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Evaluation of the Efficacy of Imidacloprid 10%/Moxidectin 2.5% (Advocate®, Advantage® Multi, Bayer) for the Prevention of *Dirofilaria repens* Infection in Dogs

Claudio Genchi[1], Marco Genchi[1], Gabriele Petry[2] (✉), Eva Maria Kruedewagen[2], Roland Schaper[2]

[1] University of Milan, Milan, Italy
[2] Bayer Animal Health GmbH, 51368 Leverkusen, Germany

Corresponding author:
Gabriele Petry
✉ E-mail: gabriele.petry@bayer.com

Abstract

The efficacy of imidacloprid 10%/moxidectin 2.5% (Advocate®, Advantage® Multi, Bayer) against experimental *Dirofilaria* (*D.*) *repens* infection in dogs was evaluated in a blinded, negative controlled randomised laboratory efficacy study. On SD (study day) 0, eight dogs received a spot-on treatment at a dose of 10 mg imidacloprid and 2.5 mg moxidectin per kg body weight. Another 8 dogs were left untreated. On SD 28 each dog was infected with approximately 75 infective *D. repens* larvae. Blood samples were collected every 4 weeks after treatment. A modified Knott test was conducted to detect mf (microfilaria). PCR analysis was performed with mf-positive blood samples. On SDs 245 and 246, all dogs were euthanised for detection of *D. repens* worms. Blood samples of all treated dogs were negative for mf at all sampling days. Blood samples of control dogs were positive for mf in 5 out of 8 control dogs. Individual mf counts ranged from 7 to 2800 mf/ml. In mf-positive blood samples, only *D. repens* was identified by PCR analysis. During necropsy *D. repens* worms could be detected in eight untreated control dogs (range: 3–21 worms per dog), whereas no worm could be detected in any of the treated dogs. These results indicate a 100% preventive efficiency of a single spot-on treatment of imidacloprid 10%/moxidectin 2.5% in dogs against experimental infection with *D. repens* (L3 larvae). The product was well tolerated in all study animals, no treatment related adverse reactions were observed throughout the study.

Introduction

Dirofilaria repens (D. repens) Railliet and Henry, 1911, is the main causative agent of subcutaneous filarial infections of dogs and cats in Europe and eastern countries (Svobodova et al. 2006; Fok 2007; Genchi et al. 2009; Miterpáková et al. 2010). Adult worms are localised mainly in subcutaneous connective tissues and in perimuscular fasciae. Most infections are asymptomatic, however it has been reported that dogs with *D. repens* can present cutaneous disorders of varying severity, such as pruritus, dermal swelling and subcutaneous nodules containing the adult or pre-adult parasites. Severe infections with allergic reactions likely due to microfilarial sensitisation have also been reported (reviewed by McCall et al. 2008; Genchi et al. 2011; Rocconi et al. 2012).

Adult female worms measure 13–17 cm in length and 460–650 µm in width, while the male worms are shorter (5–7 cm) (Anderson 1952) (Figs. 1 and 2). After mating, adult female worms release microfilariae (330–370 µm in length and 6–8 µm in width) into the blood stream of the host. Circulating microfilariae are taken up by bloodsucking female mosquitoes, mostly of the genera *Anopheles*, *Culex* and *Aedes* (Cancrini et al. 2007), and develop to the infective larval stage (L3), which are transmitted to the final host through the subsequent blood meal of the infected mosquito (Fig. 3). The development of infective larvae in mosquitoes is a temperature-dependent process and at optimal temperature of 28 °C it takes about 30 days. The prepatent period lasts about 6.5–9 months, and patency from 5 to 7 years (Weber and Hawking 1955; Anderson 2000). Dogs are the main reservoir, cats and wild carnivores such as foxes being seldom microfilaraemic (Magi et al. 2008).

