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Efficacy of oral afoxolaner plus milbemycin oxime chewables against induced gastrointestinal nematode infections in dogs

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ABSTRACT

The efficacy of oral afoxolaner plus milbemycin oxime combination chewables against induced gastrointestinal nematode infections in dogs was evaluated in six separate studies. Two studies were performed to evaluate the efficacy of the product against *Toxocara canis*, two studies evaluated the efficacy against *Toxascaris leonina*, one study evaluated the efficacy against *Ancylostoma braziliense*, and one study evaluated the efficacy against *Ancylostoma caninum*. In the *A. caninum* study, the efficacy of milbemycin oxime alone and afoxolaner alone was also evaluated. Dogs in all studies were inoculated with infective eggs or larvae and confirmed to have patent infections based on a fecal examination prior to allocation to study group and treatment.

Each study utilized a randomized block design with blocks based on pre-treatment body weight. All dogs were assigned to blocks based on body weight, and then each dog within a block was randomly assigned to treatment group. There were two groups of 10 dogs each in the *T. canis, T. leonina*, and *A. braziliense* studies: 1) an untreated (control) group and 2) a group treated with afoxolaner plus milbemycin oxime chewables (NexGard Spectra®, Merial). This group was treated at a dose as close as possible to the minimum effective dose of afoxolaner and milbemycin oxime (2.5 mg + 0.5 mg per kg body weight, respectively) once on Day 0 using whole chews. There were four groups of 10 dogs each in the *A. caninum* study: 1) untreated (control), 2) NexGard Spectra® as described above, 3) milbemycin oxime alone (dose of at least 0.5 mg per kg of body weight) and 4) afoxalaner alone (dose of at least 2.5 mg per kg body weight). For parasite recovery and counts, dogs were euthanized humanely and necropsied seven days after treatment.

The efficacy of the afoxolaner plus milbemycin oxime combination was ≥98% against *T. canis,* ≥95.8% against *T. leonina,* and 90.2% against *A. braziliense.* Efficacy of the combination against *A. caninum* was 99.7%, while the efficacy of milbemycin oxime alone was 99.6% and the efficacy of afoxolaner alone was 2.1%. Dogs treated with afoxolaner plus milbemycin oxime chewables had significantly ($p \leq 0.0002$) fewer nematodes than the untreated controls in all studies. There were no adverse events or other health problems that were related to treatment with Nexgard Spectra® in these studies.

The results of these controlled studies demonstrate the high efficacy of the afoxolaner plus milbemycin oxime chewables against a broad range of canine intestinal nematode infections.

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1. Introduction

Gastrointestinal parasites, including ascarids, hookworms, whipworms, *Giardia* sp. and *Cystoisospora* spp., are very common in dogs and cats and are among the most common infectious agents encountered by veterinarians in companion animal practice (Little et al., 2009). Despite recognized advances in the treatment and control of gastrointestinal nematode infections, these parasites are still prevalent worldwide. The prevalence in dogs in the United States ranges from 6.1% in owned dogs to 52% in shelter dogs (Blagburn et al., 1996; Little et al., 2009; Blagburn et al., 2014). Similar results have been demonstrated in owned vs stray and shelter dogs in Europe (Borecka, 2005; Orhun and Ayaz, 2006; Kozan et al., 2007; Martínez-Carrasco et al., 2007; Unlü and Eren, 2007; Ortuño et al., 2014; Simonato et al., 2015).

In addition to health concerns for pets, some ascarid and hookworm infections are also a public health concern as the fecal droppings contaminate the environment and infective development stages may cause human infections such as cutaneous larva migrans, eosinophilic enteritis, visceral larva migrans, ocular larva migrans, and other less well-defined clinical syndromes (Croese et al., 1994; Khoshoo et al., 1995; Prociv and Croese, 1996; Bowman et al., 2010; Moreira et al., 2014; Woodhall et al., 2014). The aggregation of dogs and humans in public areas, e.g., parks and playgrounds, creates the ideal interface for zoonotic infection due to the widespread fecal contamination of these places (Chorazy and Richardson, 2005; Traversa, 2012). As there are no practical methods for reducing environmental egg levels once present, prevention of contamination of the environment is the most important approach to reduce exposure to humans (Overgaauw and van Knapen, 2013). Environmental contamination can be reduced by restriction of free-roaming dogs and cats, prevention and/or quick removal of pet feces in public areas, hygiene, educating the public, and eliminating patent infections in dogs and cats through anthelmintic treatments/regular anthelmintic use (Glickman and Shofer, 1987; Overgaauw and van Knapen, 2013).

