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Short Communication

Assessment of the combination of spinosad and milbemycin oxime in preventing the development of canine *Angiostrongylus vasorum* infections

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ABSTRACT

Angiostrongylus vasorum is an increasingly reported parasite in Europe that develops in dogs after ingestion of infective third stage larvae (L3) that reside in gastropod molluscs which are needed to complete the parasite's life-cycle. Infection can produce a diversity of clinical signs, determined by involvement of the respiratory, neurological, and/or coagulation system, with a likely fatal outcome in the absence of treatment. Few drugs have been shown to reliably prevent infection, and data on treatment of infections is limited. A controlled, randomized, partially blinded laboratory study was therefore executed to evaluate the efficacy and safety of a combination tablet of spinosad/milbemycin oxime in dogs inoculated with approximately 250 *A. vasorum* L3. Sixteen healthy nematode free adult dogs were randomly allocated to two study groups of 8 dogs each. Thirty days post inoculation (dpi) all dogs in the fed state were treated: dogs in group B were treated with spinosad and milbemycin oxime at the dose rates of 45–60 mg/kg and 0.75–1.0 mg/kg bodyweight, respectively, approximately the lower half portion of the expected full unit dose range; dogs in group A were treated with placebo tablets. All dogs were euthanized and necropsied 56–58 dpi. The heart and lungs were examined to determine the presence of *A. vasorum*. All placebo group dogs were infected at necropsy with counts ranging from 22 to 98 adult worms and a geometric mean worm count of 55.2. In contrast, the geometric mean worm count in the spinosad/milbemycin oxime group was 0.7 with worm numbers ranging from 0 to 8. The results of this study demonstrate that a single treatment with the tablet combination of spinosad and milbemycin oxime administered 30 dpi provided 98.8% preventive efficacy against development of adult *A. vasorum* infections. Monthly treatments with spinosad and milbemycin oxime have the potential to prevent the establishment of infections with *A. vasorum* in dogs.

1. Introduction

Adult worms of *Angiostrongylus vasorum* (Nematoda, Metastrongylidae, Baillet 1866) live in the pulmonary arteries and the right atrium and ventricle of the heart of the final hosts, which include domestic dogs, foxes (Guilhon, 1963; Sreter et al., 2003), wolves (Segovia et al.,

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2001) and badgers (Torres et al., 2001). In dogs patency (excretion of first stage larvae) is usually between 38 and 57 days after infection but can range from 28 to 108 days after infection (Bolt et al., 1994). Clinical signs are attributed to inflammation caused by the presence of the parasite's eggs and larval stages in the lungs and can range from a cough, dyspnoea and further respiratory signs, through a bleeding diathesis that can manifest with gastrointestinal or neurological signs (Chapman et al., 2004; Schnyder et al., 2010). If not treated, infections in dogs may be progressive and potentially carry a fatal outcome (Staebler et al., 2005; Traversa et al., 2008).

Often called the French Heartworm from its first recorded incidence in France in the 1800s (Serres, 1854), the geographic range of *A. vasorum* is now known to have expanded throughout Europe. Initial observations in Ireland were reported in 1968 (Roche and Kelliher, 1968) and parasite's presence in the UK in 1975 (Jacobs and Prole, 1975), where it now appears to have spread from the area of original identification in southern England throughout the country (reviewed by Yamakawa et al., 2009). The widespread presence of the parasite is suggested by prevalence surveys performed in foxes and dogs in Italy (Poli et al., 1991; Guardone et al., 2013; Di Cesare et al., 2011); an apparent endemic focus in Denmark (Koch and Bolt, 1990); and from sporadic cases reported across Europe, including in Sweden (Ablad et al., 2003), Greece (Papazahariadou et al., 2007), Hungary (Sreter et al., 2003), Switzerland (Staebler et al., 2005) and Germany (Barutzki and Schaper, 2009; Seybold, 2011). Higher incidence rates were reported by other authors in a 4 year long epidemiological overview including samples from dogs in Germany and Denmark (Taubert et al., 2008).

