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Original Article

Efficacy of a single administration of afoxolaner (NexGard®) or fipronil plus permethrin (Frontline® Tri-Act) against *Hyalomma marginatum* ticks in dogs

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ABSTRACT

This study was conducted to assess the acaricidal efficacy of afoxolaner (NexGard®, Boehringer Ingelheim), and fipronil - permethrin (Frontline® Tri-Act, Boehringer Ingelheim) administered once to dogs experimentally infested with *Hyalomma marginatum* ticks. Twenty-four Beagle dogs were randomly allocated based on a pre-treatment *H. marginatum* infestation to an untreated control group, a NexGard® or a Frontline® Tri-Act treated groups. Treatments were administered once on Day 0 as per the products' labels. For the efficacy evaluation, dogs were experimentally infested with 30 adult *H. marginatum* ticks on Days −2, 7, 28 and 36. In-situ counts were performed at 48 h post-treatment on Day 2 and post-infestations on Days 9, 30 and 38. Ticks were removed and counted at 72 h post-treatment on Day 3 and after each tick infestation on Days 10, 31 and 39. The numbers of live ticks counted in the treated groups were significantly different than in the control group at all time-points ($p \leq 0.0006$). The efficacy was at least 97% after 48 h, and at least 99% after 72 h for both treatments. In this study both afoxolaner and fipronil/permethrin formulations demonstrated a high efficacy against adult *H. marginatum* ticks in treated dogs for at least five weeks.

1. Background

Hyalomma marginatum ticks are widely distributed in North Africa, some parts of Asia and in the Middle East (European Centre for Disease Prevention and Control and European Food Safety Authority (ECDC), 2021a; European Centre for Disease Prevention and Control and European Food Safety Authority (ECDC), 2021b; Estrada-Peña et al., 2017; Walker et al., 2014). In Europe, permanent populations of *H. marginatum* are limited to Eastern (Southern Russia, Balkans, Romania) and Southern countries surrounding the Mediterranean Basin (Spain, Portugal, Italy, Greece, Turkey) and some islands (Malta, Sicily, and Corsica). Moreover, these ticks are more and more frequently reported in non-endemic areas such as western and central European countries including France, Germany, United Kingdom, Poland, Austria, and Sweden, where immature stages were collected from migratory birds, and adult ticks recovered from sheep, horses, cattle, dogs, and humans (Capek et al., 2014; Chitimia-Dobler et al., 2019; Cuber, 2016; Duscher et al., 2018; Grandi et al., 2020; Jameson et al., 2012; Kampen et al., 2007; McGinley et al., 2021; Vial et al., 2016). This provides evidence of successful moltings into the adult stage under favorable conditions.

Hyalomma marginatum is a two-host tick. Larvae feed on small and medium-size animals such as rabbits, hares and passerine birds, then molt to nymphs which usually remain and feed on the same host, while adult ticks parasitize a second host, mainly wild and domestic ungulates (e.g. camel, cattle, deer, goat, horse, sheep), and may occasionally infest dogs and humans (European Food Safety Authority (EFSA), 2010; Estrada-Peña et al., 2017; Valcárcel et al., 2020; Walker et al., 2014). These ticks are considered very aggressive and have mainly an exophilic behavior, finding their host using the 'hunter' strategy. Indeed, adults actively seek or may even run towards a host while detecting stimuli such as vibrations, carbon dioxide and body temperature. In addition, *Hyalomma* ticks have well-developed eyes allowing them to visualize the host shadow, as part of their hunting strategy. This species produces one generation per year and shows peaks of activity in summer and autumn (immature stages), and spring (adults) in their natural environment. *H. marginatum* ticks can tolerate a wide range of relative humidity, from

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dry conditions to humid situations. They tolerate cold temperatures, but not freezing ones, and are active in the environment when temperatures are above 14–16 °C for immatures and 22 °C for adults. These ticks are consequently well adapted to a variety of habitats and a wide range of environmental conditions such as the Mediterranean climate of North Africa and Southern Europe (European Centre for Disease Prevention and Control and European Food Safety Authority (ECDC), 2021a; Valcárcel et al., 2020).

