Parasitol Res (2016) 115:1845–1851
DOI 10.1007/s00436-016-4924-6

ORIGINAL PAPER

Efficacy of oral afoxolaner plus milbemycin oxime chewables against induced infestations with *Dermacentor reticulatus* in dogs

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Received: 5 October 2015 / Accepted: 14 January 2016 / Published online: 27 January 2016

Abstract The efficacy of afoxolaner plus milbemycin oxime (AFX + MO) combination chewables (NexGard Spectra®, Merial) and AFX single-entity chewables (NexGard®, Merial) against induced infestations with *Dermacentor reticulatus* ticks was evaluated in dogs. Thirty dogs were assigned to blocks of three animals each based on pre-allocation tick counts and were randomly allocated to one of three groups: untreated (control), treated with a combination of AFX + MO chewables to be as close as possible to the minimum effective dose of AFX + MO (2.5 + 0.5 mg per kg body weight), and treated with a combination of NexGard® chewables to be as close as possible to the minimum effective dose of AFX (2.5 mg per kg body weight). Treatments were administered orally once on day 0. Starting 2 days before treatment administration, each dog was infested with approximately 50 ticks weekly for six consecutive weeks. Live ticks were counted at ~48 h post-treatment (removal count) and at ~48 h (in situ counts) and ~72 h (removal counts) following each post-treatment infestation. Treatment with both AFX + MO and NexGard® chewables rapidly eliminated the existing tick infestations (100 % efficacy) within 2 days following treatment administration. Weekly re-infestations were controlled for a minimum of 5 weeks with the efficacy ranging from 92.2 to 99.7 % based on ~48 h post-treatment in situ counts and between 99.0 and 100 % based on ~72 h post-treatment removal counts ($p < 0.0001$ at each occasion). This study demonstrated a high efficacy of both AFX + MO chewable and NexGard® chewable treatments against infestations of dogs with *D. reticulatus* ticks for at least 5 weeks. In addition, this study indicated no interference between the two compounds with respect to the acaricidal activity provided by AFX.

Keywords *Dermacentor reticulatus* · Tick · Afoxolaner · Milbemycin oxime · Dog

Introduction

Beside mosquitoes and fleas, hard ticks (Acari: Ixodidae) constitute the most important ectoparasites of dogs worldwide being adapted to various environmental conditions. Engorgement on dogs is usually painless and the amount of blood drawn trivial, but tick attachments may result in local skin reaction (e.g., redness, swelling, irritation). However, the real significance of ixodid ticks infesting dogs is the broad range of pathogens that ticks can transmit. These pathogens include viral, bacterial, and protozoan disease agents. In addition, but more of geographically restricted importance, some species of ticks are able to induce paralysis and/or toxicosis.

Increasing incidence of tick-borne infections and awareness among veterinarians, physicians, and pet owners, growing travel between countries of people and pets, and changes in the abundance and distribution of ticks have prompted several studies on the tick-domestic dog association in Europe in the recent past.

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The "brown dog tick" or "kennel tick," *Rhipicephalus sanguineus* (s. l.), constitutes probably the most widespread tick infesting dogs worldwide. *R. sanguineus* (s. l.), preferably found in urban and suburban areas, is the pre-dominant tick infesting dogs in the south temperate regions of Europe, mainly in countries bordering the Mediterranean Sea and the Black Sea (e.g., Grandes 1986; Gilot et al. 1992; Papadopoulos et al. 1996; Evstaf'ev and Tovpinec 2002; Milutinović and Radulović 2002; Papazahariadou et al. 2003; Marotel 2006; Torina et al. 2006; Xhaxhiu et al. 2009; Otranto and Dantas-Torres 2010; Mateescu et al. 2011; Omeragic 2011; Santos-Silva et al. 2011; Kirkova et al. 2013; Maia et al. 2014). The most common tick found attached to dogs in the rest of Europe including the British Isles, Scandinavia, and Iceland is the "sheep tick" or "castor bean tick," *Ixodes ricinus*, which has an exceptionally wide host range and had been recorded from many different biotopes. Other *Ixodes* species parasitizing dogs with substantial lower prevalence in Europe are *Ixodes hexagonus*, *Ixodes canisuga*, and *Ixodes persulcatus* (e.g., Jaenson et al. 1994; Papadopoulos et al. 1996; Ogden et al. 2000; Pavlović et al. 2002; Dautel et al. 2006; Marotel 2006; Földvári et al. 2007; Nijhof et al. 2007; Bona et al. 2011; Bugmyrin et al. 2011, 2013; Omeragic 2011; Smith et al. 2011; Claerebout et al. 2013; Duscher et al. 2013; Richter et al. 2013). In addition, *Dermacentor reticulatus* ("European meadow tick" or "marsh tick") has been reported recently with increasing frequency infesting dogs in western and central Europe (e.g., Dautel et al. 2006; Marotel 2006; Nijhof et al. 2007; Smith et al. 2011; Claerebout et al. 2013; Duscher et al. 2013; Krčmar et al. 2014). Some surveys from Germany, Hungary, Poland, and Romania demonstrated that *D. reticulatus* attached to dogs at a higher prevalence than *I. ricinus* or *R. sanguineus* (s. l.) (Földvári and Farkas 2005; Zygner and Wędrychowicz 2006; Tudor et al. 2010; Beck et al. 2014). Although *D. reticulatus* has probably been present in focal occurrence in several countries of Europe for a long time, the increasing observations of *D. reticulatus* on dogs indicate that the distribution of this tick has expanded substantially and is continuing across the continent including the UK (Sréter et al. 2005; Dautel et al. 2006; Bullová et al. 2009; Medlock et al. 2011; Široky et al. 2011; Karbowiak 2014; Paulauskas et al. 2014; Chitimia-Dobler 2015; Jongejan 2015).