Previous studies have demonstrated the ability of milbemycin oxime alone, as well as in combination with other actives such as ectoparasiticides or other anthelmintic compounds, to kill the common gastrointestinal parasites of dogs, including *T. canis, T. leonina, A. braziliense,* and *A. caninum* (Nolan, 2012; Schnitzler et al., 2012; Bienhoff et al., 2013). However, none of the previous combination products provided efficacy against ticks, which are an important parasite to veterinarians and pet owners globally. The new combination of afoxolaner, a newly commercialized isoxazoline insecticide and acaricide (Letendre et al., 2014; Shoop et al., 2014), and milbemycin oxime is the first to provide efficacy against ticks as well as fleas, heartworms, and gastrointestinal nematodes in a single, easy to administer chewable formulation (NexGard Spectra®, Merial; European Medicines Agency, 2015). Thus, six separate experiments were conducted to confirm the anthelmintic efficacy of this novel combination of afoxolaner and milbemycin (NexGard Spectra®) against experimentally induced gastrointestinal nematode infections in dogs.

2. Material and methods

Six separate studies were performed to test the efficacy of the afoxolaner plus milbemycin oxime combination against experimental intestinal nematode infections in dogs. Two studies were performed to evaluate the efficacy against *T. canis* (Studies 1 and 2), two evaluated efficacy against *T. leonina* (Studies 3 and 4), one study evaluated efficacy against *A. braziliense* (Study 5) and one evaluated efficacy against *A. caninum* (Study 6). In Study 6, two additional groups were included in addition to the untreated control group and the group treated with the afoxolaner and milbemycin oxime combination product. One of the additional groups was treated with milbemycin oxime alone and the other was treated with afoxolaner alone. These groups were included to evaluate the efficacy of milbemycin alone and afoxolaner alone to demonstrate lack of interference between the actives.

Each study was performed in accordance with the "World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines for evaluating the efficacy of anthelmintics for dogs and cats" (Jacobs et al., 1994), the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products VICH GL7, "Efficacy of Anthelmintics: General Requirements" (Vercruysse et al., 2001) and VICH GL19 "Efficacy of Anthelmintics: Specific Recommendations for Canines" (Vercruysse et al., 2002). All studies were conducted in compliance with Good Clinical Practices (VICH GL9). All study procedures complied with the appropriate local animal welfare regulations and were approved by either the appropriate Ethics Committee for studies conducted in South Africa or by the Merial Independent Animal Care and Use Committee for studies conducted in the United States.

All personnel that were involved in efficacy or safety data collection or assessment in all studies were blinded to treatment assignment of the animals throughout the entire study period.

2.1. Experimental animals

The studies were conducted in the United States (Studies 1, 3, 5 and 6) and in the Republic of South Africa (Studies 2 and 4). Twenty dogs were enrolled in all studies except Study 6, in which 40 dogs were enrolled. An additional two to four dogs were inoculated in each study (except Study 4) to ensure that an adequate number of dogs were infected for the study. The dogs were Beagles (Studies 1, 3, 5, and 6) or mixed-breed mongrels (Studies 2 and 4) that were purpose bred for research purposes. The dogs were of a mixed sex ratio in all studies except Study 6, in which only male dogs were used. Young dogs, approximately three to six months of age, were used in all studies. All dogs were dewormed with an oral dewormer (Drontal® Plus, Bayer Animal Health, in Studies 1, 3, and 5; Antezole® Liquid, Kyron Laboratories (Pty) Ltd., in Studies 2 and 4; Pyrantel Pamoate Suspension, Columbia Laboratories, in Study 6) 14 days before inoculation. In addition, fecal samples were collected once from all dogs prior to inoculation and examined to confirm the absence of patent infection of any gastrointestinal nematode. Animal details are presented in Table 1.

