Larvicidal effect of imidacloprid/moxidectin spot-on solution in dogs experimentally inoculated with Angiostrongylus vasorum

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Larvicidal effect of imidacloprid/moxidectin spot-on solution in dogs experimentally inoculated with Angiostrongylus vasorum

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Keywords: *Angiostrongylus vasorum* – Larvicidal treatment – Moxidectin – Imidacloprid - Dog.

1. Introduction

Domestic dogs, fox species, (Bolt et al., 1994), wolves (Segovia et al., 2001) as well as badgers (Torres et al., 2001) are susceptible hosts of *Angiostrongylus vasorum* (Nematoda, Strongylida). Intermediate hosts are snails or slugs harbouring infective third stage larvae (L3) which are ingested by the final host. The adult parasites live in the pulmonary arteries and the right ventricle of the heart. Their presence as well as eggs and larval stages in the lungs can cause severe alterations. Associated primary complaints in dogs are respiratory signs, syncopal episodes and haemorrhagic diathesis (Chapman et al., 2004), and if left untreated, the infection can be fatal. *A. vasorum* is recognised to be endemic in France, the United Kingdom, Denmark (Bolt et al., 1994), Italy (Poli et al., 1991; Traversa et al., 2008) and Ireland (Dodd, 1973) but sporadic cases have increasingly occurred in many other countries within Europe (Ablad et al., 2003; Papazahariadou et al., 2007; Sreter et al., 2003; Staebler et al., 2005) and outside Europe in Uganda (Bwangamoi, 1972), Brazil (dos Santos Lima et al., 1985), Australia (Collins et al., 1992) and in Canada (Bourque et al., 2002; Conboy, 2004). Fenbendazole (Boag et al., 2004; Chapman et al., 2004; Esteves et al., 2004) and levamisole (Soland
and Bolt, 1996) have been the most employed anthelmintics for the treatment of angiostrongylosis in the last decade. Recently new members of the macrocyclic lactones group such as milbemycin oxime (Conboy, 2004) and moxidectin (Willesen et al., 2007), which are tolerated even in ivermectin-sensitive dogs (Paul et al., 2004), have also been employed. Moxidectin has become available on the European market in a spot-on combination formulation together with imidacloprid (Advocate®, Bayer AG, Leverkusen, Germany). This topical product has a prolonged duration of activity after application (4 weeks) and has already been tested for the treatment of A. vasorum in naturally infected dogs in a single application (Willesen et al., 2007) by comparison with fenbendazole administered for 20 days. The efficacies were equally high: 85.2% (63.3-95.8) for imidacloprid/moxidectin and 91.3% (71.9-98.9) for fenbendazole, and similar to milbemycin oxime with 84.8% (Conboy, 2004). The purpose of our study was to investigate the potential of a monthly prophylactic application of imidacloprid 10% and moxidectin 2.5% spot-on solution to prevent angiostrongylosis in dogs.

2. Materials and Methods

The study was conducted as a controlled, randomized, blinded dose confirmation study according to the standards of Good Clinical Practice and based on VICH (http://www.vichsec.org/, International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products) guidelines (GL7 and GL 19). Approval by the Cantonal Veterinary Office of Zurich was obtained prior to study start in April 2008 (permission number 13/2008).

The study was carried out at the experimental units of the Vetsuisse faculty at the University of Zurich. Thirteen male and eleven female, purpose bred beagle dogs aged between 9 months and 2.3 years were included in the study. All animals were kept in groups of two to four dogs of the same sex, housed in kennels of 6.5 m² with 8 m² runs/two dogs, both with concrete floors and wooden stands for rest, cleaned daily with a high pressure cleaner at 80°C (Kärcher). They were fed commercially prepared dried dog food and had access to tap water ad libitum. Approximately three weeks before the experiment started, all dogs received a combination of febantel, pyrantel embonate and praziquantel (Drontal flavour Plus®, Bayer AG).
2.1 Experimental inoculation