In dogs, *D. repens* infection is currently diagnosed by Knott test for circulating microfilariae as, until

Fig. 1 Adult male of *Dirofilaria repens* from the untreated control group. White square indicates area of Fig. 2

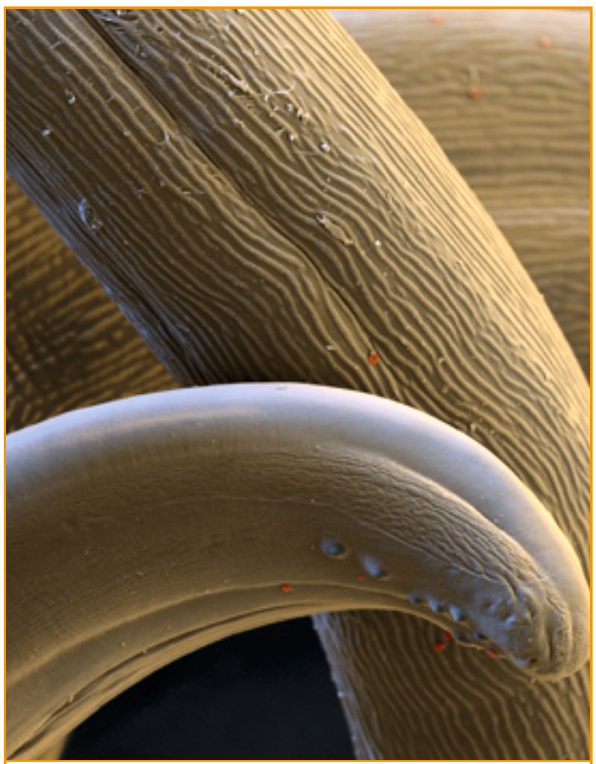

Fig. 2 Enlargement from Fig. 1 showing the typical longitudinal ridges on the cuticula