The increasing reports of this parasite and its distribution all over Europe and also in Canada, drive the need for effective treatment and even more importantly, preventing the establishment of infection. Treatments shown to be effective against infections with *A. vasorum* include the orally administered compounds fenbendazole and milbemycin oxime, and the topically applied combination of imidacloprid/moxidectin. Fenbendazole administered *per os* at 25–50 mg/kg bodyweight (BW) for 20 days has been reported to be effective (Willesen et al., 2007), while a single application of imidacloprid 10%/moxidectin 2.5% spot-on (0.1 ml/kg bodyweight) was 85.2% effective against adult *A. vasorum* (Willesen *et al.*, 2007). Administration of that topical product was reported to be effective against experimental *A. vasorum* infections when administered as a single treatment once at 4 days, and to a second group at 32 days post inoculation (Schnyder et al., 2009).

Spinosad is a novel tetracyclic macrolide insecticide recently approved as an orally administered tablet for the treatment and prevention of flea infestations in dogs and cats. A single oral treatment has been shown effective against fleas on dogs, and consecutive monthly treatments have been shown to provide >99% control of fleas in client-owned dogs (Wolken et al., 2012; Dryden et al., 2013). Milbemycin oxime (MO) is a macrocyclic lactone anthelmintic which has been shown to be effective against larval stages of the heartworm *Dirofilaria immitis*, as well as against a range of gastrointestinal parasites. The broad spectrum efficacy and safety of a combination tablet of spinosad with MO has been demonstrated in studies on flea infestations and on infections with gastrointestinal nematodes, including *Ancylostoma caninum*, *Toxocara canis*, *Toxascaris leonina*, *Trichuris vulpis* and the heartworm *D. immitis* (Snyder et al., 2011; Snyder and Wiseman, 2012; Schnitzler et al., 2012). Against *A. vasorum*, one report indicated that an oral dose of MO (0.5 mg/kg BW) administered as a single product, cleared larval excretion in 14 of 16 (87.5%) infected dogs when given weekly over four weeks (Conboy, 2004).

The purpose of this study was to investigate the prophylactic effectiveness of a single treatment of an oral combination tablet containing spinosad and MO against immature stages of *A. vasorum* in dogs. As this was a study undertaken to support a registration claim, treatment was administered using dose rates of 45–60 mg/kg BW spinosad and 0.75–1.00 mg/kg BW MO, approximately the lower half of the expected full unit dose range (45–70 mg/kg BW spinosad and 0.75–1.18 mg/kg BW MO).

2. Materials and methods

The study was conducted as a controlled, randomized, partially blinded study adhering to the standards of Good Clinical Practice and VICH (http://www.vichsec.org/, International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products) guidelines (GL7 and GL19). The study was conducted at the Institute of Parasitology, University of Veterinary Medicine, Hanover in Germany. The protocol was reviewed and approved by the laboratory's Institutional Animal Care and Ethics Committee.

2.1. Dogs

Sixteen healthy beagle dogs, 8 male and 8 female, approximately 10 months of age, were included in the study. Faecal samples were collected from each of the study dogs in the week prior to inoculation with *A. vasorum* third stage larvae (L3) to verify that none were carrying any existing gastrointestinal nematode infections. Qualitative faecal examinations were conducted using the Baermann technique (standard amount of faeces, 10 g/dog, examination for larvae after 24 h of incubation, Baermann, 1917) in combination with a sedimentation-flotation technique (Eckert, 1972). A negative nematode status was required for inclusion and enrolment into the study. All dogs were individually housed in kennels with concrete floors and wooden stands and were fed a commercial dry dog food ration with *ad libitum* access to tap water (see Table 1 for schedule of events). All personnel responsible for clinical observations or for performing nematode counts were blinded to the treatments.

2.2. Experimental inoculation

First stage larvae of *A. vasorum* were harvested from a fox infected with a field isolate, originally obtained from dogs in Denmark and passaged twice by the Faculty of Health and Medical Science, University of Copenhagen,

Table 1
Study schedule of events.

