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Assessment of the insecticidal activity of afoxolaner against *Aedes aegypti* in dogs treated with NexGard®

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Abstract– Twelve healthy dogs were studied in this parallel group, blinded, randomised, and negative controlled efficacy study. On Day -1, the 12 dogs included were ranked within sex in descending order of individual pre-treatment (Day -5) fed mosquito counts and randomly allocated by blocks of two dogs to the untreated control group or the afoxolaner-treated group. NexGard® (Merial, now part of Boehringer Ingelheim Animal Health) was administered orally on Day 0 in accordance with the European label instructions. On Days 1, 7, 14, 21 and 28, all dogs were exposed for a duration of 1 hour to 50 ± 5 unfed *Aedes aegypti* females. After each exposure, mosquitoes were collected after 1 hour and assessed for viability during collection and at 24 ± 2 hours. The arithmetic (and geometric) mean values of live fed mosquito counts at 24 hours after the exposure periods for the negative control group ranged from 33.7 (32.3) to 49.8 (49.7), indicating that this was a vigorous mosquito strain. There was no significant difference between control and treated groups in the number of live and fed mosquitoes at each 1 hour post-exposure collection time. Based on arithmetic and geometric mean values at 24 hours after each exposure, significantly fewer live fed mosquitoes were recorded in the treated group, compared to the negative control group, throughout the study ($p < 0.001$). The afoxolaner insecticidal efficacy against *A. aegypti* varied from 98% (Day 2) to 75.3% (Day 29) based on arithmetic means, and 98.7% (Day 2) to 89.8% (Day 29) based on geometric means.

Keywords: *Aedes aegypti*, insecticide, afoxolaner, NexGard®, dog

Résumé – Évaluation de l'activité insecticide de l'afoxolaner contre *Aedes aegypti* chez les chiens traités avec NexGard®. Douze chiens en bonne santé ont été étudiés dans cette étude d'efficacité en aveugle, en groupes parallèles et avec contrôles négatifs. Au jour -1, les 12 chiens inclus ont été classés par sexe par ordre décroissant de prétraitement individuel (jour -5) de comptage de moustiques nourris et répartis au hasard par blocs de deux chiens, en groupe témoin non traité et en groupe traité par afoxolaner. Du NexGard® (Merial, maintenant un membre de Boehringer Ingelheim Animal Health) a été administré par voie orale au jour 0 conformément aux instructions dela notice européenne. Aux jours 1, 7, 14, 21 et 28, tous les chiens ont été exposés pendant une durée de 1 heure à 50 ± 5 *Aedes aegypti* femelles à jeun. Une heure après chaque exposition, les moustiques ont été recueillis et leur viabilité a été évaluée à la collecte et après 24 ± 2 heures. Les valeurs moyennes arithmétiques (et géométriques) du nombre de moustiques vivants et nourris 24 heures après les périodes d'exposition pour le groupe témoin négatif variaient de 33,7 (32,3) à 49,8 (49,7), ce qui indique une bonne viabilité de la souche de moustiques. Il n'y avait pas de différence significative entre les groupes témoins et les groupes traités dans le nombre de moustiques vivants et nourris à chacune des collectes à 1 heure post-exposition. Sur la base des valeurs moyennes géométriques et arithmétiques, à 24 heures après chaque exposition, des nombres significativement plus petits de moustiques nourris et vivants ont été enregistrés dans le groupe traité par rapport au groupe témoin non traité pendant toute l'étude ($p < 0{,}001$). L'efficacité insecticide de l'afoxolaner contre *A. aegypti* variait de 98 % (jour 2) à 75,3 % (jour 29) sur la base de moyennes arithmétiques, 98,7 % (jour 2) à 89,8 % (jour 29) sur la base de moyennes géométriques.