Twenty-four adult dogs were acclimatized to the study facility for at least 7 days prior to experimental inoculation. On study day 0 all dogs were experimentally inoculated with approximately 200 L3 of *A. vasorum*. Larvae were obtained from experimentally infested snails (*Biomphalaria glabrata*). The isolate was originally gained from an inoculated dog, passed through *B. glabrata* snails and one fox before infection of the snails used in the experiment. The infection of snails was performed by placing 2 snails in containers with 100-150 L1 per snail in 15-20 ml water for 24 hours under a constant light source. The snails were then transferred to larger water tanks and maintained for 6 weeks. Just prior to inoculation of the dogs the snail shells were crushed, the snail tissue minced with scissors and then digested for 10-20 min in a mix of 1 liter tap water at approximately +43° C with 37% HCl, to which 10 g pepsin 1:10'000 IU had been added. The digested material was passed through a 180 µm sieve and allowed to settle for 30 min before the supernatant was discarded. The sediment with larvae was washed 2-3 times in tap water (Ferdushy et al., 2009, modified). The number of larvae in a sub sample was counted under a stereomicroscope and individual inoculation doses for the dogs were prepared.

Sedation of dogs was done with acepromazine i.m. (Prequillan®, Arovet, Ozzano Emilia, Italy, 0.06mg/kg BW) and buprenorphine i.m. (Temgesic®, Reckitt Benckiser Healthcare, UK, 0.007 mg/kg BW) simultaneously. Anaesthesia followed approximately 20 minutes later with propofol (Propofol® 1% MCT, Fresenius, Stans, Switzerland, 4mg/kg BW) i.v. Then a stomach tube was inserted and the inoculation dose was given intragastrically. The tube was rinsed with 20 ml warm tap water before removal. Immediately after inoculation, 1 ml of metoclopramide (Paspertin®, Solvay Pharma, Berne, Switzerland, 0.4 mg/kg i.v.) was given to each dog. Dogs were then kept separately and observed for vomiting/regurgitation until awakening. When a sleeping dog showed regurgitation, anaesthesia was prolonged with Propofolum (20 mg per dog) for a maximum of 1 hour after inoculation.

2.2 Administration of test product

All 24 dogs were randomly allocated to three study groups (n=8 each) before the first treatment at 4 days post inoculation (dpi). Treatment consisted of the administration of imidacloprid 10 mg/kg body weight (BW) / moxidectin 2.5 mg/kg BW spot-on. This dose corresponds to 0.1 ml product (Advocate®) per kg BW. Doses were based on the body weights of the day before treatment. Group 1 was treated 4 dpi, group 2 was treated 32 dpi and group 3 was left untreated.
2.3 Faecal examination

During the acclimatisation period a faecal examination was performed to demonstrate the parasitological status of the dogs with regard to helminth infections. Faecal samples were taken from each dog. They were examined once by a combined sedimentation/flotation and by a Baermann-Wetzel technique (Eckert et al., 2008). Starting from 40 dpi to study end 10 g of faeces from each dog were quantitatively examined every day for shedding of L1 of *A. vasorum* by the above mentioned Baermann-Wetzel technique. For the larval counting, 15 ml were obtained by opening the clamp placed at the end of the plastic tube, centrifuged and 1 ml of the sediment was observed under the microscope with 40x magnification.

2.4 Clinical follow-up

General health observations were performed on each dog daily by the keepers throughout the study. All dogs underwent physical examination and weighing by a veterinarian 7 days before inoculation and 3, 31 and 55 dpi. The overall physical condition and the following systems were assessed: cardiovascular, respiratory, gastrointestinal and genitourinary. Additionally, starting on 41 dpi, a deepened respiratory assessment was performed in all dogs twice weekly. The intensity of inspiratory and expiratory sounds on a scale from 0 to 3 (0= no sound; 1= slight sound; 2= moderate sound; 3= severe sound), the quality of the respiratory sound (normal / deepened normal sound / stertor / stridor / rhonchus / wheeze / crackle), abdominal involvement and cough/retch (yes or no) were listed.