Introduction into and spreading of *H. marginatum* in new areas have been attributed to migratory birds and changes in the migratory routes (Estrada-Peña et al., 2017). Because of the two-host life cycle, the duration of feeding from larvae to nymph may last from 12 to 26 days allowing for a passive transport of pre-adult stages across seas and continents (Capek et al., 2014; Pascucci et al., 2019). The potential role of migratory birds in the dispersion and introduction of infected ticks in Europe is under a constant surveillance (Duscher et al., 2018; Gale et al., 2011; Hagman et al., 2014; Jameson et al., 2012; Palomar et al., 2016; Pascucci et al., 2019). Recent modelling work indicated the possibility of the establishment of permanent *H. marginatum* populations in Western Europe including France, Belgium, the Netherlands, South-Eastern United Kingdom, and large zones in Central Europe (Estrada-Peña et al., 2021). Human activity, such as importation of livestock may also play a role in tick spreading as they may carry large infestations (European Centre for Disease Prevention and Control and European Food Safety Authority (ECDC), 2021a; Estrada-Peña et al., 2017). Climate and environmental changes are important factors for the establishment of these ticks in new habitats, with the added risk to import the pathogens they may transmit (Estrada-Peña et al., 2012; Estrada-Peña et al., 2015).

Hyalomma marginatum is known to be a vector of several pathogens of human and veterinary medical importance. It is considered as the most important vector of Crimean-Congo haemorrhagic fever virus to humans (European Centre for Disease Prevention and Control and European Food Safety Authority (ECDC), 2021a; European Food Safety Authority (EFSA), 2010; Hoogstraal, 1979; World Organisation for Animal Health (OIE), 2000). It is a recognized vector of *Rickettsia aeschlimannii*, a *Rickettsia* belonging to the spotted fever group (Duscher et al., 2018; McGinley et al., 2021; Palomar et al., 2016; Sajid et al., 2018; Sentausa et al., 2014). In addition, its ability to transmit West Nile virus has been shown under laboratory conditions (Formosinho and Santos-Silva, 2006).

Dogs, and particularly those living or traveling into endemic areas, may be opportunistic hosts for *H. marginatum* ticks. Until now, the competence of *H. marginatum* ticks infesting and feeding on dogs had not been studied.

The objective of this study was to assess the therapeutic and persistent efficacy of afoxolaner (NexGard®, Boehringer Ingelheim) and fipronil - permethrin (Frontline® Tri-Act, Boehringer Ingelheim) against experimental infestations with adult *H. marginatum* over a period of five weeks. NexGard is a palatable insecticidal and acaricidal oral formulation that has been developed to provide a convenient monthly option for pet owners and veterinarians to control flea, tick and mite infestations (Beugnet et al., 2015a; Beugnet et al., 2015b; Carithers et al., 2016; European Medicines Agency, 2020; Halos et al., 2015; Hampel et al., 2018; Lebon et al., 2018). Additionally, it has been recently demonstrated effective against *Ixodes hexagonus* tick infestation (Lebon et al., 2019). Frontline Tri-Act spot-on formulation is indicated for treatment and prevention of flea and tick infestations (Halos et al., 2016; Beugnet et al., 2015c). Besides its insecticidal-acaricidal activity against fleas and ticks, Frontline Tri-Act has also been shown to provide repellent activity against mosquitoes, stable flies, sandflies and three tick species (i.e. *D. reticulatus*, *I. ricinus,* and *R. sanguineus*) (Dumont et al., 2015a; Dumont et al., 2015b; Dumont et al., 2015c; Dumont et al., 2015d; Fankhauser et al., 2015a; Fankhauser et al., 2015b). Moreover, the reduction of the risk of infection with *Leishmania infantum* via transmission by sandflies was demonstrated in two field studies (Papadopoulos et al., 2017; Papadopoulos et al., 2020).

2. Methods

2.1. Design

The study design followed the European Medicine Agency (EMA) and the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines (European Medicine Agency Committee for Medicinal Products for Veterinary Use, 2007; Marchiondo et al., 2013).

This study was a randomized, blinded and negative controlled laboratory study which complied with Good Clinical Practices (VICH GL9). The containment of the dogs complied with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) prior to conduct of the study.

2.2. Animals

Adult purpose-bred Beagle dogs were acclimatized to the study conditions for 14 days. During the acclimation period, dogs were examined by a veterinarian and bodyweight was measured. An initial *H. marginatum* tick infestation was conducted to evaluate the suitability of each animal to the infestation and for random allocation to the three study groups. Twenty-four healthy dogs, 12 males and 12 females, aged 5.4 to 7.3 years and weighing 8.25 to 16.70 kg were included. None of the dogs used in this study was treated with any topical or systemic acaricide/insecticide in the 12 weeks prior to treatment.

