

# Evaluation of the Adulticidal Efficacy of Imidacloprid 10%/Moxidectin 2.5% (w/v) Spot-on (Advocate<sup>®</sup>, Advantage<sup>®</sup> Multi) against *Dirofilaria repens* in Experimentally Infected Dogs

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## Abstract

This study aimed to evaluate the efficacy of imidacloprid 10%/moxidectin 2.5% (w/v) spot-on (Advocate<sup>®</sup>/Advantage<sup>®</sup> Multi, Bayer) against adult *Dirofilaria repens* in a blinded, placebo-controlled randomised laboratory study. Twenty-four Beagle dogs were experimentally infected with approximately 75 infective *D. repens* larvae each on study day (SD) 0. Treatment was initiated on SD 228 after patency had been confirmed in 21 dogs, using a modified Knott Test. Eleven dogs received monthly treatments with imidacloprid/moxidectin at the minimum therapeutic dose (10 mg/kg imidacloprid and 2.5 mg/kg moxidectin) for six consecutive months and 12 control dogs were treated with a placebo formulation.

Approximately one month after the last treatment, all dogs were euthanised and necropsied for the detection of *D. repens* worms. Eleven control dogs harboured live adult *D. repens* (range 2–11, geometric mean 5.44). Eight of 11 imidacloprid/moxidectin-treated dogs were free of live worms. The live worm count was reduced by 96.2% (range 0–1, geometric mean 0.21). The majority of dead worms were encapsulated and degenerated. After the first treatment, Knott Tests were negative in all imidacloprid/moxidectin-treated dogs and this status was maintained in 10 dogs until study end. One dog showed a low microfilariae count (1 and 4/mL) on four occasions but was also negative before necropsy. The treatment was well tolerated by all study animals. It is concluded that six consecutive

monthly treatments with imidacloprid/moxidectin spot-on are effective and safe against adult *D. repens* and provide an option for preventing the further spread of this zoonotic parasite.

## Introduction

In recent years human dirofilariosis has been considered an emerging zoonosis in Europe (Genchi et al. 2011). The majority of human cases are attributed to *Dirofilaria repens*, a mosquito transmitted filarial nematode, which causes subcutaneous dirofilariosis in dogs, cats and wild canids (Simón et al. 2012). It has been shown not only that infection rates are increasing in well-known endemic areas in southern Europe, but also that the disease is spreading towards northern and eastern areas formerly free of the infection (Genchi et al. 2011, Tasić-Otašević et al. 2015). Recently, Tappe et al. (2014) described the first autochthonous human case in Germany, after a series of cases in dogs in previous years had indicated the occurrence of *D. repens* there (Hermosilla et al. 2006, Pantchev et al. 2009, Sassnau et al. 2009). Since the parasite has also been repeatedly detected in a local mosquito population in Germany, a stable local transmission is now assumed (Czajka et al. 2014; Kronefeld et al. 2014).

Adult *D. repens* live and mate mainly in the subcutaneous tissue and perimuscular fasciae and female worms release their offspring, the microfilariae (MF), into the blood stream. After being taken up by a blood feeding mosquito, these microfilariae develop into infective third stage larvae (L3) and are then transmitted to the next host during a subsequent blood meal (Webber and Hawking 1955). Dogs represent the main reservoir, and thus the only effective way to protect humans from the disease is to control the infection in dogs (Genchi et al. 2010). Difficulties in putting this into practice arise from the fact that infected dogs are often asymptomatic and

hence remain undiagnosed, or symptoms that may occur, such as pruritus, dermal swelling, hyperpigmentation or subcutaneous nodules are misinterpreted. In addition, options to treat dogs against *D. repens* are limited since no adulticidal drug is available.