All dogs enrolled in each study underwent a physical examination prior to treatment and were considered to be suitable for inclusion into the study. All dogs were housed individually starting at least prior to treatment through the end of the study. All dogs within each study were housed in identical housing conditions in the same facility. All dogs received a commercial dry food diet and fresh water was freely available at all times.

2.2. Experimental infections of dogs

Each dog in Studies 1 and 2 was inoculated via the oral route with approximately 100 (Study 1) or 150 (Study 2) *T. canis* infective eggs 56, 55, and 54 days prior to treatment. The parasite strain used in Study 1 was isolated directly from a donor dog from Michigan, US in 2012 and the strain used in Study 2 was isolated from a naturally infected dog in Virginia, US in 2007 and was passaged once in the laboratory prior to use in this study. Dogs in Studies 3 and 4 were inoculated via the oral route with approximately 200 *T. leonina* infective eggs 77, 76, and 75 days prior to treatment. The strain used in Study 3 was isolated directly from a donor dog in Michigan, US in 2012 and Study 4 utilized a strain isolated in North

Table 1
Characteristics of study animals and results of pre-treatment fecal examination.

Study/Group	Sex[a]	~Age (months)	Pre-treatment[b] Body weight (kg)	Actual dosage [mg/kg body weight, mean (range)]		Pre-treatment[c] fecal examination
				Milbemycin oxime	Afoxolaner	EPG[d] range
Study 1—*Toxocara canis*						
Untreated	5 M, 5 F	4.0–4.1	6.30–8.50	NA[e]	NA	199–1854
Oral AFX + MO[f]	5 M, 5 F	4.0–4.1	5.85–8.30	0.60 (0.50–0.72)	3.00 (2.50–3.61)	69–2530
Study 2—*Toxocara canis*						
Untreated	6 M, 4 F	3.7–3.8	5.21–8.63	NA	NA	133–4533
Oral AFX + MO	4 M, 6 F	3.7–4.3	5.38–8.35	0.63 (0.50–0.75)	3.14 (2.51–3.73)	133–8600
Study 3—*Toxascaris leonina*						
Untreated	4 M, 6 F	4.7–4.8	5.25–9.40	NA	NA	24–451
Oral AFX + MO	6 M, 4 F	4.8–4.9	5.85–9.35	0.61 (0.53–0.73)	3.05 (2.66–3.63)	59–1422
Study 4—*Toxascaris leonina*						
Untreated	7 M, 3 F	5.2–6.3	7.46–12.78	NA	NA	67–1000
Oral AFX + MO	7 M, 3 F	5.2–6.3	7.08–13.27	0.58 (0.51–0.66)	2.89 (2.53–3.29)	200–1200
Study 5—*Ancylostoma braziliense*						
Untreated	7 M, 3 F	5.0–5.7	6.65–11.45	NA	NA	39–991
Oral AFX + MO	4 M, 6 F	5.0–5.7	7.20–10.25	0.58 (0.51–0.69)	2.88 (2.57–3.45)	28–495
Study 6—*Ancylostoma caninum*						
Untreated	10 M	3.7–4.6	4.50–8.40	NA	NA	709–2046
Oral AFX + MO	10 M	3.7–4.6	4.85–7.95	0.65 (0.53–0.77)	3.25 (2.64–3.87)	985–1725
Oral MO[g]	10 M	3.7–4.6	4.90–8.25	0.65 (0.52–0.77)	NA	814–1963
Oral AFX[h]	10 M	3.6–5.0	5.00–7.70	NA	3.60 (2.94–4.52)	1014–2127

[a] M = male, F = female.
[b] Day-3, Day-2 or Day-1 prior to treatment (= Day 0).
[c] Day-10, Day-9, Day-8, Day-7, or Day-4 prior to treatment (= Day 0).
[d] EPG = eggs per gram of feces.
[e] NA = Not applicable.
[f] AFX + MO = afoxolaner (minimum 2.5 mg/kg) + milbemycin oxime (minimum 0.5 mg/kg).
[g] MO = milbemycin oxime (minimum 0.5 mg/kg).
[h] AFX = afoxolaner (minimum 2.5 mg/kg).