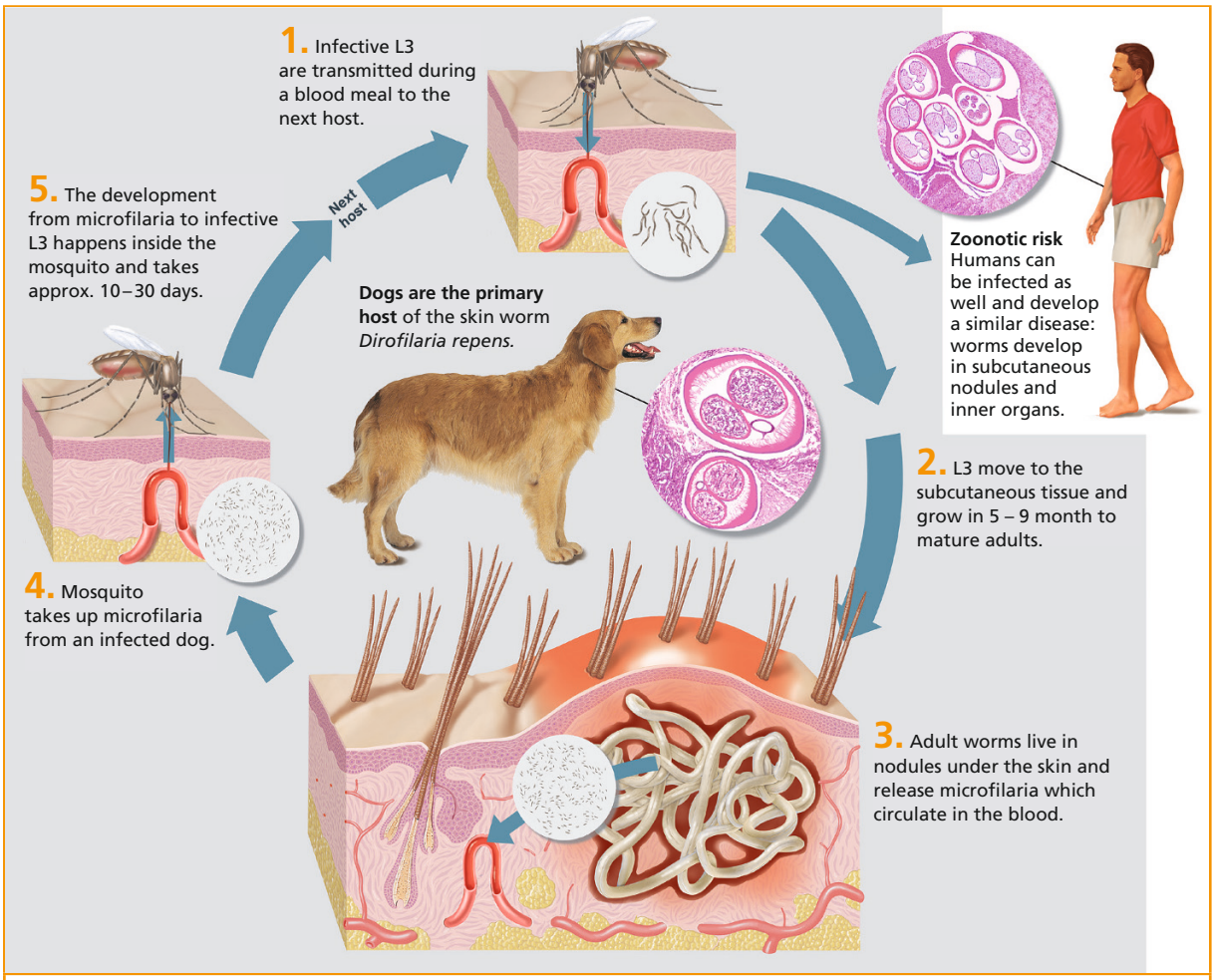

Fig. 3 Schematic representation of the life cycle of *Dirofilaria repens*

now, no serological test kits for the detection of circulating antibodies or antigens are available for veterinary practitioners. As a consequence, considering the few cases with clinical symptoms of suspicion, most cases run undiagnosed and infected dogs continue for long time to act as active reservoirs of infection, mainly throughout the movement and relocation of dogs from endemic areas of South and Southeast Europe to northern countries such as Germany, Poland and Hungary (Genchi et al. 2009; Pantchev et al. 2009; Sassnau et al. 2009).

Although subcutaneous dirofilariosis is considered of minor clinical concern in animals because the frequent lack of abnormalities, the parasite can cause benign to severe/very severe infections in humans. Overall, more than one thousand of human infections have been reported until now (Pampiglione and Rivasi 2000; Genchi et al. 2011; Simón et al. 2012). In most cases, *D. repens* is not able to develop to adult, sexually mature stage in human hosts, and infection is characterised by the presence of preadult stages located in subcutaneous tissues of different body areas, close to the original mosquito bite, although four cases of microfilaraemic zoonotic infections have been until now reported in Europe (Nozais et al. 1994; Petrocheilou et al. 1998; Supriaga et al. 2004; Kucsera 2011, personal communication). Besides subcutaneous and ocular localisation, the parasite has been shown to infect viscera as well as female breast

and male genitalia (e.g. scrotum, penis, spermatic cord and epididymis). Furthermore, several pulmonary localisations have been reported. Deep tissue localisation of the parasitic nodule usually leads to a clinical suspicion of neoplasia and requires biopsy or more invasive surgery for differential diagnosis through histology and morphologic identification of the parasite, thus causing emotional distress and costly intervention. In some cases, infections have been described as mimicking cervical intradural Langerhans cell histiocytic tumors, or scrotal tumors, or as leading to severe consequences due to intraperitoneal localisation (reviewed by Genchi et al. 2011; Simón et al. 2012).