Macrocyclic lactones, which are labelled to prevent heartworm disease and are used in higher doses to clear dogs of the microfilariae of *Dirofilaria immitis*, showed variable results against *D. repens*. In field studies, ivermectin at the recommended dose for heartworm prevention effectively prevented patent infections with *D. repens* when administered within 30 days after the start of the transmission season (Marconcini et al. 1993; Pollono et al. 1998). In some European countries ivermectin is registered for this indication. However, in studies with dogs experimentally infected four times at intervals of 15 days and treated 30 and 60 days after the first infection, efficacy of ivermectin was incomplete. Four times the recommended dose for heartworm prevention reduced worm burdens by 87.9% (Cancrini et al. 1989). For treatment against microfilariae monthly doses of 6 µg/kg ivermectin were not as effective against *D. repens* as they were against *D. immitis* (Venco et al. 2004). Sassnau et al. (2009) successfully treated microfilaraemic dogs with weekly administrations of 50 µg/kg ivermectin in combination with daily doxycycline for a period of 6 weeks. Similar results were obtained by Gianelli et al. (2013) with doxycycline for 30 days and low dose ivermectin (6 µg/kg) twice a month for 6 months. Treatment with doxycycline has been shown to weaken or even kill filarial nematodes by killing their endosymbionts (*Wolbachia*). Although it is not yet clear whether there is true synergism or just an additive effect, the combination of doxycycline/ivermectin has a much higher efficacy than ivermectin alone against *D. immitis* (Kramer and Genchi 2014).

The efficacy of selamectin at the recommended dose for heartworm prevention has been de-

monstrated in a field study (Genchi et al. 2002). While 41% of control dogs became infected, all treated dogs were negative following the transmission season. The microfilaricidal effect has been evaluated by Jacsó et al. (2010) treating naturally infected dogs monthly or twice a month for a period of 6 or 9 months. Overall, 65% of the dogs were cleared of microfilariae. A single case describes the elimination of microfilariae by application of 0.4 mg/kg doramectin 5 days after treatment with melarsomine (Baneth et al. 2002).

The ability of oral milbemycin oxime to prevent infections with *D. repens* was tested in one field study. None of the dogs treated monthly with the recommended dose became microfilaraemic, while 6 of 114 control dogs became infected. All positive dogs were successfully treated with milbemycin oxime, but the exact treatment protocol was not described (Di Cesare et al. 2014). Oral and injectable formulations of moxidectin have shown preventive efficacy against *D. repens* in field studies (Rossi et al. 2002, 2004), and the efficacy of an injectable sustained-release formulation was confirmed in experimentally infected dogs (Genchi et al. 2010). In some European countries the formulation is licensed for the prevention of skin lesions and dermatitis caused by *D. repens*. The preventive efficacy of moxidectin in a spot-on formulation of imidacloprid/moxidectin (Advocate<sup>®</sup>/Advantage<sup>®</sup> multi, Bayer) has been demonstrated in a field trial (Traversa et al. 2013) and was confirmed in a laboratory study with dogs receiving a challenge infection 4 weeks after a single treatment at the minimum therapeutic dose. No worms were detected at necropsy in any of the treated dogs (Genchi et al. 2013). The potential of the spot-on formulation to eliminate microfilariae has been investigated by various authors. In a study by Traversa et al. (2011) a single application cleared microfilariae in all 18 treated dogs. 16 dogs stayed negative throughout the observation period of 5 to 6 months after treatment.

Two dogs had recurring microfilaraemia 2 and 3 months after treatment respectively. In another field study all 18 treated dogs became a microfilaraemic after the first treatment and stayed negative throughout an observation period of 3 to 4 months after treatment (Hellmann et al. 2011). Fok et al. (2010) evaluated different treatment protocols comprising multiple applications, *i.e.*, once a month for 3 or 6 months or every 2 weeks for 6 months. Thirty-eight out of 44 treated dogs were free of microfilariae 2 weeks after the initial treatment, and after one month only one dog still showed a low microfilaraemia. After the second treatment, all dogs were negative and no recurrence of microfilaraemia was detected throughout an observation period of 6 months after the last treatment. Similar results were obtained by Paran et al. (2011) with 4 consecutive monthly treatments. All 11 dogs were negative for microfilariae of *D. repens* after the first treatment and, again, this status persisted for 6 months after the last treatment. In the European Union imidacloprid/moxidectin spot-on is registered for the prevention of skin dirofilariosis and for the reduction of *D. repens* microfilariae.

Currently no marketed product with an adulticidal claim against *D. repens* exists. Surgical removal of adult worms may be performed when the worms are located in nodules under the skin but not when they reside in deep tissues or even in visceral cavities. In addition, it can be difficult to locate the worms under the skin as they tend to alter their position. The only licensed active against adult heartworms is melarsomine, an arsenic drug which needs multiple intramuscular administrations for adequate efficacy. While some case reports describe the use of melarsomine against skinworms (Baneth 2002), the efficacy of the drug against *D. repens* has not been confirmed in clinical studies (Genchi, personal communication).