The animals were kept individually with visual and auditory contact with conspecifics and were housed in an indoor animal unit, environmentally monitored for temperature and humidity. All the animals were observed daily from Day −14 to Day 39 for their general health.

2.3. Allocation to study groups

The study followed a randomized block design based on pre-treatment live attached tick count. The 24 dogs were randomly allocated to the three study groups: Group 1 - untreated control; Group 2 - treated with NexGard® (afoxolaner), and Group 3 - treated with Frontline Tri-Act® (fipronil - permethrin). Groups were homogenous and comparable at baseline regarding pre-treatment live attached tick counts ($p = 0.9536$) as no statistically significant differences were found between the dogs of the three groups.

2.4. Treatment, tick infestations and counts

Each dog was experimentally infested on Day −7 with a tick removal and count on Day −5 for selection and randomization purposes, on Day −2 to assess immediate curative efficacy, and on Days 7, 28 and 36 to assess the preventive (i.e. sustained) efficacy. For infestation, dogs were sedated and placed into individual infestation chambers for up to one hour to facilitate tick infestation. Each dog was infested with 30 (balanced sex ratio) viable, adult, unfed *H. marginatum* ticks by depositing ticks on the dogs' flank. Elizabethan collars were fitted to the dogs during each infestation and were removed at the removal tick counts. A one-year laboratory-bred isolate of *H. marginatum* originating from field collected ticks in Morocco was used for the experimental infestations.

On Day 0, Group 2 dogs were treated once with NexGard as per label instruction, providing minimum dose of 2.7 mg afoxolaner per kg body weight. Group 3 dogs were treated once with Frontline Tri-Act, as per label instruction, providing a minimum dose of 6.76 mg/kg fipronil and 50.48 mg/kg permethrin. Dogs from Group 1 remained untreated and served as negative control group.

In-situ thumb counts by palpation and visual observation of the ticks on the dogs were performed at 48 h after treatment on Day 2 and after each tick exposure on Days 9, 30 and 38. At 72 h post-treatment or post-exposure, all ticks were removed and counted (i.e. Days 3, 10, 31 and

Table 1
Tick counts and efficacy results.

Tick counts	Tick challenge day	Group[1] 1	Group 2			Group 3		
		AM[2]	AM	% Efficacy[3]	*p*-value[4]	AM	% Efficacy	p-value
+48 h (thumb counts)	−2	18.1	0.0	100	0.0004	0.1	99.3	0.0005
	7	17.5/17.6[5]	0.5	97.1	0.0007	0.0	100	0.0004
	28	17.5	0.1	99.3	0.0006	0.0	100	0.0004
	36	19.3	0.0	100	0.0004	0.1	99.4	0.0006
+72 h (removal counts)	−2	18.5	0.0	100	0.0004	0.0	100	0.0004
	7	17.1	0.0	100	0.0004	0.0	100	0.0004
	28	17.5	0.0	100	0.0004	0.0	100	0.0004
	36	18.3	0.0	100	0.0004	0.1	99.3	0.0005

[1] Group 1 = Untreated Control; Group 2 = NexGard®; Group 3 = Frontline Tri-Act®.
[2] AM = tick arithmetic mean.
[3] Percent efficacy = [(C − T)/C] × 100, where C and T are AM of Group 1 and Group 2 counts (live attached), or AM of Group 1 and Group 3 counts (live free + attached) respectively.
[4] p- value = Wilcoxon rank sum test.
[5] On Day 9 (i.e. 48 h after Day 7 infestation), one live free tick was counted on one dog from Group 1. AM Group 1: live free + attached = 17.6; live attached = 17.5.

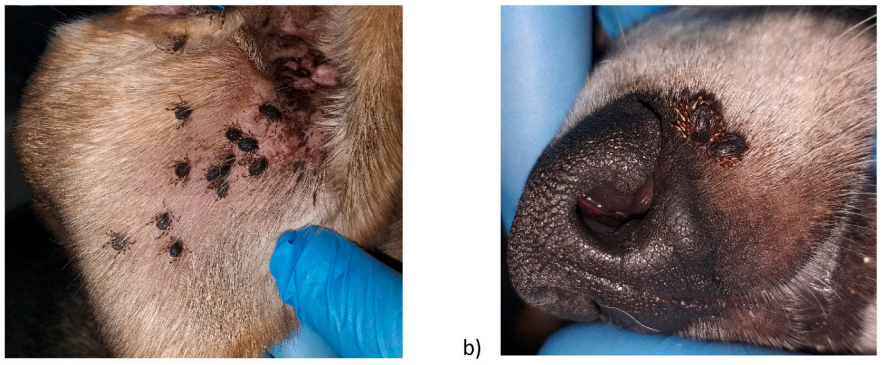

Fig. 1. a) Numerous *Hyalomma* ticks attached to dog's ear and b) two *Hyalomma* ticks attached at nose periphery.