Introduction

Recently, a new class of insecticides/acaricides, the isoxazolines, have demonstrated very good efficacy against fleas and ticks [19]. Afoxolaner is an isoxazoline administered monthly to protect dogs against fleas and ticks (NexGard®, Merial, now part of Boehringer Ingelheim Animal Health) [2,3,8,10]. It is administered at a minimum dose of 2.5 mg/kg. Recent studies have demonstrated its activity against other arthropods, including

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Demodex canis, the agent of canine demodicosis, *Sarcoptes scabiei* var. *canis* and *S. scabiei var. suis*, the agent of sarcoptic mange in dogs and swine, respectively, as well as *Otodectes cynotis*, the agent of ear mange in dogs and cats [1,4,5].

After oral administration, afoxolaner is absorbed quickly, with peak plasma levels (Cmax) reached between 2 to 4 hours after administration [14,15]. Plasma protein binding is more than 99%, which explains the long half-life, 10–14 days on average [14,15]. Due to its strong binding to plasma proteins, its activity is systemic and exposure is related to the ingestion of blood or inflammatory fluids by the biting insect.

In addition to its activity against well-known blood-feeding ectoparasites like fleas and ticks, or resident ectoparasites like *Demodex, Sarcoptes,* and *Otodectes*, it is probable that afoxolaner would also have a certain level of insecticidal activity against other blood-feeding arthropods like mosquitoes. Insecticidal efficacy following a blood meal might not prevent pathogen transmission from the female mosquito, but it could have a further effect by killing the mosquitoes before a new bite, and/or by reducing the mosquito population in a restricted area like a household where treated dogs are living. It could therefore have an indirect action on the rate of vector-borne pathogen transmission within the household.

Aedes aegypti mosquitoes are endemic in tropical areas around the globe, but have expanded into sub-tropical areas and even some warm temperate locations, although the species seems less adaptable to temperate climate than *Aedes albopictus* [13]. It is now found in many parts of the world including South and Central America, the southern USA, Africa, India, tropical islands, South-East Asia, Northern Australia, and sporadically in the Mediterranean zone [13]. *A. aegypti* is a major vector of several diseases of animals and/or humans, e.g. heartworm disease due to *Dirofilaria immitis* in dogs, equine encephalitis viruses, West Nile virus, Dengue virus, Chikungunya virus, Zika virus, and yellow fever virus [9,12]. The objective of this study was to assess the insecticidal activity that afoxolaner may have against *A. aegypti* mosquitoes.

Materials and methods

The design and conditions of this study were approved by the South African and ClinVet animal welfare ethics committees, and were performed in accordance with the Good Clinical Practices of the European Agency for the Evaluation of Veterinary Medicinal Products (CVMP/VICH GL9, July 2000; CVMP/VICH GL19, July 2001). This study was a parallel group, blinded, randomised, negative controlled efficacy study. It was conducted with two groups of six dogs each.

Male and female dogs were included in the study if they had been acclimatised to the study conditions for at least 8 days; they were clinically healthy as verified by a veterinarian on Day -8; they were ≥ 6 months at the time of inclusion (Day -1); females were not pregnant; they had not been treated with a long-acting topical or systemic acaricide/insecticide during the 12 weeks preceding Day 0.

The animals were kept individually in cages and no physical contact between dogs was possible. However, animals still had visual and auditory contact with conspecifics. During the acclimatisation period (Day -8 to Day -1), an initial *A. aegypti* mosquito challenge was performed on Day -5 to evaluate the receptivity of each dog to experimental infestation and for random allocation of the dogs to the study groups. The 12 dogs included in the study were randomly allocated to two groups (untreated control group and afoxolaner-treated group), based on total counts of fed mosquitoes 1 hour after the initial challenge. In addition, veterinary clinical examination was performed on Day -8 for enrolment purposes, and weighing of all dogs was performed on Day -1 for appropriate dose determination. All the dogs were observed daily from Day -8 to Day 28 for their general health.

On Day 0, all dogs assigned to the treated group received afoxolaner. The dogs were treated orally with NexGard® (2.27% w/w afoxolaner chewable tablets) in accordance with European label instructions [8]. All dogs weighed from 10 to 25 kg and were treated with a chewable tablet containing 68 mg of afoxolaner. The dogs were observed hourly for 4 hours after administration to detect possible adverse reactions.