Studies that evaluated the microfilaricidal properties of imidacloprid/moxidectin spot-on showed long-term suppression of microfilaraemia,

suggesting that the treatment might also have a killing effect on adult stages of *D. repens* (Fok et al. 2010). The present study was therefore designed to test this hypothesis under controlled laboratory conditions.

## Materials and methods

The study was performed as a monocentric, placebo-controlled, randomised and blinded efficacy study. Investigations were conducted in accordance with VICH Guideline 9 “Good Clinical Practice” (July 2001) and the recommendations given in VICH Guideline 7 “Efficacy of anthelmintics: general requirements” (December 2000), VICH Guideline 19 “Efficacy of anthelmintics: Specific Recommendations for Canine” (July 2002) and the WAAVP guidelines for evaluating the efficacy of anthelmintics for dogs and cats (Jacobs et al. 1994) were followed. Since these guidelines do not include any specific recommendations for *D. repens*, the infection protocol was based on scientific knowledge and experience from previous experimental studies. The reliability of the infection protocol had been confirmed in studies on the prevention of experimental *D. repens* infection in dogs (Genchi et al. 2010, Genchi et al. 2013).

### Study animals

Twenty-four purpose-bred Beagle dogs (12 male/12 female) were included in the study. At study start the dogs were 10 months old and weighed between 7 and 12.2 kg. Twenty-three dogs were finally evaluated. They had been vaccinated according to their age against all major canine infectious diseases. Revaccination was not conducted during the study to avoid interference with the treatment. Treatment with drugs that could influence the study results, especially macrocyclic lactones, was not allowed for a period of at least three months before study start. All dogs were acclimatised for at least nine days before infection. They were housed in indoor

kennels in social groups of three to six animals of the same sex. After the first treatment, dogs of the same treatment group were housed together except for a period of two days after each treatment when all dogs were housed singly to avoid cross-contamination between dogs. The dogs were fed a standard commercial dog food and water was available *ad libitum*. They received toys and chewing material for environmental enrichment, had daily access to an outdoor exercise area and human interaction periods at least once a day. Before onset of patency, protective measures (nylon fabric) were taken to avoid the migration of mosquitoes into the animal maintenance area. Husbandry complied with the “Commission recommendation on guidelines for the accommodation and care of animals used for experimental and other scientific purposes” (European Commission, 2007/526/EC, 18 June 2007).

### Health observations

General health observations were conducted daily and the dogs were physically examined at the start of the acclimatisation period, before infection and prior to the first treatment. Only healthy dogs were included in the study. On treatment days, clinical assessments were performed before treatment and approximately 1, 4 and 24 hours after treatment for the detection of adverse effects. Body weights were determined on all treatment days for dose calculation and at least every 4 weeks throughout the study period.

### Experimental infections

On study day (SD) 0 each dog was injected subcutaneously with approximately 75 infective *D. repens* larvae (L3). Preparation of infection material was performed as previously described (Genchi et al. 2010). In brief, 4-day-old *Aedes aegypti* mosquitoes were fed on blood collected from a naturally *D. repens*-infected dog in Italy. The heparinised blood was maintained at 37 °C in a feeding apparatus and the mosquitoes were allowed to feed for

60 minutes. After 28 days, shortly before the experimental infection, infective third stage larvae were collected and maintained in RPMI-medium. For each infection dose approximately 75 larvae were manually collected using a glass pipette and transferred to tubes containing 1.5 mL RPMI-medium.

### Allocation and treatment

On SD 227, the dogs were randomly allocated to two study groups based on sex and their highest microfilariae count of 4 Knott tests performed between SD 164 and 220. After randomisation each group consisted of 12 dogs with equal sex distribution. Dogs in the treatment group were dosed with the recommended minimum therapeutic dose of imidacloprid (10 mg/kg) and moxidectin (2.5 mg/kg), corresponding to 0.1 mL spot-on formulation per kg body weight. In the control group a placebo spot-on formulation was used at the same amount of 0.1 mL spot-on formulation per kg body weight to mimic the appearance of the moxidectin/imidacloprid spot-on. Six consecutive treatments were performed at 4-weeks intervals (SD 228, 256, 284, 312, 340 and 368).

Three dogs, that were still microfilariae-negative on SD 227, were also included in the treatment phase and randomly allocated to the study groups. However, irrespective of the study group the dogs were allocated to, all microfilariae-negative dogs were treated with placebo, *i.e.* there were two negative dogs in the placebo group and one in the imidacloprid/moxidectin group.