Apart from avoidance of dogs accessing tick habitats and physical removal of ticks attaching to dogs, regular use of acaricidal products is the key element of successful treatment and control of canine tick infestation. Topical preparations with non-systemic compounds which kill ticks through direct contact to the active ingredient after dispersal over the body surface historically were the only available products for tick control on dogs. Recently, orally administered, systemically acting compounds of the isoxazoline class, including afoxolaner, were developed and authorized as ectoparasiticides for dogs (Letendre et al. 2014; Shoop et al. 2014). Laboratory studies demonstrated that NexGard® chewable tablets (afoxolaner at minimum 2.5 mg per kg body weight) are highly efficacious against existing and new infestation of all three major species of ticks infesting dogs in Europe for at least 4 weeks (Dumont et al. 2014; Kunkle et al. 2014). Modeling of plasma concentrations and efficacy data suggested that *Dermacentor* ticks may be the least sensitive ectoparasites to afoxolaner (European Medicines Agency 2014). *D. reticulatus* therefore was selected to confirm the therapeutic and preventive efficacy against ticks of a novel beef-flavored chewable formulation containing afoxolaner in combination with the macrocyclic lactone, milbemycin oxime (NexGard Spectra®, Merial). The evaluation was done in direct comparison to the afoxolaner single-entity formulation, NexGard® chewables, in order to confirm the clinical equivalence of the two products.

Material and methods

The design of the study was in accordance with the "World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines for evaluating the efficacy of parasiticides for the treatment, prevention and control of flea and tick infestation on dogs and cats" (Marchiondo et al. 2013). The study was conducted in compliance with VICH GL9, entitled *Good Clinical Practice*, and in compliance with local animal welfare legislation and was approved by both the Merial and an Independent Animal Care and Use Committee. This was a blinded study, i.e., all personnel involved in collecting efficacy data and making health observations were masked to the treatment assignment of the animals.

Experimental animals

The study was conducted in the Republic of South Africa and included 30 male and female Beagle or mixed breed domestic dogs (mongrels), older than 7 months and weighing more than 6.97 kg at the time of treatment (details are presented in Table 1). No dog had been treated with any ectoparasiticide within 3 months before inclusion into the study. All dogs enrolled in the study were subjected to a physical examination before treatment and considered in good health and suitable for inclusion into the study.

The animals were housed individually during the entire study and acclimated to the study conditions for 7 days prior to treatment. All dogs received a commercial dry food diet once daily as per manufacturer's recommendation, and fresh water was freely available all the time. The environmental conditions were identical for all animals within the study.