The only effective and safe measure to protect humans from the risk of infection is to control subcutaneous dirofilariosis in dogs. Several macrocyclic lactones (ivermectin, milbemycin oxime, moxidectin and selamectin) have been shown to be fully efficacious to prevent heartworm (*Dirofilaria immitis*) infection both in dogs and cats throughout experimental and field studies (reviewed by McCall et al. 2008), but only few studies have been carried out against *D. repens*. Most studies have been conducted as field studies. Oral ivermectin (Marconcini et al. 1993; Pollono et al. 1998), topical selamectin (Genchi et al. 2002a) and oral moxidectin (Rossi et al. 2002) have been reported to be effective to prevent *D. repens*-patent infections in dogs living in endemic areas when administered every 30 days during *Dirofilaria* transmission season, while one injection of moxidectin as a sustained release formulation is able to protect from infection throughout the entire transmission season (Rossi et al. 2004). In most studies, the efficacy was assessed comparing the results with the infection prevalence observed in the previous year(s).

In 1989, Cancrini et al. (1989) carried out a study in experimental infected dogs with ivermectin as a preventative. Dogs were infected 4 times, 15 days apart, with *D. repens*-infective larvae (estimated total challenge 484–919 infective larvae) and treated orally with ivermectin at dose rates of 6 µg, 12 µg and 24 µg/kg body weight (b.w.) given 30 and 60 days after the first infection. At necropsy 8 months later, the efficacy was 86.6%, 92.8% and 87.9%, respectively.

Recently, Genchi et al. (2010) have shown the full efficacy of moxidectin at the dose of 0.17 mg/kg b.w. in a microsphere sustained release injectable formulation when administered six months after experimental infection of dogs.

The present study was carried out to assess the efficacy of an imidacloprid 10%/moxidectin 2.5% spot-on formulation (Advocate®, Advantage® Multi, Bayer) to prevent *D. repens* infection in experimentally infected dogs.

Material and methods

The study was conducted as a randomised, placebo-controlled and blinded efficacy study designed and conducted in accordance with the standards of VICH GL7 (Efficacy of anthelmintics: general requirements, 11/2000), VICH GL9 (Good Clinical Practice, 06/2000) and VICH GL19 (Effectiveness of anthelmintics: specific recommendations for canine, 06/2000). Because the guidelines do not include any specific information on *D. repens*, the animal infection was carried out on the basis of previous experimental studies on the development of this filarial species in the dog (Webber and Hawking 1955; Genchi et al. 2010) and in accordance with the WAAVP guidelines for evaluating the efficacy of anthelmintics for dogs and cats (Jacobs et al. 1994).

Efficacy criterion

The study was designed as an efficacy study. Therefore the primary criterion for efficacy was the result of the necropsy *D. repens* counts. The primary variable evaluated was the efficacy of the combination of imidacloprid 10%/moxidectin 2.5% against infective larvae of *D. repens*. The efficacy evaluation was assessed through the number of adult worms recovered 8 months after dog infection (245–246 days). A statistically significant difference between the

mean value of the treatment group and the control group ($p<0.05$) and 90% geometric mean percent reduction relative to the untreated control group was required for efficacy of the treatment.
Further parameters were connected with the general welfare of the dogs, their suitability for inclusion in the study and the safety evaluation of the combination of imidacloprid 10%/moxidectin 2.5% (clinical assessments).

Study design

Sixteen Beagle dogs, 8 male (7.6–8.7 kg body weight (b.w.) and 8 female (6.3–7.4 kg b.w.) dogs, aged 5 to 6 months at beginning of the study, were included in the study. After acclimatisation for 7 days, on study day –3 dogs were blocked by body weight and gender and randomly allocated to two study groups (8 dogs per group). Dogs were housed in groups of 2 animals of the same sex in pens according to the "Commission recommendation on guidelines for the accommodation and care of animals used for experimental and other scientific purposes" (2007/526/EC – 18/06/07). On study day –7, blood samples were taken from each dog and examined by Knott test for circulating microfilariae (Knott 1939; Genchi et al. 2007).
On study day 0, 8 dogs were treated with the combination of imidacloprid 10%/moxidectin 2.5% at a dose of 10 mg imidacloprid and 2.5 mg moxidectin per kg b.w. (group 1), while 8 dogs (placebo-treated, group 2) served as control. On study day 28, all dogs were experimentally infected with approximately 75 infective larvae of *D. repens*. Overall, the study lasted 245–246 days, when dogs were humanely euthanised for necropsy and pre-adult and adult worms were collected and counted. After treatment, blood samples were individually taken at study days 28, 56, 84, 112, 120, 140, 168, 196, 224 and 238 and examined by Knott test. Microfilaraemic samples were examined by PCR, using primers for identification of different filarial species, including *D. repens* as previously described by Gioia et al. (2010).

