. Microfilaria counts on day 0 ranged from 2.0 to 15,550 mf/ml in T01a and from 3.0 to 8,939.0 mf/ml in T03. As the statistical analysis of nematode count reduction data has recently been discussed (Dobson et al. 2009), the data were first of all tested for skewedness. As it was confirmed that the treatment arm data were skewed, it was justified to apply a natural logarithmic transformation {ln (count + 1)} to the microfilaria count data prior to analysis. After log-transformation, the distribution became normal and the planned statistical tests were conducted.

To prove the preventive efficacy of a product is often difficult. Bowman et al. (1992) reported that preventive products containing ivermectin or milbemycin oxime licensed in the US for the treatment of dirofilariosis due to *Dirofilaria immitis* are not given at dosages designed to be completely

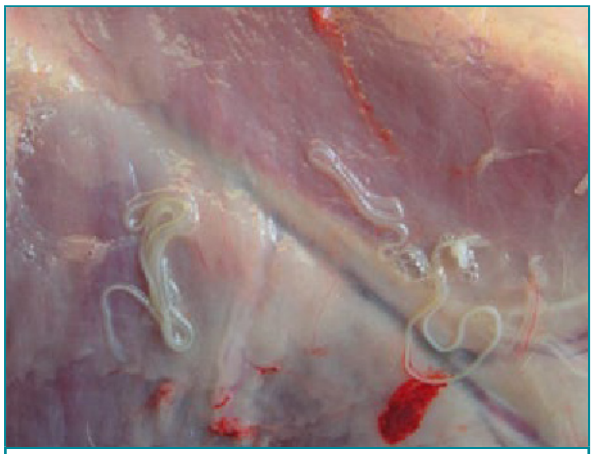

Fig. 1 Massive subcutaneous dirofilariosis in an autopsied dog naturally infected by *Dirofilaria repens*. The length of parasites varies from 7 to 12 cm

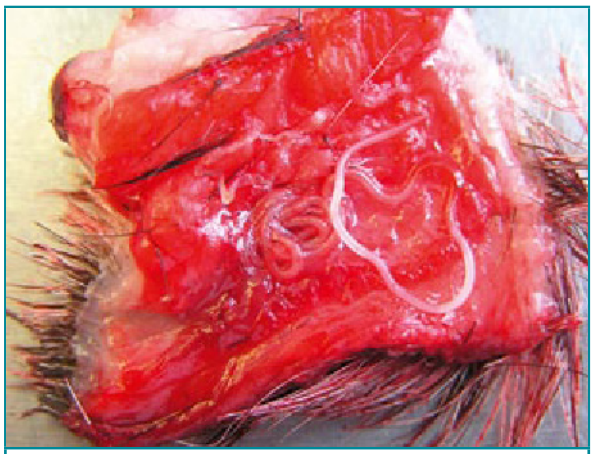

Fig. 2 Removing of adult *Dirofilaria repens* from a naturally infected dog by means of surgery

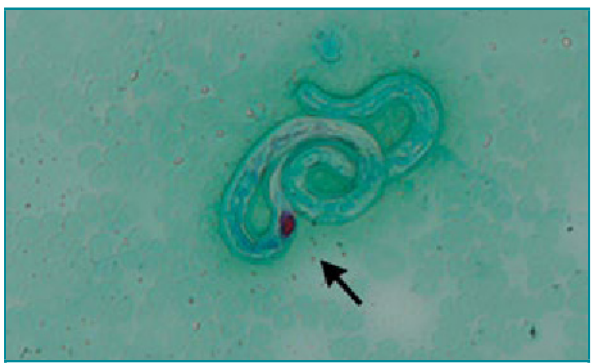

Fig. 3 Microfilaria of *D. repens* stained with acid phosphatase reaction and showing a purple spot (arrow) close to the posterior end which is a diagnostic criterium for differentiation between *D. immitis* and other microfilariae. Parasite length ~ 320 µm