Carolina, US in 2010 and maintained in the laboratory until used in this study. Dogs in Study 5 were each inoculated with approximately 100 third-stage *A. braziliense* 28, 27 and 26 days prior to treatment with a strain isolated from cats in Florida, US and maintained in dogs and cats in the laboratory. In Study 6, each dog was inoculated orally with *A. caninum* larvae from a strain isolated from a naturally infected dog in Tennessee, US and passaged in the laboratory for use in this study. The exact number of larvae used per dog in Study 6 is unknown due to an error during preparation of the inoculum, however each animal received the same inoculum volume from a common infective source, so all dogs received approximately the same number of *A. caninum* larvae.

2.3. Selection of animals based on fecal egg counts

Only animals with patent infection as confirmed by coproscopic screening of fecal samples collected prior to treatment were included. Fecal samples were collected once between three and ten days prior to treatment and fecal egg counts were performed by either a quantitative centrifugal fecal flotation (Studies 1, 3, 5 and 6) or a standard McMaster's technique (Studies 2 and 4) to confirm that all enrolled dogs were shedding eggs of the target nematode. In studies in which additional dogs were inoculated, the dogs with the lowest fecal egg counts or with health concerns were excluded. Pre-treatment fecal egg count ranges are listed in Table 1.

2.4. Experimental design, treatment, and parasite recovery and counts

All studies were conducted under separate but similar protocols utilizing a randomized block design. For allocation to treatment groups, blocks of two dogs each (four dogs each in Study 6) were formed sequentially, based on decreasing pre-treatment body weight. Within blocks, dogs were randomly allocated to one of the treatment groups via coin toss (Studies 1, 3, and 5), a computer based randomization procedure (Studies 2 and 4), or via lottery (Study 6). Animals were randomly allocated to two groups for Studies 1 through 5: 1) untreated (control) or 2) treated with afoxolaner plus milbemycin oxime chewables (NexGard Spectra®, Merial). In Study 6, dogs were randomly allocated to one of four treatment groups: 1) untreated (control); 2) treated with afoxolaner plus milbemycin oxime chewables (NexGard Spectra®, Merial); 3) treated with milbemycin alone chewables; or 4) treated with afoxolaner alone chewables.

For treatment with afoxolaner plus milbemycin oxime, three sizes of chewable tablets were used: 0.5 g, 1 g, and 2 g chewables containing 9.375 mg + 1.875 mg, 18.75 mg + 3.75 mg, and 37.5 mg + 7.5 mg of afoxolaner plus milbemycin oxime, respectively. In Study 6, milbemycin was administered in chewables of two sizes: 0.5 g (containing 1.875 mg milbemycin) and 1 g (containing 3.75 mg milbemycin) and afoxolaner was administered in chewables of 0.5 g containing 11.3 mg afoxolaner. Chewables of the different sizes were combined as appropriate in order to achieve dosing of the dogs as close as possible to the minimum effective dose of 2.5 mg/kg afoxolaner or 0.5 mg/kg milbemycin oxime. The resulting actual dose ranges are listed in Table 1. In all studies, food was removed from all dogs on the afternoon prior to treatment and dogs were fasted until approximately four hours after the treatment was administered. Treatments were administered orally once on Day 0 and all dogs were observed for health problems and adverse events hourly for four hours post-treatment and thereafter at least once daily until the end of the study.

To obtain a total gastrointestinal nematode count, all study animals were euthanized humanely and necropsied seven days after treatment. The contents of the whole gastrointestinal tract (stomach, small intestine, and large intestine including cecum) were collected. For isolation and counting of nematodes, organ contents were washed over appropriate sized sieves to remove most of the

Table 2
Nematode counts and therapeutic efficacy in dogs of oral afoxolaner plus milbemycin oxime chewables, milbemycin oxime alone chewables, and afoxolaner alone chewables against induced infections of nematode parasites.