2.5 Post mortem examination

On day 56-59 post inoculation all dogs were euthanized and dissected to determine the worm burden by reverse lung perfusion applying a method modified from a procedure described for foxes (Monrad et al., 2007). The dogs were treated with heparin sodium i.v. (350 IU/kg BW) and euthanized approximately 3 minutes later by an intravenous pentobarbital (Eutha® 77, Provet, Lyssach, Switzerland) overdose. The thorax was opened. Aorta, vena cava caudalis and vena cava cranialis were clamped off. A plastic pipe was then placed into the truncus pulmonalis and blood was collected into plastic tubes as long as it was flowing. Then a probe was placed into the left ventricle and an isotonic perfusion liquid (sodium citrate, 15 g/L + NaCl, 8.6 g/L dissolved in tap water) at approximately +37 °C was pumped into the left ventricle, through the pulmonary veins, the lung
capillaries and the pulmonary arteries to the pulmonary trunk, from which was led via the above plastic pipe into glass bottles. Approximately two litres of blood diluted with isotonic perfusion liquid were collected and maintained at approximately +37°C until sieving. The blood and the diluted blood were poured onto a fine sieve (≤ 100 μm mesh size). All worms and worm fragments were collected, counted and sexed. In the case of worm fragments we counted the total number of heads and the number of female and male tails (Rosen et al., 1970). The number of worms recorded from the fragments was the total number of heads or the sum of male and female tails (whichever was greater). Viability was checked by observing the movements of the worms in a lined petri dish.

The extent and severity of pneumonic change was assessed semi-quantitatively using a scale from 0 to 3 (0 = normal; 1 = slight; 2 = moderate; 3 = severe). Tissue samples from affected areas were fixed in formalin for subsequent histological examination. The sections were graded according to the extent of pneumonic alteration, frequency of thrombi and the presence of parasites using the same scale. Adult worm recovery was performed by flushing large arteries and veins, bronchi and bronchioli with isotonic solution. Finally, the lung tissue was sliced up finely and flushed again. Additionally, chopped lung tissue was then processed by the Baerman-Wetzel technique in order to isolate L1.

2.5. Statistics

The primary efficacy criterion in response to treatment with Advocate (groups 1 and 2) was the number of worms at necropsy. A one-sided Wilcoxon-Mann-Withney test was applied to compare with the untreated group 3 (significance level = 0.025 adjusted for multiple testing with a-priori ordered hypothesis). Additionally the correlation between the geometric mean of L1 excreted in faeces and the number of adult worms recovered at necropsy was tested with Pearson’s correlation coefficient (= r)

3. Results

The excretion of L1 of *A. vasorum* started from 47 dpi in 4 control dogs of group 3. All dogs of this group excreted L1 at least once before euthanasia. Prepatency varied between 47-55 days. Starting from the day of patency until study end, the mean daily larval output per dog varied between 0.03-8.8 (mean: 3.5, standard deviation, SD: 2.8) larvae per gram of faeces (LPG).

Non-respiratory symptoms (diarrhoea, enlarged mandibular lymph nodes, eye or external genitalia discharge) were observed in several controls before and after experimental inoculation in all groups.
Bloody diarrhoea with reduced feed consumption was observed in one dog of group 3 13 and 14 dpi. The respiratory assessment starting from 41 dpi twice weekly until study end revealed occasional slight inspiratory and expiratory sounds in all groups. Moderate respiratory sounds were auscultated twice in group 3 (both 55 dpi) and once in group 2 (41 dpi). This latter dog from group 2 also showed deepened normal respiratory sounds, cough/retch and stertor once. In group 3 deepened normal respiratory sounds were frequently auscultated in two dogs, stridor once in two dogs and rhonchus once in one dog.