microfilaricidal. Some 10 % to 20 % of dogs with patent infections that are placed on preventatives will continue to have circulating microfilariae for many months after beginning product administration. Arther et al. (2005) reported 100 % efficacy of 2.5 % moxidectin as well as of a combination of 10 % imidacloprid and 2.5 % moxidectin topical solution applied once on day 0 to dogs to prevent heartworm disease caused by *Dirofilaria immitis* artificially challenge with 50 third stage larvae on day –30 and –45 and necropsy 110–119 days post treatment.
In a field study, the success is mainly dependent on the natural challenge with the target pathogen. For *Dirofilaria repens* this is particularly due to the temperature-dependent activity of the vectors being more than 60 mosquito species (Svobodova et al. 2006). Although the area where dogs were living enrolled in this study was confirmed to harbour many positive dogs (Dobešová et al. 2009) – as confirmed by the number of patients enrolled in T01a and T03 – it appears that a low infection pressure was present throughout the study period of summer 2009 to autumn 2010. Due to the very low infection rate confirmed in the untreated dogs group, the preventive efficacy could not be proven. However, none of the animals in the preventive group treated monthly with the IVP became positive.

Conclusion

Advocate® was effective in the treatment of dogs infected with microfilaria of *Dirofilaria repens*. Due to the low rate of natural infections in dogs in the control group during the years 2009 and 2010, the preventive efficacy could not be proven, although no dog in the group treated with Advocate® for up to 18 months became infected.

Acknowledgements

The authors are sincerely grateful to the dog owners that permitted their animals to participate in this study after granting informed owner consent.

Compliance statement

This study was conducted in the Czech Republic with the express authorisation of the State Control Institute for the Control of Veterinary Medicinal Products following the current regulations of the Czech Republic and the EU.

Disclosure statement

This clinical study was completely funded by Bayer Animal Health GmbH, Monheim, Germany, thereof J. Heine is an employee. Klifovet AG is an independent Contract Development Organisation, which was contracted to manage the conduct of this study. K. Hellmann is the managing director, G. Braun an employee. V. Svobodova and R. Paran-Dobesova work at the Department of Pathological Morphology and Parasitology of the University of Veterinary and Pharmaceutical Sciences of Brno, Czech Republic. All authors voluntarily publish this article and do not have a personnel interest in this trial other than publishing the scientific findings they have been involved in by planning, setting-up, monitoring, conducting and analysing this study.

References

Arther RG, Bowman DD, Slone RL, Travis LE (2005) Imidacloprid plus moxidectin topical solution for the prevention of heartworm disease (*Dirofilaria immitis*) in dogs. Parasitol Res. 2005, Oct; Res 97(Suppl 1):76–80.

Baneth G, Volansky Z, Anug Y, Favia G, Bain O, Goldstein RE, Harrus S (2002) *Dirofilaria repens* infection in a dog: diagnosis and treatment with melarsomine and doramectin. Vet Parasitol 105:173–178.

Bowman DD, Johnson RC, Ulrich ME et al. (1992) Effects of long-term administration of ivermectin and milbemycin oxime on circulating microfilariae and parasite antigenemia in dogs with patent heartworm infections. In: Proceedings of the Heartworm Symposium, Austin, TX, 1992, pp 151–158.

Dobešová R, Svobodova V (2009) Progressive spread of *Dirofilaria* infections in dogs along rivers in the Southeastern Czech Republic. Second European *Dirofilaria* Days, Salamanca, Spain, p 190.

Dobson RJ, Sangster NC, Besier RB, Woodgate RG (2009) Geometric means provide a biased efficacy result when conducting faecal egg count reduction test (FECRT). Vet Parasitol 161:162–167.

Duscher G, Feiler A, Wille-Plazzai W, Bakonyi T, Leschnik M, Miterpáková M, Kolodziejek J, Nowotny N, Joachim A (2009) Nachweis von Dirofilarien in österreichischen Hunden. Berl Münch Tierärztl Wochenschr 122:199–203.

EMEA/CVMP/816/00-Final: Guideline on statistical principles for veterinary clinical trials.

Fok É, Szabo Z, Farkas R (1998) The first autochthonous case of a dog infected with *Dirofilaria repens* in hungary. Kisal Latorvoslás 4:218–219.

Fok É, Jascó O, Szebeni Z, Györffy A, Südkösd l, Lukács Z, Schaper R (2010) Elimination of *Dirofilaria* (syn. *Nochtiella*) *repens* microfilariae in dogs with monthly treatments of moxidectin 2.5%/imidacloprid 10% (Advocate®, Bayer) spot-on. Parasitol Res 106(5):1141–1149.