Study day	Event
Day-37 to Day-30	Dog acclimatization pre-treatment faecal samples collected
Day-30	Physical examinations Infection of all dogs with 250 third-stage larvae of *Angiostrongylus vasorum*
Day 0	Physical examination pre- and post-treatment dosing
Days 4, 7, 12, 14, 19, 22	Thoracic auscultation for respiratory assessments
Days 26–28 (56–58 days post-inoculation with L3)	Necropsy

Denmark. The larvae were shipped to the trial facility for infection of snails (*Achatina fulica*). Third stage larvae were harvested from the snails by peptic digestion.

For infection with *A. vasorum*, general anaesthesia was performed on Day-30 with intravenous acepromazine (Vetranquil®, 0.4 ml/kg BW) and propofol (Narcofol®, 0.6 ml/kg BW). Each anaesthetized dog was inoculated by stomach tube with approximately 250 L3, and then was closely monitored to verify that regurgitation of the inoculation dose did not occur.

2.3. Randomization and treatment

Using a standard statistical program (SAS® version 9.2) each dog was randomly assigned, without blocking, to one of two treatment groups. Group B dogs were treated orally with a tablet containing spinosad and MO at respective dose rates of 45–60 mg/kg BW and 0.75–1.0 mg/kg BW, receiving therefore approximately the lower half portion of the dose range (45–70 mg/kg BW and 0.75–1.18 mg/kg BW). Group A dogs were treated with a placebo tablet containing no active ingredient, but identical in appearance to those containing active drug.

Treatments were administered 30 days post inoculation (dpi) on Day 0 to dogs according to their randomization to a treatment group. To optimize absorption of spinosad and MO, tablets are recommended to be given with food. Therefore, on the evening prior to dosing, food was removed from the animal housing areas and on the following morning each dog was allowed to consume approximately 25% of the manufacturer's recommended daily amount of a palatable dry dog food, based on body weight. Study tablets were then administered, after which all dogs were offered the remainder of their standard daily maintenance diet.

2.4. Clinical examinations

Clinical examinations were performed before inoculation and then during the pre-treatment phase. On the day of treatment (Day 0), dogs were observed pre-dosing, at 1 and 2 h (±15 min.) post dosing, and again at 4 and 8 h (±30 min.) post dosing by the animal care taker. On subsequent days, two veterinarians trained in a standardized facility procedure recorded their clinical observations, including respiratory assessments. From the day after dosing (31 dpi), twice weekly auscultatory respiratory assessments were performed with the quality (normal/deepened normal sound/stertor/stridor/rhonchus/wheeze/crackle), and intensity of inspiratory and expiratory sounds graded on a scale from 0 (no abnormal sound) to 3 (severe sound). In addition, faecal sampling was performed from 40 dpi until a dog was confirmed to be shedding larvae using the methods described above (Baermann, 1917).

2.5. Necropsies

Dogs were euthanized and necropsied on Days 26–28 post-treatment (56–58 dpi). Post-necropsy examinations were carried out following Schnyder et al. (2009). For each dog, the heart and lungs were examined in detail to determine the presence or absence of adult *A. vasorum*. All worms were counted and identified to gender and stage of development, whenever possible. If worm fragments were present, they were only included in the count if the head was present.

2.6. Statistics

Efficacy calculations were based on the total number of adult worms recovered from each dog at necropsy in the treated and control groups to determine whether or not treatment had prevented development of infections with adult *A. vasorum*. To show adequate infection, a minimum of five *A. vasorum* must have been present in at least 6 control dogs. For each treatment group, the total number of adult *A. vasorum* along with the group geometric mean (GM) was calculated.