### Blood sampling

A modified Knott test was used for the detection of circulating MF of *D. repens*. Blood samples were taken from all 24 dogs in the late afternoon (4:00 p.m. to 6:00 p.m). For study inclusion the dogs had to be negative for *D. repens*. The onset of patency was monitored on SD 164, 178, 192 and 220 post infection. After the first treatment, the course of microfilaraemia was followed monthly until study end (SD 256, 283, 311, 339, 367 and 395). Microfilaraemia was quantified by screening the whole

sediment or, if the density was high, by counting a representative sample. Species confirmation was done by PCR (Polymerase Chain Reaction) analysis. Three primer pairs were used: pan-filaria primers (Rishniw et al. 2006) that detect different *Dirofilaria* species (*D. repens*, *D. immitis*, *D. reconditum*), different *Brugia* species (*B. pahangi*, *B. malayi*, *B. timori*) as well as *Acanthocheilonema dracunculoides* and *Onchocerca volvulus*, and primer pairs specific for *D. repens* and *D. immitis* (Favia et al. 2000; Hermosilla et al. 2006). In the case of a weak positive PCR result with pan-filaria primers and a negative result with *D. repens* species-specific primers, a sequence analysis was performed to verify the species.

### Necropsy

Thirty-five to 36 days after the last treatment (SD 403/404), the dogs were euthanised and subsequently necropsied. The dogs were completely skinned and the subcutaneous tissue and fat, muscular fasciae and scrotum were carefully visually inspected for the presence of adult and pre-adult *D. repens* worms and any nodules suspected of containing worms or worm fragments. Thereafter, the abdominal and thoracic cavities were opened and also examined for worms. Skin and carcasses were then immersed in warm water (approximately 38 °C) for 20 minutes. After that, the skin was re-examined for the presence of parasites. The water from the basins was passed through sieves and the retained material was also examined for the presence of *D. repens*. Worms were collected in Petri dishes containing warm isotonic saline solution, identified for developmental stage and sex and counted. The mobility of the worms was checked in order to distinguish between live (mobile) and dead (immobile) worms. In addition, the worms and nodules were investigated under a stereomicroscope for any other characteristics and specific findings such as signs of degeneration.

### Efficacy determination and statistical analysis

The adequacy of infection was determined according to VICH Guidelines 7 and 19. These require a

minimum of 6 animals in the control group with at least 5 worms each.

The efficacy was based on live worm counts in the study groups and calculated according to VICH Guideline 7 and the WAAVP guideline for evaluating the efficacy of anthelmintics for dogs and cats (Jacobs et al. 1994) as follows:

<b>% Effectiveness (reduction) = (N1-N2)/N1 x 100</b>
N1: geometric mean (GM) of live <i>D. repens</i> counts in the control group
N2: geometric mean (GM) of live <i>D. repens</i> counts in the treated group

Due to the presence of worm count values of “0”, all counts were modified by adding 1 prior to log transformation and subtracting 1 from the antilog value. The non-parametric Wilcoxon rank sum test (two-tailed,  $\alpha = 0.05$ ) was used to test for a treatment group (imidacloprid/moxidectin vs. placebo) effect. Live, dead and the total worm counts as well as microfilariae counts on all testing dates were evaluated. The analyses were performed using SAS software 8.2 (SAS Institute, Cary, North Carolina, USA, 2001).

## Results

### Knott Test and PCR

On SD 164 eleven of 24 dogs were microfilaraemic. This number increased to 21 positive dogs on SD 220 and treatment was therefore initiated on SD 228. Two negative dogs were allocated to the control group. The Knott Test in these two dogs was positive on SD 256, and at necropsy they were shown to be adequately infected. Consequently, these dogs were included in the efficacy calculations. The third negative dog was allocated to the treatment group to give an equal number of dogs in the study groups. However, this dog was treated with the placebo solution and was therefore excluded from the efficacy calculation. PCR analysis of the 21 positive samples on SD 220 identified

*D. repens* DNA in 19 samples. In two samples with a weak positive PCR result with the pan-filaria primers and a negative result with the *D. repens* species specific primers, the species was confirmed by sequence analysis. In two samples (dog nos. 101 and 121) microfilaraemia on SD 220 was too low to detect DNA.