Dogs were challenged with 50 ± 5 *Aedes aegypti* unfed female mosquitoes on Day -5 for randomisation purposes, and then on Days 1, 7, 14, 21 and 28 to assess insecticidal activity. Mosquitoes were assessed for viability and feeding status during collection 1 hour after exposure and on Days 2, 8, 15, 22 and 29 (24 hours after exposure).

To perform the mosquito challenge, the dogs were sedated using medetomidine (Domitor®, Zoetis), and placed into a mosquito proof net (dimensions: 81 cm × 58 cm × 58 cm). The whole body of the dog was thus exposed to the mosquito challenge. The mosquito net used allowed both exposure of dogs to the parasites and collection of parasites after the challenge, without mosquitoes escaping during the process. A Clinvet laboratory-bred strain of *A. aegypti* of US origin was used in the infestation challenges.

Food was removed at least two hours prior to sedation of animals or animals were fasted overnight if required by scheduling constraints. The 50 ± 5 female mosquitoes were released into the net and they were carefully vacuumed after 1 hour.

At the end of the exposure period, atipamezole (Antisedan®, Zoetis) was used to reverse the effects of the sedation in dogs.

One hour after challenge, the mosquitoes were collected using an aspirator and they were then assessed as live, fed or unfed, moribund or dead. Mosquitoes were classified as live if they exhibited normal behaviour and were capable of coordinated locomotion and flight upon external stimuli. Mosquitoes were classified as moribund if they were only capable of some movement, but exhibit abnormal, obviously impaired behaviour, and were not capable of coordinated locomotion or flight upon external stimuli.

Table 1. Comparison of body weights between dogs and mosquito counts obtained 1h after exposure at Day -5 for allocation purposes.

	Day	Statistic	Control dogs	Afoxolaner-treated dogs
Body weight (kg)	Day -1	n	6	6
p-value: 0.8655		Mean	16.37	16.03
		SD	2.467	3.999
		Median	16.00	14.70
		Minimum	13.8	12.4
		Maximum	19.2	23.6
Mosquito count 1h after exposure	Day -5	n	6	6
p-value: 0.5008		Mean	53.3	54.0
		SD	2.25	0.63
		GeoMean	53.3	54.0
		Median	53.0	54.0
		Minimum	50	53
		Maximum	57	55

p-value: One-way ANOVA with a treatment effect.

Prior feeding by dead mosquitoes was assessed following the collection, by placing the dead mosquito on tissue paper and squashing the abdomen with a spatula or similar suitable object to assess if a blood meal was taken.

Live and moribund mosquitoes were incubated in suitable containers at 24.3 °C to 28.1 °C for 24 hours (± 2 hours). During this period the mosquitoes had access to a 10% sucrose solution, or a suitable alternative. The mosquitoes were again assessed for viability following the 24-hour (± 2 hours) incubation period and then assessed for feeding as described above. All live and moribund mosquitoes were immobilised in a freezer prior to the feeding assessments.

The dead mosquito counts observed after each challenge are the sum of the dead mosquitoes counted at 1 hour (Table 1) and the dead mosquitoes counted at 24 hours (Table 2).

Insecticidal activity calculations were based on both arithmetic and geometric mean values. Geometric mean efficacy calculations were based on the geometric mean values of the mosquito (count + 1) data. One (1) was subsequently subtracted from the result to obtain a meaningful value for the geometric mean of each group.

The primary efficacy of afoxolaner against *A. aegypti* mosquitoes was calculated using the total live fed mosquitoes at 24 hours after each mosquito challenge, according to the formula below:

Insecticidal efficacy (%) against mosquitoes = 100 x (Mc − Mt)/Mc, where:

Mc = mean number of live fed mosquitoes in the control group at 24 hours after challenge;

Mt = mean number of live fed mosquitoes in the treated group 24 hours after challenge.

The groups were compared using an ANOVA (Proc GLM procedure in SAS) with a treatment effect on both untransformed and logarithmic transformed mosquito (count + 1) data. SAS Version 9.3 TS Level 1M2 was used for all the statistical analyses.