Table 1 Characteristics of the study animals and actual dosage of treatments

Group	Sex	Breed	Age	Pre-treatment (day −4) body weight, kg	Actual dosage (mg/kg body weight, mean [range])	
					AFX	MO
Control, untreated	5 M, 5 F	5 Be, 5 Mo	7 months–10.3 years	12.66–19.83	Not applicable	Not applicable
AFX + MO	6 M, 4 F	6 Be, 4 Mo	1.7–8.5 years	6.97–16.28	2.89 (2.62–3.05)	0.58 (0.52–0.61)
NexGard® (AFX)	7 M, 3 F	5 Be, 5 Mo	1.5–5 years	11.57–18.48	2.74 (2.54–2.93)	Not applicable

M male, *F* female, *Be* beagle, *Mo* mongrel, *AFX* afoxolaner, *MO* milbemycin oxime

Selection of animals based on pre-allocation tick infestation

For selection of dogs for inclusion into the study, 40 dogs were infested with 50 (±5) unfed *D. reticulatus* ticks during the acclimation period (day −6). On day −4, ticks were removed from the dogs, counted, and categorized to establish total live-attached tick counts, and the 30 dogs with the highest tick counts were selected for the study. Each dog included in the study harbored between 22 and 44 live-attached ticks corresponding to a retention rate ranging from 44 to 88 % which was greater than the minimum 20 % retention rate recommended for inclusion of animals in tick efficacy studies by WAAVP (Marchiondo et al. 2013)

Experimental design, treatment, and tick counts

For allocation to treatment groups, blocks of three dogs each were formed sequentially, based on decreasing pre-allocation (day −4) live-attached *D. reticulatus* tick counts, and dogs were allocated at random to one of three treatment groups: untreated (control), treated with a combination of afoxolaner plus milbemycin oxime chewables (NexGard Spectra®, Merial), and treated with a combination of NexGard® (afoxolaner; Merial) chewables.

For treatment, two sizes of each type of chewables were used: 0.5 and 1 g afoxolaner plus milbemycin oxime chewables containing, respectively, 9.375 + 1.875 and 18.75 + 3.75 mg of afoxolaner plus milbemycin oxime and 0.5 and 1.25 g NexGard® (afoxolaner) chewables containing, respectively, 11.3 and 28.3 mg of afoxolaner. Chewables 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 afoxolaner plus 0.5 mg milbemycin oxime or 2.5 mg afoxolaner per kg body weight, respectively. Treatments were administered orally once on day 0 to the dogs after feeding. Dogs were observed for health problems and adverse events at approximately hourly intervals for 4 h post-treatment and thereafter at least once daily until the end of the study.

On days −2, 7, 14, 21, 28, and 35, all dogs were infested with each 50 (±5) unfed adult *D. reticulatus* ticks with approximately equal sex ratio. Ticks used in this study were from a laboratory-maintained population that had been established from ticks originating from field locations in Europe.

Counting of live ticks was performed by parting and feeling through the dog's hair with the finger tips. When a suspected tick was found, the hair was further parted, visual confirmation of the tick's presence was made, and the tick was removed on day 2 (~48 h after treatment) and on days 10, 17, 24, 31, and 38 (~72 h after each infestation). After an area was cleared by this method, a flea comb was applied to the area for secondary confirmation of tick removal. In situ (thumb) tick counts were performed in the same manner on days 9, 16, 23, 30, and 37 (~48 h after infestation each), but ticks were only visually inspected after detection and not removed, and dogs were not combed. The viability (living/dead status) of the ticks was determined in situ and at removal as described elsewhere (Marchiondo et al. 2013).

Data analysis

To evaluate the therapeutic efficacy (efficacy against day −2 infestation) and the efficacy in the prevention of *D. reticulatus* tick infestations (efficacy against day 7, 14, 21, 28, and 35 infestations), the live tick count of each dog was transformed to the natural logarithm of (count +1) for calculation of the geometric mean by treatment group at each time point. Percent efficacy of each treated group with respect to the control group was calculated using the formula $[(C-T)/C]\times 100$, where C is the geometric mean for the control group and T is the geometric mean for the treated group. The log counts of each treated group were compared to the log counts of the untreated control group and each other using a two-factor analysis of variance with blocks considered random effects. The mixed procedure in SAS® version 9 was used for analyses. Only those treatment groups being compared were included in the model. Testing was two-sided at the significance level $\alpha = 0.05$.

Results

No adverse events or other health problems were observed throughout the study, indicating that the treatment with afoxolaner plus milbemycin oxime chewables and NexGard® (afoxolaner) chewables was well accepted.

The dogs weighed between 6.97 and 18.48 kg at the time of treatment, and based on the combination of chewables, the dose of afoxolaner that they received therefore varied between 2.62 and 3.05 mg/kg or 2.54 and 2.93 mg/kg for the animals treated with afoxolaner plus milbemycin or NexGard® (afoxolaner), respectively (Table 1).