39). Upon removal, ticks were categorized as live or dead and free or attached.

2.5. Statistical analyses

To evaluate the acaricidal efficacy, arithmetic means of live tick counts were calculated by group at each time-point. Percent effectiveness for each treated group was calculated using the Abbott's formula: [(C - T) / C] x 100, where C = arithmetic mean for the control group and T = arithmetic mean for the treated group at a specific time point. According to the efficacy guidelines (European Medicine Agency Committee for Medicinal Products for Veterinary Use, 2007; Marchiondo et al., 2013), only live attached ticks are included in the efficacy calculation regarding systemic acting molecules, like afoxolaner, needing tick attachment and feeding. For topical products acting by contact, like fipronil-permethrin, both free and attached live ticks are included in the efficacy calculation.

The tick counts between the groups were compared using a non-parametric Wilcoxon rank sum test. Pre-treatment live, attached tick counts recorded during acclimation were also compared for group homogeneity at the time of inclusion using a one-way ANOVA. All statistical comparisons were two-sided at the 0.05 significance level.

3. Results and discussion

This study demonstrated that under experimental conditions, dogs were suitable host for *H. marginatum* infestations as a large proportion of ticks attached and fed on dogs. The average retention rates in the untreated control dogs ranged from 57 to 62% (means of 17.1 to 18.5, respectively) at 72 h assessment time-points (Table 1). The adult ticks were mostly collected on dogs around the head, especially on and in ears (Fig. 1). Only one live free tick was observed in one dog of the control group on Day 9. Except this single live free tick, all live ticks removed from the dogs were attached to the animals, confirming that dogs can be appropriately infested with *H. marginatum* under experimental conditions.

There were no adverse events related to treatment observed throughout the study.

In both treated groups, the curative efficacy against established tick infestations was 100% at 72 h post-treatment (Day 3). The sustained acaricidal efficacy against the subsequent tick challenges was >97.1% for both products at all time-points (Table 1). The numbers of live ticks in the treated groups were significantly different than the control group at all time-points ($p \leq 0.0006$).

According to EMA and WAAVP guidelines, the recommended tick infestation level is ~50 unfed adult ticks per animal. Here, the infestations were limited to 30 ticks per animal as this species is known to be aggressive and to induce severe skin irritation and damage, especially in case of repeated infestations. Such lesions were observed by the authors during an initial model validation with 50 ticks on few dogs (data not shown). For the same risk of skin lesions and ethical reason, the tick infestations were not performed on a regular weekly basis but on Day −2 to assess treatment of existing tick infestations and then on Days 7, 28 and 36. Following such design, no tick-induced dermatological lesions were observed.

Both NexGard and Frontline Tri-Act provided high efficacy against *H. marginatum* ticks and results are consistent with published data assessing the efficacy against other tick species. Veterinarians will be able to recommend these products in the case of risk of *Hyalomma* infestation.

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Availability of data and materials

All relevant data generated or analyzed during this study are included in this published article.

Ethics approval

The study protocol was approved by the Clinvet's Institutional Animal Care and Use Committee (IACUC) prior to conduct the study.

Consent for publication

Not applicable.

Disclaimer

NexGard® and Frontline® Tri-Act are registered trademarks of Boehringer Ingelheim Animal Health. Any references in this article to these trademarks are informative only and not intended for commercial purposes.

Authors' contributions

MM, WL and FB: Conceptualization; Methodology.LM, FEA: Data curation; Formal analysis; Investigation. WL, FB: Project administration; Supervision. WL and FB: Validation; Visualization; Writing - original draft; Writing - review & editing.

Declaration of Competing Interest

The authors LM, FEA and MD declare that they have no conflict of interest.

The authors WL and FB are employees of Boehringer Ingelheim Animal Health, Lyon France.

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