After randomization there were no statistically significant differences in the geometric mean values of the pre-treatment microfilariae counts between the study groups. Four weeks after the first treatment, all dogs treated with imidacloprid/moxidectin spot-on were amicrofilaraemic and this status was maintained until study end, except for one dog that showed low microfilariae counts (*i.e.* 4 and 1 MF/mL) between SD 283 and 367. In the control group microfilariae counts were positive in 9 of 12 dogs from SD 256 to 339. Two dogs with low microfilaraemia (ID 105 and 109) were negative on the last two evaluation dates. One dog (ID 121) with a positive Knott Test between SD 178 and 220 was negative for the rest of the evaluation period. On all post-treatment evaluation dates there was a statistically significant difference between the treated and the control group (Table 1).

### Efficacy evaluation

Due to exclusion of one dog, 12 dogs were evaluated in the control group and 11 in the treatment group. Nine of 12 control dogs harboured 5 or more live worms at necropsy (Fig. 1). Hence, the requirements for the adequacy of infection with *D. repens* were fulfilled. With the exception of one dog (ID 105), live *D. repens* were isolated from all control animals (range 2–11). Eight of 11 dogs treated with imidacloprid/moxidectin spot-on were free of live worms at necropsy. In the remaining three, one live female *D. repens* was found in each dog. Based on geometric means of live worm counts, the efficacy was 96.2% ( $p < 0.0001$ ).

The number of dead worms ranged between 0 and 4 (geometric mean 1.41) in the control group and between 2 and 16 (geometric mean 5.94) in the

Table 1 Pre and post treatment individual microfilariae counts in the study groups

Group	Dog ID	SD 164	SD 178	SD 192	SD 220	SD 256	SD 283	SD 311	SD 339	SD 367	SD 395
Advocate®	101	0	2	6	2	0	0	0	0	0	0
	102	16	122	370	340	0	0	0	0	0	0
	103	4	33	660	2810	0	0	0	0	0	0
	106	0	0	4	35	0	0	0	0	0	0
	110	2	30	30	32	0	0	0	0	0	0
	112	2	16	45	83	0	0	0	0	0	0
	115	0	23	500	440	0	0	0	0	0	0
	117	0	89	910	4170	0	0	0	0	0	0
	119	0	0	310	420	0	0	0	0	0	0
	122	107	510	3710	6100	0	0	0	0	0	0
123	5	99	810	1320	0	4	1	1	1	0	
<b>Geometric mean</b>		<b>2.29</b>	<b>21.45</b>	<b>175.66</b>	<b>299.30</b>	<b>0.00</b>	<b>0.16</b>	<b>0.07</b>	<b>0.07</b>	<b>0.07</b>	<b>0.00</b>
Placebo	104	2	6	25	62	229	1540	1080	610	1400	140
	105	0	0	7	10	3	3	7	1	0	0
	107	0	11	66	123	43	544	1150	250	740	770
	108	9	117	630	2420	2370	4930	4800	1000	2800	1500
	109	0	0	0	0	4	54	40	1	0	0
	113	6	31	2630	2700	271	6040	2100	2050	3100	5200
	114	0	4	3	76	18	507	960	520	60	580
	116	9	195	800	910	55	740	570	600	110	65
	118	0	23	310	1100	279	3450	2100	700	560	490
	120	2	126	1620	3020	145	9050	9600	2100	1500	270
	121	0	34	350	2	0	0	0	0	0	0
124	0	0	0	0	6	71	354	1540	450	5880	
<b>Geometric mean</b>		<b>1.07</b>	<b>12.39</b>	<b>68.69</b>	<b>91.52</b>	<b>41.98</b>	<b>358.50</b>	<b>397.78</b>	<b>175.19</b>	<b>128.95</b>	<b>129.97</b>
<b>p-value</b>		<b>0.5468</b>	<b>0.6600</b>	<b>0.4219</b>	<b>0.3551</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>0.0006</b>	<b>0.0003</b>

treated group. There was a statistically significant difference between the study groups, with the treated dogs harbouring significantly more dead worms ( $p = 0.0013$ ). The majority of dead worms were either encapsulated, degenerated or both (Fig. 2 and 3). Histopathology of subcutaneous nodules revealed signs of inflammation and a capsule/demarcation containing structures which were likely of parasitic

origin or identified as worms. To some extent mineralisation was present (Fig. 4 and 5). The total number of worms (live + dead) ranged between 2 and 14 (geometric mean 7.72) in the control group and between 2 and 16 (geometric mean 6.25) in the treated group. There was no statistically significant difference in the total worm counts between the study groups ( $p = 0.5127$ ) (Table 2).