As summarized in Table 2, mean number of live *D. reticulatus* ticks in the untreated control dogs ranged from 20.1 to 29.0 for the in situ counts conducted ~48 h following post-treatment infestations and ranged from 26.2 to 32.1 for the removal counts established ~48 h following treatment (day 2) or ~72 h following post-treatment (re-)infestations. Mean retention rate of the experimental tick infestation was >40 % at each occasion (and ≥26 % for each individual dog). Thus, the dogs demonstrated an adequate level of infestation as per WAAVP guidance which indicates that a minimum retention rate of ticks should be at least 20 % to allow for a valid assessment of tick efficacy (Marchiondo et al. 2013), and the study was therefore considered valid.

The *D. reticulatus* tick counts of the dogs in the groups treated with afoxolaner plus milbemycin and with NexGard® (afoxolaner) were significantly ($p < 0.0001$) lower compared to the untreated control group on each of the assessment days throughout the study. Afoxolaner plus milbemycin oxime and NexGard® (afoxolaner) chewable tablet treatments eliminated all ticks present on the dogs at treatment within 2 days of treatment; thus, therapeutic efficacy of both treatments was 100 %. Weekly re-infestations were controlled for at least 5 weeks with the efficacy ranging from 92.2 to >99 % based on ~48 h post-treatment in situ counts and achieving 99 to 100 % based on ~72 h post-treatment removal counts (Table 2). With the exception of thumb counts on day 23, there was no evidence that the two treated groups had different tick counts at the 5 % significance level (individual results not shown).

Discussion

In order to provide dog owners and veterinarians with products to be used conveniently for the treatment and/or control of multiple ectoparasites and endoparasites which may infest or infect dogs concurrently, the parasiticidal spectrum of NexGard® chewable tablets was extended through the combination of afoxolaner with the macrocyclic lactone,

Table 2 *Dermacentor reticulatus* live tick counts and percentage efficacy relative to untreated controls for dogs treated with either afoxolaner plus milbemycin oxime chewables or NexGard® (afoxolaner) chewables

Study day	Control, untreated ($n = 10$ dogs)	Afoxolaner + milbemycin oxime chewables, once on day 0 ($n = 10$ dogs)		NexGard® (afoxolaner) chewables, once on day 0 ($n = 10$ dogs)	
	GM [AM] ticks (range)	GM [AM] ticks (range)	%Efficacy[a]	GM [AM] ticks (range)	%Efficacy
2[b]	31.3 [32.4] (20–47)	0	100*	0	100*
9[c]	27.3 [28.2] (19–47)	0.2 [0.4] (0–3)	99.2*	0.2 [0.3] (0–2)	99.3*
10[b]	24.8 [26.2] (13–46)	0	100*	0	100*
16[c]	24.0 [25.1] (16–39)	0.1 [0.1] (0–1)	99.7*	0.1 [0.2] (0–2)	99.5*
17[b]	25.4 [26.6] (14–36)	0	100*	0	100*
23[c]	19.7 [20.1] (15–25)	0.5 [0.6] (0–1)	97.4*	0.1 [0.2] (0–1)	99.2*
24[b]	28.5 [28.9] (19–35)	0	100*	0	100*
30[c]	27.4 [28.1] (16–40)	0.3 [0.4] (0–2)	99.0*	1.0 [1.5] (0–7)	96.4*
31[b]	26.6 [27.3] (18–37)	0	100*	0.1 [0.1] (0–1)	99.7*
37[c]	28.0 [29.0] (18–43)	1.1 [1.3] (0–3)	96.0*	2.2 [2.8] (0–8)	92.2*
38[b]	30.9 [32.1] (16–44)	0	100*	0.3 [0.5] (0–3)	99.0*

GM geometric mean tick count (calculated by averaging the log tick counts, taking the anti-logarithm, and then subtracting 1), *AM* arithmetic mean tick count

[a] Percent efficacy = $100 \times [(C - T) / C]$, where C is the GM tick count of the controls and T is the GM tick counts of the dogs treated with afoxolaner plus milbemycin oxime chewables or NexGard® (afoxolaner) chewables

[b] Removal tick count (~48 h post-treatment, day 2, or ~72 h after [re-]infestation, days 10, 17, 24, 31, and 38)

[c] In situ tick count (~48 h after re-infestation, days 9, 16, 23, 30, and 37)

*$p < 0.0001$, comparison of the log tick count for the treated group to the log tick count of the controls

milbemycin oxime, in a novel braised beef-flavored chewable formulation (NexGard Spectra®). This formulation, with respect to its composition, is similar to the afoxolaner single-entity chewable tablet formulation except for the active ingredients. Milbemycin oxime was proven to be safe in dogs (Jung et al. 2002) and, when administered to dogs orally at a dose of 0.5 mg per kg body weight, to be efficacious against adult intestinal nematode infections and to prevent heartworm disease by interrupting the life cycle of *Dirofilaria immitis* (Nolan and Lok 2012). At that dose and route of administration, milbemycin oxime did not demonstrate activity against fleas and ticks in dogs (Snyder and Wiseman 2012; Merial, unpublished data). The interrelationship between milbemycin oxime and afoxolaner, however, was not known.