Results

The weight of dogs varied from 13.8 to 19.2 kg in the control group (mean = 16.37 kg) and from 12.4 to 23.6 kg (mean value 16.02 kg) in the treated group. No statistically significant differences were recorded between the pre-treatment fed mosquito counts at Day -5 ($p = 0.5008$) nor the body weights ($p = 0.8655$) of the dogs in the two groups, which indicated homogeneity between the dogs included in each group (Table 1).

No adverse events were recorded after treatment or during the study duration [6].

The live, moribund and dead status of the mosquitoes was assessed at all time-points (Tables 2 and 3). The collection of live mosquitoes at 1 hour post-exposure indicated a mortality of 1.86 to 8% during the contact time between dogs and mosquitoes (Table 2). There was no significant difference between the control group and the treated group in the numbers of live or dead mosquitoes at the end of the 1 hour exposure (Table 2). No moribund mosquitoes were observed at 1h.

The arithmetic mean values of live fed mosquito counts at 24 hours after the challenge period for the negative control group ranged from 33.7 to 49.8, indicating that this was a vigorous mosquito strain. The insecticidal and acaricidal activities, following the European Medicine Agency guideline (EMA) [7] and the World Association for the Advancement of Veterinary Parasitology (WAAVP) [16], should be based on the comparison of the number of live arthropods collected from control and treated animals (Table 3). Arithmetic and geometric mean values of live fed mosquito counts and efficacies are summarised in Tables 3 and 4.

Based on both arithmetic and geometric mean values of live fed mosquitoes at 24 hours, significantly fewer live fed mosquitoes were recorded for the afoxolaner treated group compared to the negative control group, throughout the study ($p < 0.001$). Based on arithmetic and geometric mean values of live fed mosquitoes at 24 hours, the insecticidal efficacy of NexGard® was 98.2% (98.7% based

Table 2. Mosquito status assessment during collection at 1h post-challenge.

Group	Dog ID	Day 1			Day 7			Day 14			Day 21			Day 28		
		Live	Moribund	Dead	Live	Moribund	Dead	Live	Moribund	Dead	Live	Moribund	Dead	Live	Moribund	Dead
Control dogs	2A9 928	53	0	0	49	0	2	53	0	3	52	0	2	49	0	2
	2AA 13E	51	0	3	45	0	4	53	0	2	55	0	1	62	0	1
	5A6 23A	54	0	0	48	0	2	35	0	13	50	0	2	56	0	1
	5BF 5E2	54	0	1	46	0	1	51	0	2	47	0	3	50	0	2
	5CC 399	52	0	2	48	0	0	53	0	2	51	0	0	51	0	1
	DF7 F0E	52	0	0	45	0	5	51	0	4	39	0	11	57	0	0
Arithmetic means		52.7	0.0	1.0	46.8	0.0	2.3	49.3	0.0	4.3	49.0	0.0	3.2	54.2	0.0	1.2
% Dead mosquitoes				1.86%			4.68%			8.02%			6.13%			2.16%
Afoxolaner-treated dogs	284 46C	51	0	1	50	0	3	53	0	2	50	0	6	54	0	4
	289 EAA	55	0	0	49	0	0	49	0	0	51	0	0	48	0	0
	2AC 6FC	57	0	0	37	0	7	45	0	4	50	0	2	54	0	0
	5CE 690	31	0	2	46	0	0	49	0	5	45	0	2	48	0	4
	B2C 501	44	0	2	51	0	1	52	0	2	50	0	2	55	0	1
	E19 6E0	51	0	1	49	0	1	54	0	1	53	0	3	50	0	0
Arithmetic means		48.2	0.0	1.0	47.0	0.0	2.0	50.3	0.0	2.3	49.8	0.0	2.5	51.5	0.0	1.5
% Dead mosquitoes				2.03%			4.08%			4.37%			4.78%			2.83%

Table 3. Counts of mosquitoes and assessment of their status 24h post-exposure (Days 2 8 and 15).