The mean worm counts were determined and compared post-treatment between treated (spinosad/MO combination) and control (placebo) groups. Efficacy was calculated based on GM using the formula:

$$\%\text{Efficiency} = \left\{ \frac{\text{mean}_{\text{control}} - \text{mean}_{\text{treated}}}{\text{mean}_{\text{control}}} \right\} \times 100$$

To demonstrate prevention, both of the following criteria must have been satisfied:

1. Efficacy ≥90% against *A. vasorum* with the combination tablet, based on GM.
2. A statistically significant difference ($p < 0.05$, two-sided) between the control group and the combination treated group based on analysis of transformed counts of adult *A. vasorum*.

A logarithmic transformation (ln[count + 1]) was applied to the post-treatment counts for each dog to address skewness of the data, as well as zero counts. Back transformed geometric means were calculated by $e^x - 1$,

where $\bar{x}$ equals log transformed treatment mean. Transformed GM worm counts were analyzed with a general linear mixed model with fixed effect treatment. A contrast between spinosad/MO and the placebo groups was conducted to confirm effectiveness. A covariance structure allowing for heterogeneous error variance between treatment groups was considered via a test for heterogeneity but was not deemed necessary in the final analysis.

3. Results and discussion

There were no adverse events within the 8 h post treatment period, and no abnormal respiratory signs were detected in any of the dogs treated with spinosad/MO. Auscultation revealed minor intensity respiratory sounds in two dogs in the placebo group, one on Days 7 and 12, and one on Day 12. No other physical abnormalities were observed in any dog during the study period, and a pre-necropsy physical examination of each dog did not detect any clinical abnormalities.

Faecal samples taken each day in the treatment phase, starting 40 post inoculation until a dog was positive, revealed that all dogs in group A (placebo) were shedding larvae between 47 and 54 dpi, with larval counts ranging from 2 to 46 per 10 g of faeces. The objective of the faecal counts was to demonstrate patency, and it is likely that subsequent samples would have yielded increasingly higher counts. At no point during the study were larvae detected in the faeces of dogs in the treated group B.

Necropsy examinations for the presence of *A. vasorum* demonstrated adequate infection in all eight control (placebo-treated) dogs, with adult worm counts ranging from 22 to 98, and the GM worm count was 55.2. In the spinosad/MO group, no worms were found in 5 dogs; counts in the 3 dogs in which *A. vasorum* were detected were 1, 2 and 8. The GM worm count in the spinosad/MO group was 0.7, which, relative to the placebo group, met the required reduction in counts to demonstrate effectiveness. The objective of the study was met as the results demonstrated a significant difference between the control and treated groups ($p < 0.0001$). The GM efficacy of spinosad/MO in the treated group versus the placebo-treated group was 98.8%.

Gross necropsy findings were similar to the findings of Schnyder et al. (2009). Macroscopic changes in the lungs varied considerably between the groups but were consistent within each group. Generally, the lungs appeared pale and anaemic, likely a result of the necropsy procedure of flushing the blood out of the lungs. In the lungs of dogs in the spinosad/MO group, there were faintly visible lung changes observed as a pattern of disseminated pale pink coalescing, slightly consolidated, raised foci. In some cases these foci were associated with darker red haemorrhagic areas, but usually had a yellow tinge from degrading haemorrhages (Fig. 1). In contrast, the lungs of all the dogs in the control group (Fig. 2) were severely affected with large confluent areas that were firm, raised, and discoloured from pale beige to yellow to dark red. Fresh haemorrhages, alternating with pale non-aerated areas confirmed severe damage to the lungs, consistent with those that have been described as a consequence of an established lungworm infection (Schnyder et al., 2009).