In all dogs from the untreated control group adult parasites were recovered in a range of 49-165 (mean 99, SD 42.8, Table 1) in the post mortem examination. Their viability was confirmed by active movements. There was a strong positive correlation (Pearson’s r = 0.7127) between the geometric mean of the number of worms recovered at necropsy and L1 excreted in faeces. The overall number of male worms (440/788, 55.8%, C.I. 52.3-59.3) was significantly higher than the number of female worms (348/788, 44.2%, C.I. = 40.7-47.7%). The recovery rate of adult parasites as compared with the inoculation dose in this group was 24.5-82.5% (mean: 49.5%). In the lungs processed by the Baermann-Wetzel technique, a mean of 2019 (SD: 1211) L1 were isolated from lungs of dogs from group 3. In contrast, in dogs of group 1 and 2 no parasites were found in the post mortem examination and no larvae were recovered from the processed lungs. Differences concerning the number of worms at necropsy between the treatment groups (1 and 2) and the control group (3) were statistically significant (p<0.0001).

The spectrum of macrosopic and histological changes in the lungs varied considerably between the groups but was highly consistent within each group (Table 2). Generally, the lungs were nearly white since most of the blood had been flushed out. All dogs of group 1 treated 4 dpi had dappled pink to beige blotches of varying size on the pleural surface in all the lobes due to irregular blood content. There was no consolidated pneumonic tissue and the lymph nodes were normal. These findings were confirmed histologically; four tissue samples from each lung were free of pathological changes.

All animals in group 2 treated 32 dpi had a disseminated pattern of pale pink coalescing, slightly consolidated, raised foci (Fig. 1a-b). In 3 dogs these foci were associated with dark red haemorrhagic areas but otherwise the foci usually had a yellow tinge from degrading haemorrhages. Fibrous tags were observed (Fig. 1b). One dog had several foci with an opaque centre. The lymph nodes were not enlarged in this group either. Upon histological examination 7 dogs had disseminated remnants of granulomatous inflammation, 4 of these had arterial thrombi, but parasites were totally absent (Fig.
In contrast, the lungs of all the animals of group 3 were severely affected; large confluent areas were firm, raised, and discoloured from pale beige to yellow to dark red. Haemorrhagic patches were frequent. Some pale beige foci had a central opaque centre (Fig. 2a). The lung lymph nodes were consistently enlarged (Fig. 2b). Thrombi, often with incorporated larvae and eggs, were frequently observed in the histological sections. The arterial walls were thickened. Multiple granulomas consisting mainly of macrophages, multinucleated giant cells and lymphocytes had accumulated around larvae and eggs and were associated with pneumocyte proliferation, haemorrhage and intracellular haemosiderin (Fig. 2c). Additionally, sections of adult specimens of *A. vasorum* in arteries were observed (Fig. 2d).

4. Discussion

After intragastric inoculation of 200 L3 and rinsing with 20 mL of warm tap water, no vomiting was observed and therefore no re-dosing was necessary. A recovery rate of up to 82.5% adult worms/inoculated L3 in dogs of the control group showed that the experimental infection was successful. There is no explanation for the imbalance of sex (55.8 % male and 44.2 % female worms). The prepatent period was 47-55 days and is comparable with other observations of 35-60 days (Rosen et al., 1970). The mean larval output between 47-59 dpi in this group was low (mean LPG of 3.5, SD: 4.3); similar results with a mean LPG of 7.7 (SD: 6.7) between 31-60 dpi were obtained in a study where dogs received 100 L3/kg BW (Oliveira-Junior et al., 2004).

As described in early biological studies (Guilhon and Cens, 1973), four days after inoculation with infectious L3 of *A. vasorum*, larvae have moulted into L4 and are mainly situated in the mesenterial lymph nodes, while around 32 dpi larvae have developed into immature adults situated already in their definitive localisation. Hence, in our inoculated dogs treated 4 dpi (group 1) or 28 days later (group 2) with imidacloprid 10% and moxidectin 2.5% spot-on L4 and immature adults were exposed to treatment. This permitted the evaluation of monthly administration of Advocate® (Bayer AG) to prevent clinical angiostrongylosis. In the presented randomized and blinded experimental study, the efficacy of Advocate® was 100% concerning the development of adult *A. vasorum* as shown by *post mortem* analysis. This result was confirmed by faecal and lung analysis by the Baermann-Wetzel technique, since L1 were only recovered in faeces and lungs of the control group.