Throughout the study, the dogs were fed once per day with a commercial diet and drinking water was supplied *at libitum*. General health observations were carried out on all dogs daily from study day –7 to study day 0 (treatment day) and again at 1 and 4 hours post treatment and daily until the end of the study. Dogs were weighted prior to feeding on study days –7 and –3 and again each 2–3 weeks throughout the entire study.

Preparation of *D. repens*-infective larvae and experimental infection

Infective larvae were obtained as previously described by Genchi et al. (2010). Briefly, approximately 1.500 female pupae of *Aedes aegypti* were allowed to emerge as adults into a carton and 4-day-old mosquitoes were used for producing infective larvae. Heparinised blood was collected from a naturally *D. repens*-infected dog. Infected blood was maintained at 37 °C in a feeding apparatus and mosquitos were allowed to feed. *D. repens*-infective larvae were collected from mosquitoes after 28 days and put into 16 tubes, each containing approximately 75 larvae in 1.5 ml of fresh RPMI-1640 medium. On study day 28 of the study, each dog was injected subcutaneously with 75 larvae in accordance with the WAAVP Guidelines (Jacobs et al. 1994 and Vercruysse et al. 2002).

Statistical analysis

The efficacy was assessed based on the number of *D. repens* worms found at necropsy in the treated dog group compared to the untreated dog group. Efficacy was calculated based on geometric means (GM). Percent efficacy was calculated as [(GM control – GM treated)/GM control] x 100. The statistical analysis was carried out with the non-parametric Wilcoxon-Mann-Whitney-U Test (two-sided, alpha = 0.05) for the group comparisons. The medical relevance of the differences between groups was quantified using the Mann-Whitney superiority measure (MW) and its two-sided 95.0% confidence interval as corresponding effect size. The MW measure (0.0 to 1.0) reflects the probability

that a randomly selected patient of the test group is better off than a randomly selected patient of the control group (Coldiz et al. 1988).

Results

Dogs in group 1, treated with the combination of imidacloprid 10%/moxidectin 2.5%, were negative at all Knott test examinations carried out at the different times of the study. In group 2 (placebo-treated) no *D. repens* microfilariae were found in 3 dogs at any study time. On study day 196, 3 dogs were microfilaraemic. The individual number of microfilariae per ml was 10, 22 and 229. On study day 224, 5 dogs were microfilaraemic; the individual number of microfilariae/ml was 174, 105, 1,410, 370 and 7. On study day 238, the same dogs were still positive with an individual number of microfilariae per ml of 510, 870, 2,800, 700 and 30, respectively (Table 1) showing a time-dependent increase of microfilaria counts. PCR analysis on the microfilaraemic blood samples confirmed that the infection was caused by *D. repens*.