Parasite	Study	NI/NG[a]	GM[b] (Range)	NI/NG	GM (Range)	Efficacy(%)[c]	P-Value[d]
		Untreated (control)		Oral AFX + MO[e]			
Toxocara canis	1	10/10	12.6 (5–27)	2/10	0.3 (0–4)	98.0	<0.0001
Toxocara canis	2	10/10	22.4 (12–50)	0/10	0.0	100.0	<0.0001
Toxascaris leonina	3	10/10	20.5 (12–32)	5/10	0.9 (0–9)	95.8	<0.0001
Toxascaris leonina	4	10/10	26.9 (1–65)	4/10	1.1 (0–17)	96.0	0.0002
Ancylostoma braziliense	5	10/10	68.5 (20–141)	10/10	6.7 (2–29)	90.2	<0.0001
Ancylostoma caninum	6	10/10	535.3 (458–675)	6/10	1.8 (0–8)	99.7	<0.0001
				MO[f]			
				6/10	2.0 (0–20)	99.6	<0.0001
				AFX[g]			
				10/10	523.9 (449–598)	2.1	0.6636

[a] NI/NG: Number of dogs infected with adult worms/Number of dogs in Group.
[b] GM: Geometric mean nematode count (based on transformation to ln [count + 1]).
[c] Efficacy = 100 [(geometric mean untreated (control) – geometric mean Oral AFX + MO)/geometric mean untreated (control)].
[d] Two-sided *p*-value comparing the worm burden of the Oral AFX + MO group with the Untreated (control) group.
[e] AFX + MO: afoxolaner (minimum 2.5 mg/kg) + milbemycin oxime (minimum 0.5 mg/kg).
[f] MO: milbemycin oxime (minimum 0.5 mg/kg).
[g] AFX: afoxolaner (minimum 2.5 mg/kg).

debris. Counts of parasites were made on total gastrointestinal contents. The nematodes recovered were identified based on their morphology.

2.5. Data analysis

Parasite counts for the target parasite in each individual study were transformed to the natural logarithm of (count + 1) for calculation of geometric means for each treatment group. Efficacy for each treated group was calculated as the percent efficacy using the formula $100 \times [(C - T)/C]$, where C is the geometric mean among untreated controls and T is the geometric mean among the treated animals. The log-counts of the treated groups were compared to the log-counts of the untreated control group using an F-test adjusted for the allocation blocks. The Mixed procedure in SAS® Version 9 was used for this analysis with Treatment Group listed as the fixed effect and allocation blocks listed as the random effect. Testing was two-sided at the significance level $\alpha = 0.05$.

3. Results

No adverse events or other health problems were observed during Studies 2, 3, 4, and 5. In Study 1, at the 1-h post-treatment observation, one untreated control dog had vomited and one afoxolaner plus milbemycin treated dog had loose stool containing blood and mucus. In Study 6, vomiting, bloody diarrhea, and inappetence were observed in animals from all groups starting approximately two weeks after inoculation. Retrospectively, these abnormalities were consistent with inadvertent inoculation of greater numbers of *A. caninum* larvae than intended. The incidence of abnormal health events in these young dogs was similar among animals in all groups, both before and after treatment.

VICH GL19 requires that at least six dogs in each untreated (control) group harbour at least five adult nematodes to consider that the animals were adequately infected and the study was valid. This requirement was met in all six studies reported here as at least nine dogs in each untreated (control) group met the requirements for adequate infection. The results of the individual studies including parasite counts and calculation of percent efficacy are summarized in Table 2.

Dogs treated with afoxolaner plus milbemycin oxime chewables or milbemycin oxime chewables alone had significantly ($p \leq 0.0002$) fewer nematodes than the untreated (control) dogs in all studies. The efficacy of the afoxolaner plus milbemycin oxime combination chewables was ≥98% against adult *T. canis* (Studies 1 and 2), ≥95.8% against adult *T. leonina* (Studies 3 and 4) and 90.2% against adult *A. braziliense* (Study 5). Efficacy of the afoxolaner plus milbemycin oxime combination chewables against adult *A. caninum* was 99.7% while the efficacy of milbemycin oxime only chewables was 99.6% (Study 6). Adult *A. caninum* counts of dogs treated with afoxolaner only chewables did not differ from the counts of the untreated (control) animals ($p = 0.6636$; Study 6) and thus confirmed that, as expected, afoxolaner has no anthelmintic efficacy against *A. caninum*.