**Fig. 1** Live adult *D. repens* in the abdominal cavity on the serosa



**Fig. 2** Dead adult *D. repens* in capsule on the perimuscular fascia following skin removal. Right half of the capsule was cut open.

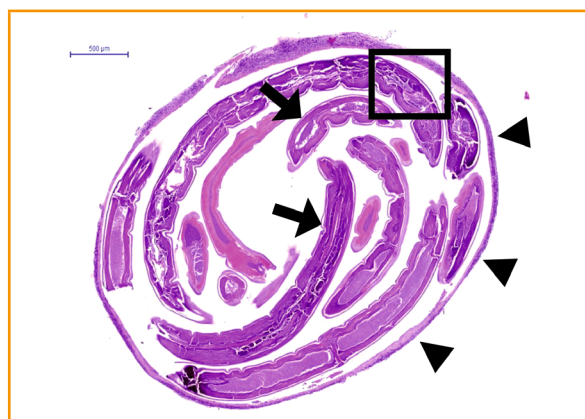


**Fig. 3** Remnant of dead adult *D. repens* on the perimuscular fascia following skin removal

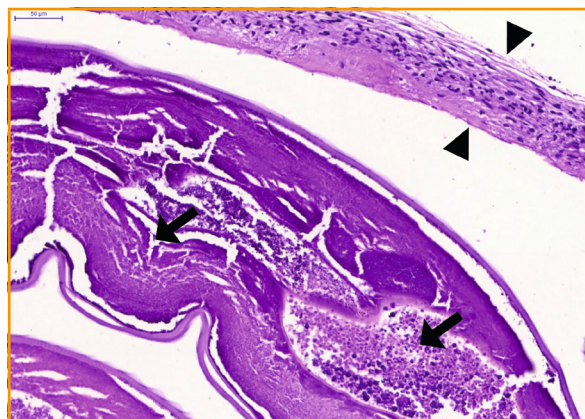
The majority of worms were found in the subcutaneous tissue. Five control animals harboured a total of 8 live and 1 dead worm in visceral cavities. Six worms were found in the abdominal cavity and 3 in the thoracic cavity. One dead worm was found in the abdominal cavity of a treated dog.

**Health observations**

Treatment with imidacloprid/moxidectin spot-on was well tolerated in all dogs. No clinical signs of



**Fig. 4** HE colored micrograph of a dead adult *D. repens* in a capsule. Arrowheads show the thin slightly inflamed fibrous capsule build around the worm by the dog. Arrows show cuts of the worm. Frame shows area of Fig. 5 (scale bar 500 µm)



**Fig. 5** HE colored micrograph of a dead *D. repens* in a capsule (Enlargement of Fig. 4). Arrowheads show the slightly inflamed fibrous capsule. Arrows show degenerating inner structures of the worm with beginning mineralization (scale bar 50 µm)



Table 2 Individual *D. repens* counts at necropsy

Dog ID	Live <i>D. repens</i>					Dead <i>D. repens</i>					Live + dead	
	male	female	n.d.	sum	geo. mean/ Efficacy	male	female	n.d.	sum	geo. mean	sum	geo. mean
<b>Imidacloprid/moxidectin</b>												
101	0	0	0	0	0.21/ 96.2 %	0	0	8 <sup>a</sup> /8 <sup>c</sup>	16	5.94	16	6.25
102	0	0	0	0		6 <sup>a</sup>	7 <sup>a</sup>	0	13		13	
103	0	1	0	1		0	0	3 <sup>c</sup>	3		4	
106	0	0	0	0		0	2 <sup>a</sup>	0	2		2	
110	0	0	0	0		0	0	1 <sup>a</sup> /8 <sup>c</sup>	9		9	
112	0	0	0	0		0	0	1/2 <sup>a</sup> /1 <sup>c</sup>	4		4	
115	0	0	0	0		0	0	1 <sup>a</sup> /3 <sup>c</sup>	4		4	
117	0	0	0	0		0	0	1 <sup>a</sup> /1 <sup>c</sup>	2		2	
119	0	1	0	1		0	0	3 <sup>a</sup> /2 <sup>c</sup>	5		6	
122	0	1	0	1		0	0	1/3 <sup>a</sup> /1 <sup>b</sup> /4 <sup>c</sup>	9		10	
123	0	0	0	0		0	0	2/9 <sup>a</sup> /1 <sup>b</sup> /1 <sup>c</sup>	13		13	
<b>Placebo</b>												
104	4	6	0	10	5.44	0	0	1 <sup>c</sup>	1	1.41	11	7.72
105	0	0	0	0		0	2 <sup>a</sup>	0	2		2	
107	3	3	0	6		0	0	3 <sup>c</sup>	3		9	
108	2	9	0	11		0	0	0	0		11	
109	3	5	2	10		0	0	1/3 <sup>c</sup>	4		14	
113	2	4	0	6		0	0	0	0		6	
114	1	1	0	2		0	0	3 <sup>c</sup>	3		5	
116	2	3	0	5		0	4 <sup>a</sup>	0	4		9	
118	2	1	1	4		0	0	1 <sup>c</sup>	1		5	
120	0	10	0	10		0	0	0	0		10	
121	3	5	0	8		0	1 <sup>a</sup>	2 <sup>a</sup>	3		11	
124	2	5	0	7	0	0	1 <sup>c</sup>	1	8			