Genchi C, Poglayen G, Kramer L, Casiraghi M, Venco L, Brianti E (2002) Efficacy of selamectin in the prophylaxis of *Dirofilaria repens* infestations in dogs. Veterinaria 16(1):69–71.

Genchi M, Pengo G, Genchi C (2010) Efficacy of moxidectin microsphere sustained release formulation for the prevention of subcutaneous filarial (*Dirofilaria repens*) infection in dogs. Vet Parasitol 170:167–169.

Hermosilla C, Pantchev N, Dyachenko V, Gutmann M, Bauer C (2006) First autochthonus case of canine ocular *Dirofilaria repens* in Germany. Vet Rec 158:134–135.

Kershaw O, Dyachenko V, Gruber AD (2009) Kutane Dirofilariose beim Hund in Deutschland. Dtsch Tierärztl Wochenschr 116(7):273.

Marconcini A, Magi M, Hecht Contin B (1993) The value of ivermectin in the prophylaxis of *Dirofilaria repens* infestation in naturally infected dogs. Parassitologia 35:67–71.

McCall J (2005) The safety-net story about macrocyclic lactone heartworm preventives: a review, an update, and recommendations. Vet Parasitol 133:197–206.

Rishniw M, Barr SC, Simpson KW, Frongillo MF, Franz M, Domingues-Alpizar JL (2006) Discrimination between six species of canine microfilaria by a single polymerase chain reaction. Vet Parasitol 135:303–313.

Rossi L, Ferroglio E, Agostini A (2002) Use of moxidectin tablets in the control of canine subcutaneous dirofilariosis. Vet Rec 150:383.

Rossi L, Ferroglio E, Agostini A (2004) Use of an injectable sustained-release formulation of moxidectin to prevent canine subcutaneous dirofilariosis. Vet Rec 154:26–27.

Sassnau R, Dyachenko V, Pantchev N, Stöckel F, Dittmar K, Daugschies A (2009) *Dirofilari repens* Befall in einem Schlittenhunde-Rudel im Land Brandenburg. Tierärztl Prax Ausg K Kleintiere Heimtiere 37: 95–101.

Sasaki Y and Kitagawa, H (1993) Effects of milbemycin D on microfilarial number and reproduction of *Dirofilaria immitis* in dogs. J Vet Med Sci 55:763–769

Svobodova V, Svobodova Z, Beladicova V, Valentova D (2005) First cases of canine dirofilariosis in Slovakia, a case report. Vet Med Czech 50(11):510–512.

Svobodova Z, Svobodova V, Genchi C, Forejtek P (2006) The first report of authochthonous dirofilariosis in dogs in the Czech Republic. Helminthologica 43(4):242–245.

Svobodova Z, Svobodova V, Genchi C (2007) *Dirofilaria repens* infection in dogs in the Czech Republic. In: Genchi C, Rinaldi L, Cringoli G (eds), Mappe Parasitologie 8 – *Dirofilaria immitis* and *Dirofilaria repens* in dog and cat and human infection. Rolando Editore, Neapel, Italy, pp 199–200.

Traversa D, Aste G, Di Cesare A, Paoletti B, Di Tommaso M, Di Giulio M, Pampurini F, Tunesi C, Boar A (2011) Efficacy of a single administration of a spot-on solution containing imidacloprid 10 %/moxidectin 2.5 % in eliminating *Dirofilaria repens* microfilariae in naturally infected dogs. Vet Parasitol. doi:10.1016/j.vetpar.2011.02.018.

Trotz-Williams LA, Trees AJ (2003) Systematic review of the distribution of the major vector-borne parasitic infections in dogs and cats in Europe. Vet Rec 152:97–105.

Vasylyk NS (2004) Morhological and functional changes and adaptations and compensatory reactions in dog's organism at dirofilariosis. Dissertation, National Agrarian University, Kiew, pp 1–22.

VICH guideline 7: Efficacy requirements for anthelmintics, overall requirements (Step 7, CVMP/VICH/832/99-corr) 1999.

VICH guideline 9: Good clinical practice (CVMP/VICH/595/98 – Final) 2000.

VICH guideline 19: Efficacy of anthelmintics: specific recommendations for canines (Step 7, CVMP/VICH/835/99 – Final) 1999.

WAAVP (World Association for the Advancement of Veterinary Parasitology): Guidelines for evaluating the efficacy of anthelmintics for dogs and cats (Accepted August 31, 1993).