In the present dose confirmation study, afoxolaner plus milbemycin oxime chewables (NexGard Spectra®) and afoxolaner single-entity chewables (NexGard®) demonstrated both safety and high efficacy against canine infestation by *D. reticulatus* ticks. Both treatments delivered 100 % efficacy against established infestation within 48 h of treatment and prevented experimental re-infestations for a minimum of 5 weeks. The efficacy recorded in this study is equivalent to results observed in previously reported laboratory studies which tested the efficacy of single-entity afoxolaner chewables (NexGard®) against *D. reticulatus* (Dumont et al. 2014). The data also demonstrated that the presence of milbemycin oxime in the afoxolaner plus milbemycin oxime combination formulation did not interfere with the efficacy of afoxolaner against *D. reticulatus*. The results of this clinical study also support the results of additional studies which indicated that the simultaneous administration of afoxolaner plus milbemycin oxime does not affect the pharmacokinetic profile of afoxolaner (Letendre et al. 2016). The safety and efficacy of afoxolaner plus milbemycin oxime chewable tablets observed in the present experimental study are complemented by the results of a multicenter field study conducted in seven countries in Europe. In this field study, treatment with afoxolaner plus milbemycin oxime chewables showed >95 % efficacy over the 30-day observation period in dogs exposed to natural challenge of dogs with ticks including *Haemaphysalis concinna*, *D. reticulatus*, *I. hexagonus*, *I. ricinus*, and *R. sanguineus* (s. l.), with *D. reticulatus* being recorded on dogs in France, Germany, and Hungary (Knaus et al. 2015).

Generally, the vectoral capacity of ticks varies widely. *D. reticulatus* is a vector of several pathogens including tick-borne encephalitis virus, *Francisella tularensis* and zoonotic rickettsiae (*Rickettsia conorii* and *Rickettsia slovaca*), but the most important pathogen transmitted to dogs is the protozoan *Babesia canis* (Salman and Tarrés-Call 2013). Because of the expansion in distribution within Europe and the increasing number of records of infestation in domestic dogs (see Introduction; Petney et al. 2012), *D. reticulatus* ticks pose a greater threat to dogs than in the past. Considering the pathogenicity of *B. canis* babesiosis, treatments which do not only effectively reduce the tick burden of infested animals but provide a preventive effect on the transmission of tick-borne disease pathogens either through repellent activity or killing ticks prior to transmission of pathogens are particularly appreciated. Treatment with afoxolaner single-entity chewable tablets (NexGard®) was recently reported to prevent the infection of dogs with *B. canis* transmitted through infected *D. reticulatus* ticks (Beugnet et al. 2014). Results of the present study demonstrated that the speed of kill of *D. reticulatus* is similar following treatment of dogs with both afoxolaner single-entity and afoxolaner plus milbemycin oxime combination chewable formulations (NexGard® and NexGard Spectra®, respectively). Therefore, it can be assumed that the administration of afoxolaner plus milbemycin oxime chewables may have the same capability to prevent the transmission of *B. canis* as does have the treatment with the afoxolaner single-entity chewable formulation.

In conclusion, in this study, orally administered afoxolaner plus milbemycin oxime chewables proved safe in dogs and demonstrated a high level of efficacy against established infestation and weekly experimental challenge with *D. reticulatus* ticks for at least 5 weeks following treatment. The excellent efficacy against ticks and fleas (European Medicines Agency 2015) in combination with the treatment and control of intestinal nematode infections (Fankhauser et al. 2016; Rehbein et al. 2016) and prevention of heartworm disease as demonstrated in recently conducted studies using *D. immitis* isolates from endemic areas in Southern Europe (Tielemans et al. 2015) allows afoxolaner plus milbemycin oxime chewables to offer a new broad spectrum parasiticide which covers the common external and internal parasites of dogs.

Compliance of ethical standards

Conflict of interest The work reported herein was funded by Merial, Inc., GA, USA. All authors are current/were employees or were contractors of Merial.

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