Although no adult *A. vasorum* were recovered in dogs of group 2 treated 32 dpi, the *post-mortem*
examinations showed alteration of lungs and pneumonia and thrombi were regularly present, but no parasitic stages were found. This, together with the clinical symptoms, demonstrates the altered lungs were affecting the health of the dogs of group 2 during prepatency. The parasitic stages responsible for these lesions are immature L5 and young adults that did not join sexual maturity yet, stages that are already living in pulmonary arterioles starting from approximately 10 and 30 dpi, respectively (Neff, 1971; Guilhon and Cens, 1973). In contrast, a very early treatment at 4 dpi as performed with dogs in group 1 anticipated further larval development and prevented lung lesions as compared with group 2.

The technique of reverse lung perfusion has been adapted from a method described for captive foxes (Monrad et al., 2007) and was therefore for the first time applied in dogs. It had the advantage that recovered worms were intact and therefore viability and gender of worms could be determined. In a previous study with culled foxes no difference was found in the rate of recovery of *A. vasorum* using dissection or flushing followed by dissection (Morgan et al., 2008). However, as distinguished from our study, the foxes obviously were not pretreated with the anticoagulant heparine and, after removal of heart and lungs, the organs were frozen at -20°C.

Adverse effects of imidacloprid/moxidectin or fenbendazole in naturally infected dogs with *A. vasorum* were mainly diarrhoea (in 14.8 and 21.7% of dogs respectively) and vomiting (18.5 and 13.0% respectively) (Willesen et al., 2007). However, gastrointestinal signs seemed to occur in 20% of untreated naturally infected dogs, as reported in a summary of 45 cases (Ridyard, 2005). In our study mild diarrhoea and other non-respiratory symptoms were sporadically present in treatment and control groups and also before experimental inoculation and were considered as irrelevant. However, bloody diarrhoea and reduced feed consumption 13-14 dpi in one dog of group 3 may indicate a correlation with migrating immature adults. At this time the parasitic stages have mostly completed their migration from the abdominal lymph nodes to the liver and only small numbers are still entering the right ventricle and arterial vessels of the lungs (Guilhon and Cens, 1973). The liver is thus heavily compromised between 10-25 dpi, which has a potentially negative impact on appetite and coagulation in affected animals, as noted in this dog.

Macroscopic and histological analyses of the lesions confirmed the severe damages in the lungs in dogs of the control group 3 as compared with the reduced pathological findings in group 2. The absence of clinical findings in animals of group 1 is confirmed by the very faint lesions noted in the lungs of these dogs.
5. Conclusion

This is the first experimental study demonstrating an anthelmintic larvicidal activity of imidacloprid/moxidectin spot-on (Advocate®, Bayer AG, Leverkusen, Germany) against *A. vasorum* in dogs. Its application was shown to eliminate effectively L4 and immature *A. vasorum* stages. A monthly anthelminthic treatment interval (as recommended as control strategy for dirofilarioses and other helminths; e.g. see ESCCAP.org) further prevents the establishment of adult *Angiostrongylus* stages in the right ventricle and pulmonary arteries and therefore prevents severe clinical signs of angiostrongylosis. Additionally, treatment of travelling dogs during the prepatency can limit the spreading particularly of *A. vasorum* from endemic into non-endemic areas.

Acknowledgements

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References


Fig. 1-2: Pathological lesions and histology of lungs from dogs inoculated with 200 third stage larvae of *Angiostrongylus vasorum* and treated (group 2) with imidacloprid/moxidectin spot-on solution (Advocate®) or left untreated (group 3).

Fig. 1a-c: Lungs of dogs from group 2 (treated 32 dpi).