4. Discussion

Roundworms and hookworms are important parasites of dogs worldwide. While the incidence of these parasites varies based on age of dog, level of care, and geographic location, it is well accepted that there is still a continuous need to monitor dogs for intestinal parasites and treat infected animals with effective parasiticides. The use of monthly heartworm preventives can also be an important option, especially in *Dirofilaria immitis* endemic regions, as these products also kill gastrointestinal nematodes.

The studies presented here demonstrate that the combination of afoxolaner and milbemycin oxime (NexGard Spectra®, Merial) effectively controls the common roundworms (*T. canis, T. leonina)* and hookworms (*A. caninum, A. braziliense*) of dogs. In this set of studies, parasites of US origin were used to induce infections in dogs. The results are complemented by and consistent with results of other studies in dogs from Europe or South Africa with naturally acquired infections of adult *T. canis, T. leonina, A. braziliense,* and/or *A. caninum* (Rehbein et al., 2016a).

The anthelmintic efficacy of milbemycin oxime is documented for oral products containing the compound individually, as well as when it is combined with other actives that provide complementary anthelmintic efficacy or activity against fleas (Nolan, 2012; Schnitzler et al., 2012; Bienhoff et al., 2013). The results of the studies presented here confirm the excellent efficacy of Nexgard Spectra® chewables against experimental infections of adult *T. canis, T. leonina, A. braziliense,* and *A. caninum*. These results also indicate that there is no interference between the two compounds with respect to the anthelmintic activity. One of the six studies reported here specifically investigated the lack of interference of the combination on the anthelmintic activity of milbemycin oxime

against *A. caninum* by including groups treated with each active alone as well as a group treated with the combination product. This study confirmed the lack of interference as there was no difference in the efficacy of the combination product (99.7%) and the milbemycin oxime only treatment (99.6%). Similar results in terms of comparable efficacy levels were established in three exploratory studies using the afoxolaner plus milbemycin oxime combination and milbemycin oxime alone chewable formulations. In these studies, efficacy against naturally acquired adult *A. caninum* was 95.7% and 96.5% with the combination and milbemycin oxime only chewable, respectively; 98.8% (combination) and 94.5% (milbemycin oxime alone) against naturally acquired adult *Trichuris vulpis*; and >99% (combination) and 100% (milbemycin oxime alone) against induced adult *T. canis* (Merial, unpublished results).

These studies, along with other controlled studies in dogs with naturally acquired infections of adult *T. canis, T. leonina, A. braziliense, A. caninum*, and *Trichuris vulpis* (Rehbein et al., 2016a), demonstrate that the novel afoxolaner plus milbemycin oxime chewable formulation is an efficacious and safe treatment of canine adult intestinal nematode infections. In addition, the results of the controlled laboratory studies have been confirmed in multi-center field studies conducted in ten countries of Europe and in Japan where dogs treated with the novel afoxolaner plus milbemycin oxime chewable formulation had >97% fecal egg count reductions for *Toxocara* sp., *Toxascaris* sp., hookworms, *Trichuris* sp. and *Capillaria* sp. nematodes after a single treatment (Rehbein et al., unpublished results; Otsuki et al., unpublished results).

In conclusion, orally administered afoxolaner plus milbemycin oxime chewables were proven to be highly efficacious against experimental infections of dogs with the common roundworms and hookworms. This efficacy, in combination with the proven efficacy against *T. vulpis* (Rehbein et al., 2016a), prevention of heartworm disease (Tielemans et al., unpublished results) and treatment and control of infestation with fleas and ticks (European Medicines Agency, 2015; Rehbein et al., 2016b), demonstrates that afoxolaner plus milbemycin oxime chewables offer broad spectrum protection covering the common external and internal parasites of dogs.

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Conflict of interest

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