At necropsy, all dogs were examined for preadult and adult *D. repens* worms. All placebo treated dogs were found to be infected with worms (range: 3 to 21 worms). Data counts are shown in Table 2. Seven out of 8 dogs had 5 or more adult worms in subcutaneous tissues. At necropsy on study day 245 and 246, no worm was found in any of the imidacloprid 10%/moxidectin 2.5%-treated dogs (group 1, efficacy 100%). The imidacloprid 10%/moxidectin 2.5%-treated group was highly

Table 1 Number of *Dirofilaria repens* microfilariae/ml in placebo-treated dogs from study day 196 to study day 238 after experimental infection

Dog ID	Study day 196	Study day 224	Study day 238
9478	0	0	0
8544	10	174	510
9800	22	105	870
8838	139	1410	2800
9583	0	370	700
9257	0	0	0
9176	0	7	30
9893	0	0	0

Table 2 Number of *Dirofilaria repens* worms found in the placebo-treated dogs on study day 245/246 after experimental infection

	male worms		female worms		necrotic worms	total live worms	total dead worms	total worm counts
Dog ID	live	dead	live	dead				
9478	2	0	1	0	0	3	0	3
8544	2	0	2	0	1	4	1	5
9800	3	0	3	0	2	6	2	8
8838	7	4	8	1	1	15	6	21
9583	4	1	4	0	1	8	2	10
9257	2	0	3	0	1	5	1	6
9176	7	0	8	0	2	15	2	17
9893	3	0	7	0	2	10	2	12

statistical significant superior versus the untreated control group (p = 0.0002, MW = 1.0).
Throughout the study, no adverse reactions were observed in any of the dogs.

Discussion

Moxidectin has shown a potent prophylactic activity in preventing heartworm (*D. immitis*) infections when administered either at 1 or 2 months post infection in experimentally infected dogs (McTier et al. 1992). Effective prophylaxis has also been shown in dogs naturally exposed to heartworm-infected mosquitoes treated orally either monthly at 1 µg/kg and 3 µg/kg b.w. or bimonthly at 3 µg/kg b.w. (McCall et al. 1992). The sustained release injectable formulation (0.17 mg/kg b.w.) has been shown to be effective for at least 6 months both in field studies (Genchi et al. 2002b) and after experimental heartworm infections (Lok et al. 2005). Moxidectin is more lipophilic in nature than ivermectin and it is stored in the fat, which may act as a drug reservoir (Vanapalli et al. 2002). Compared to ivermectin, moxidectin has a higher distribution volume and a longer half-life elimination (Vanapalli et al. 2002). This may facilitate its distribution from the bloodstream to different tissues and longer residence time for the drug in the body (Alvinerie et al. 1995; Vanapalli et al. 2002). *D. repens* larval stages and the adult worms are permanent residents in subcutaneous tissues, which are rich of connective and fat tissues. Therefore, the high lipophilic nature of moxidectin is probably the reason of the full efficacy observed in this study, treating experimentally infected dogs topically with a combination of imidacloprid 10%/moxidectin 2.5%, confirming the previous data observed with an injectable formulation (Genchi et al. 2010). The efficacy was assessed in comparison with placebo-treated dogs. Only 5 of 8 control dogs had a patent infection with an increasing number of circulating microfilariae during the course of infection, whereas all control dogs were infected with living worms. Advocate® was well tolerated in all treated animals as no side effects were observed. The lack of clinical alterations after the experimental infection confirms that most *D. repens* infections are clinically undetectable. In conclusion, imidacloprid 10%/moxidectin 2.5% (Advocate®, Advantage® Multi, Bayer) can be considered an effective and safe preventative product against *D. repens* infections in dogs.

Ethical standards

The study was conducted as a randomised, placebo-controlled and blinded efficacy study designed and conducted in accordance to the standards of VICH GL7 (Efficacy of anthelmintics: general requirements, 11/200), VICH GL9 (Good Clinical Practice, 06/2000) and VICH GL19 (Effectiveness of anthelmintics: specific recommendations for canine, 06/2000). All institutional and national guidelines for the care and use of laboratory animals were followed.

Conflict of interest

C Genchi and M Genchi were employed by the University of Milan, Italy. G Petry, EM Kruedewagen and R Schaper were employed by Bayer Animal Health GmbH, Germany. The study was sponsored by Bayer Animal Health GmbH.

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