n.d. = sex not determinable; <sup>a</sup> encapsulated; <sup>b</sup> degenerated; <sup>c</sup> encapsulated+degenerated

intolerance were observed. The whole study population stayed clinically healthy throughout the study period. Minor findings like scratch or bite wounds, salivation and vomiting were observed in individual animals on single occasions. Soft to firm nodules or partly thickened skin on at least one occasion were observed in 5 treated dogs and in 2 control animals. The first observations of these signs were made on SD 164. After treatment the incidence of these findings increased slightly in the imidacloprid/moxidectin treated group. At study

end these signs were present in one control animal but in none of the treated dogs.

## Discussion

This study demonstrated the adulticidal efficacy of six consecutive monthly treatments with imidacloprid/moxidectin spot-on (Advocate<sup>®</sup>/Advantage multi<sup>®</sup>, Bayer) against *D. repens* in experimentally infected dogs. The adequate infection of the control

group proved the reliability of the infection model and the infection rate was within ranges formerly experienced with this infection dose (Genchi et al. 2010; Genchi et al. 2013.) The onset of patency was earlier than expected. According to the literature the prepatent period of *D. repens* is 27–34 weeks (Manfredi et al. 2007). Webber and Hawking (1955) identified the first positive blood samples at 25 weeks post infection. In the actual study, however, 11 dogs were already positive at 23 weeks post infection.

Due to missing data in the literature it was not clear what picture could be expected at necropsy and how adult *D. repens* killed by an anthelmintic would present. It became obvious that most dead worms were encapsulated or degenerated or both, but still clearly identifiable at necropsy. The achieved efficacy of 96.2% was therefore based on the “live worm count”. The total number of worms (live + dead) did not significantly differ between the study groups, indicating a similar degree of initial infection in both groups before treatment was started. The monthly treatment was well tolerated by all dogs. The higher incidence of soft to firm nodules in/under the skin in the imidacloprid/moxidectin group can be explained by the fact that dying worms apparently tend to coil up (Fig. 2 and 3) and that these “worm clusters” are then demarcated as a response from the surrounding tissue and form typical granulomatous inflammations (Fig 4 and 5). Although these structures could be identified at necropsy, the nodules had obviously become too soft and too small to be detected during the clinical veterinary examination directly before necropsy of the treated group.

In previous studies that evaluated the microfilaricidal efficacy of an imidacloprid/moxidectin spot-on against *D. repens*, it was supposed that multiple applications might also have an adulticidal effect (Fok et al. 2010). However, these assumptions were made on the basis of the sustained absence of microfilariae and/or disappearance of clinical symptoms. To the best of our knowledge, this is the

first study to demonstrate the adulticidal efficacy of an anthelmintic against *D. repens* by necropsy. This study was necessary, as no other test is available to detect the presence or absence of adult skinworms, and testing for microfilariae does not provide reliable data due to the fact that infections with premature worms or unisex infections cannot be detected. Finally, clearance of microfilariae is not a measure of adulticidal efficacy. As has been shown for *D. immitis*, microfilariae may reappear even a long time after treatment with macrocyclic lactones has been withdrawn (reviewed by Bowman and Mannella 2011). In fact, this observation was also made in a study that evaluated the efficacy of a single administration of imidacloprid/moxidectin spot-on in eliminating *D. repens* microfilariae. After all dogs had been tested negative, two dogs were microfilaraemic again two and three months after treatment (Traversa et al. 2011).