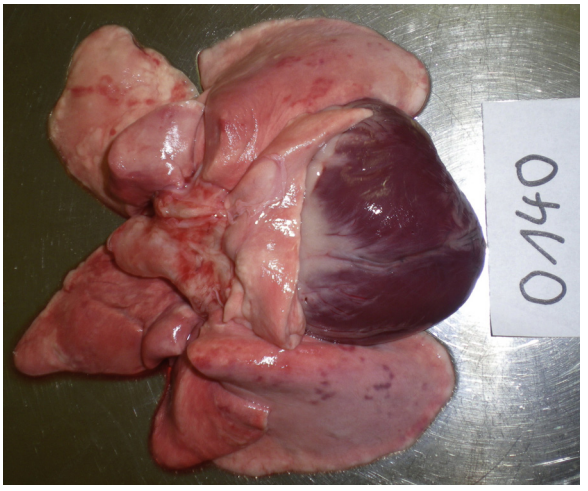

Fig. 1. Lung lobes and heart from a dog inoculated with 250 third stage larvae of *Angiostrongylus vasorum* and treated with the spinosad/MO combination 30 days later, having 0 worm counts at necropsy performed 26 days after treatment.

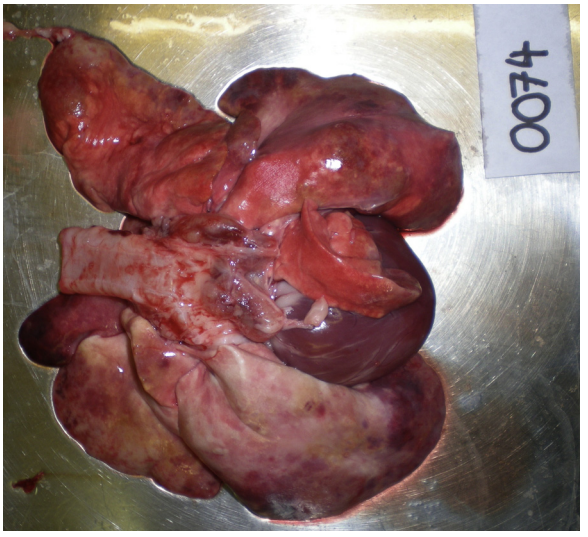

Fig. 2. Lung lobes and heart from a dog of the placebo-treated group inoculated with 250 third stage larvae of *Angiostrongylus vasorum*. This dog showed respiratory signs on Days 7 and 12 after treatment and had 93 worms at necropsy performed 26 days after placebo administration.

4. Conclusion

Traditionally, treatment of *A. vasorum* infections has been complicated by the need for repeat-treatment regimens, and only a single study has demonstrated the potential for prevention of the establishment of adult infections (Schnyder et al., 2009). In that study, the full topical dose of imidacloprid/moxidectin administered under laboratory conditions was found to be completely effective, indicating that monthly applications according to label recommendations should offer a reliable means of preventing

the effects of *A. vasorum* infection. Because of reported concerns that some owners or veterinarians have expressed about topically applied products, including the effects of inclement weather and bathing, and the difficulties some owners appear to have in applying a full topical dose directly to a dogs skin, it is important to have alternative treatments that can be administered orally (Snyder et al., 2007; Dryden et al., 2013).

The results of this study indicate that the combination tablet of spinosad/MO provides such an oral alternative. A single treatment with the flavoured combination tablet containing spinosad and MO, at the lower end of the expected label dose range for this formulation, was found to be >98% effective in preventing the development of infections with adult *A. vasorum* in study dogs. Additionally, a single treatment with the combination product substantially reduced the subsequent pulmonary damage caused by *A. vasorum* infections, relative to the pathology observed in control dogs. Such pathology is most likely due to the production of first stage larvae once adult *A. vasorum* have become established.

As such, regular monthly treatment with the spinosad/MO chewable tablets is expected to prevent dogs from developing clinical or subclinical disease associated with *A. vasorum* infection. By preventing development of infection to the adult stage, this treatment has the potential to interrupt the parasite life cycle and to help limit the environmental accumulation of infective larval stages and thus snails will not become infected.

Conflict of interest statement

This study as reported herein was funded by Elanco Animal Health. The authors from Hanover, Zurich, and Frederiksberg C, were contracted to perform this study; the remaining authors are current employees of Elanco Animal Health and assisted with the study design, study conduct, data analysis, and review of the manuscript; however, there were no conflicting interests that may have biased the work reported in this paper.

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