1a) All lung lobes contained large yellow haemosiderin stained areas and consolidated, raised foci (dog 15).

1b) One lobe was attached to the mediastinum by a fibrous tag and presented dark red haemorrhagic areas (dog 10).

1c) Lung tissue showing granulomatous inflammation with macrophages, multi-nucleated (foreign body) giant cells and lymphoplasmacellulare infiltrates, probably in occluded blood vessels. The adjacent alveoli are partially compressed and contain diffuse accumulations of macrophages and round cells (dog 10).

Fig. 2a-d: Lungs of dogs from group 3 (untreated control group).

2a) This dog had extensive patches of fresh haemorrhages interspersed with pale areas that were consolidated and no longer aerated (dog 1).

2b) The enlarged lung lymph nodes are plainly visible (dog 1).

2c) Lung tissue showing totally solidified alveoli, granulomatous inflammation, larval stages of *A. vasorum* and a large organised thrombus in an artery, also containing larval stages and a wide spectrum of inflammatory cells (dog 6).

2d) Adult specimen of *A. vasorum* within an artery. One end is partially embedded in an older thrombus. The surrounding tissue is consolidated and heavily infiltrated with inflammatory cells (dog 2).
Table 1: Adult *Angiostrongylus vasorum* recovered at *post mortem* examination 56-59 days after experimental inoculation with 200 third stage larvae in the untreated control group (group 3).

<table>
<thead>
<tr>
<th>Dog no.</th>
<th>In blood (m/f)(^a)</th>
<th>In the lung (m/f)(^{a,b})</th>
<th>Total no. of parasites (m/f)(^{a,b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>153 (77/76)</td>
<td>12 (9/3)</td>
<td>165 (86/79)</td>
</tr>
<tr>
<td>2</td>
<td>49 (29/20)</td>
<td>0</td>
<td>49 (29/20)</td>
</tr>
<tr>
<td>3</td>
<td>100 (48/52)</td>
<td>6 (0/2)(^b)</td>
<td>106 (48/54)(^b)</td>
</tr>
<tr>
<td>4</td>
<td>96 (55/41)</td>
<td>2 (1/1)</td>
<td>98 (56/42)</td>
</tr>
<tr>
<td>5</td>
<td>52 (27/25)</td>
<td>5 (1/4)</td>
<td>57 (28/29)</td>
</tr>
<tr>
<td>6</td>
<td>132 (75/57)</td>
<td>2 (2/0)</td>
<td>134 (77/57)</td>
</tr>
<tr>
<td>7</td>
<td>125 (79/46)</td>
<td>4 (2/2)</td>
<td>129 (81/48)</td>
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<tr>
<td>8</td>
<td>54 (35/19)</td>
<td>0</td>
<td>54 (35/19)</td>
</tr>
<tr>
<td>Total</td>
<td>761 (425/336)</td>
<td>27 (15/12)</td>
<td>792 (440/348)(^b)</td>
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<tr>
<td>Mean (SD)</td>
<td>48.0 (19.0)</td>
<td>3.9 (3.7)</td>
<td>99.0 (42.8)</td>
</tr>
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</table>

\(^a\): m/f: male / female parasites.

\(^b\): Count refers to total number of whole parasites and heads or tails, whichever was greater. Parasite heads could not be sexed.
Table 2: Semi-quantitative grading of the histological findings in the lungs of dogs from group 2 (treated 32 dpi) and group 3 (untreated): - = normal; + = slight; ++ = moderate; +++ = severe.

<table>
<thead>
<tr>
<th>Dog no.</th>
<th>Macroscopic findings</th>
<th>Histologic findings</th>
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<tr>
<td></td>
<td></td>
<td>Pneumonia</td>
<td>Thrombi</td>
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<tr>
<td>Group 2 / Advocate® 32 dpi (n=8)</td>
<td></td>
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<tr>
<td>9</td>
<td>+</td>
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a: yellow foci, haemorrhagic patches, pneumonic lobes.