The adulticidal efficacy of macrocyclic lactones, especially ivermectin, against filarial nematodes has been extensively studied in heartworm disease (see McCall 2005 for a summary). While larval stages (L3/L4) are effectively killed, long-term administration is necessary for premature and adult worms, and the older the worms are when first exposed to macrocyclic lactones, the longer it takes them to die. Three-months-old larvae require up to one year and mature adults 2.5 years of monthly application of preventive doses of ivermectin (6 µg/kg) to provide efficacy of at least 95% (McCall et al. 2008). Also, higher doses of ivermectin (200 µg/kg) and shorter treatment intervals did not greatly enhance the efficacy (McCall 2005). This so-called “slow-kill” method is not recommended in heartworm disease as the infection persists throughout this long period and pathology progresses. Additionally there are concerns that the stand-alone therapy with ivermectin has the potential for selection of resistant subpopulations of heartworms (AHS 2014). The effect of imidacloprid/moxidectin spot-on against adult heartworms has not been tested in the context of an efficacy study. But as the product is registered for the treatment of *D. immitis*

microfilariae, the aspect of adulticidal efficacy has been addressed by conducting overdose target animal safety studies in dogs with existing heartworm infections. Laboratory studies with doses up to 5 times the recommended dose (up to 13.6 times the minimum therapeutic dose) given 3 times at 2-week intervals showed no significant differences in the numbers of adult heartworms collected at necropsy compared to a placebo-treated control group (EMA 2009).

In contrast to *D. immitis*, the study presented here demonstrated a high efficacy of imidacloprid/moxidectin topical solution against adult *D. repens*. Besides possible differences in susceptibility to moxidectin between the two species, this can probably be explained by two facts that are directly related: the location of the adult worms and the pharmacokinetic profile of moxidectin. While adult *D. immitis* float in the blood stream of the right heart and pulmonary artery, *D. repens* migrates mainly in the subcutaneous tissue. Moxidectin is widely distributed into tissues and, due to its high lipophilicity, is mainly deposited in fat (Vanapalli et al. 2002). Adipose tissue may therefore act as a drug reservoir that contributes to the long persistence of moxidectin in the body. Compared to ivermectin and doramectin, it has the longest elimination half-life (19 days in the dog) (Vanapalli et al. 2002). *Dirofilaria repens*, dwelling in tissue rich in fat, is thus subjected to long-term exposure to high doses of moxidectin, which could account for the strong killing effect demonstrated in this study. In addition, it has been demonstrated that continuous monthly application of the formulation will lead to constant high levels of the drug in serum and tissue, which explains the far-reaching capabilities of the formulation to control in particular tissue dwelling parasites such as skin-burrowing *Demodex* mites (Paterson et al. 2014) and lung nematodes (Conboy et al. 2009).

In contrast to classical heartworm preventives, imidacloprid/moxidectin spot-on has demonstrated high efficacy against microfilariae in this study and in several other studies before. In many cases dogs

were cleared of microfilariae with a single application. The topical solution was originally developed as a broad-spectrum anthelmintic against gastrointestinal nematodes. Compared to third and fourth stage larvae of *D. immitis* and *D. repens*, these endoparasites require much higher doses of macrocyclic lactones to be effectively killed. Consequently the recommended minimum dose is many times higher than the 3 µg/kg moxidectin or 6 µg/kg ivermectin provided by classical heartworm preventives.

It can be concluded, that treatment with imidacloprid/moxidectin spot-on for six consecutive months is safe and efficacious against adult *D. repens* in dogs. Together with the new findings from this study the spot-on now provides a complete option for the control of subcutaneous dirofilariosis, including prevention, elimination of microfilariae and treatment of adult stages. Spot-on treatment is a convenient application method and thus the formulation has the potential to enhance owners' compliance in protecting their dogs in endemic areas and treating *D. repens*-positive dogs, even in cases where the infection is asymptomatic. Eliminating the parasite from the reservoir is of capital importance to prevent the further spread of the disease and to protect animals as well as human beings from becoming infected.

#### Ethical Standards

The study was performed in compliance with current national laws and regulations.

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The study was funded by Bayer Animal Health GmbH, Germany.

#### Conflict of Interest

At the time the study was conducted Claudio Genchi and Marco Genchi were employed by the University of Milan. Gabriele Petry and Roland Schaper are employees of Bayer Animal Health GmbH. Bettina Lawrenz is an employee of Bayer Pharma AG. Holger Schmidt is the owner of BioMedVet Research GmbH.

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