Group	Dog ID	Day 2						Day 8						Day 15					
		Live	Moribund	Dead	Fed (live + dead + moribund)	Unfed (live + dead + moribund)	Live Fed	Live	Moribund	Dead	Fed (live + dead + moribund)	Unfed (live + dead + moribund)	Live Fed	Live	Moribund	Dead	Fed (live + dead + moribund)	Unfed (live + dead + moribund)	Live Fed
Control dogs	2A9 928	53	0	0	53	0	53	48	0	3	46	5	45	53	0	3	52	4	49
	2AA 13E	51	0	3	47	7	46	42	0	7	45	4	42	50	0	5	38	17	35
	5A6 23A	53	0	1	53	1	52	43	0	7	47	3	41	22	8	18	31	17	20
	5BF 5E2	52	1	2	51	4	48	43	0	4	44	3	41	38	1	14	37	16	25
	5CC 399	51	0	3	49	5	47	44	0	4	45	3	41	53	0	2	32	23	32
	DF7 F0E	47	0	5	42	10	38	43	0	7	42	8	40	41	6	8	49	6	41
Arithmetic means		51.2	0.2	2.3	49.2	4.5	47.3	43.8	0..0	5.3	44.8	4.3	41.7	42.8	2.5	8.3	39.8	13.8	33.7
Afoxolaner-treated dogs	284 46C	14	3	35	38	14	0	10	0	43	41	12	0	3	18	34	50	5	0
	289 EAA	2	3	50	55	0	2	11	0	38	37	12	0	10	21	18	49	0	10
	2AC 6FC	0	8	49	57	0	0	3	0	41	35	9	0	11	7	31	33	16	0
	5CE 690	2	5	26	30	3	2	3	0	43	43	3	0	3	7	44	48	6	0
	B2C 501	6	2	38	37	9	0	0	0	52	51	1	0	21	23	10	44	10	11
	E19 6E0	1	5	46	52	0	1	1	0	49	49	1	0	14	27	14	41	14	2
Arithmetic means		4.2	4.3	40.7	44.8	4.3	0.8	4.7	0.0	44.3	42.7	6.3	0.0	10.3	17.2	25.2	44.2	8.5	3.8

Table 3 (continued). Counts of mosquitoes and assessment of their status 24h post-exposure (Days 22 and 29).

Group	Animal ID	Day 22						Day 29					
		Live	Moribund	Dead	Fed (live + dead + moribund)	Unfed (live + dead + moribund)	Live Fed	Live	Moribund	Dead	Fed (live + dead + moribund)	Unfed (live + dead + moribund)	Live Fed
Control dogs	2A9 928	52	0	2	53	1	51	49	0	2	50	1	48
	2AA 13E	54	0	2	56	0	54	44	3	16	61	2	43
	5A6 23A	46	2	4	52	0	46	56	0	1	57	0	56
	5BF 5E2	32	10	8	46	4	30	50	0	2	51	1	49
	5CC 399	51	0	0	41	10	41	51	0	1	52	0	51
	DF7 F0E	28	5	17	47	3	26	57	0	0	52	5	52
Arithmetic means		43.8	2.8	5.5	49.2	3.0	41.3	51.2	0.5	3.7	53.8	1.5	49.8
Afoxolaner-treated dogs	284 46C	20	9	27	36	20	0	6	12	40	51	7	0
	289 EAA	15	1	35	33	18	0	5	4	39	46	2	3
	2AC 6FC	14	11	27	39	13	1	7	6	41	51	3	4
	5CE 690	9	21	17	39	8	1	2	10	40	50	2	2
	B2C 501	26	8	18	42	10	17	52	0	4	53	3	50
	E19 6E0	32	8	16	49	7	32	24	17	9	41	9	15
Arithmetic means		19.3	9.7	23.3	39.7	12.7	8.5	16.0	8.2	28.8	48.7	4.3	12.3

Table 4. Insecticidal efficacy based on live fed mosquitoes counted at 24 hours post-exposure.

Day	Control Group	Afoxolaner-treated Group		
	Arithmetic (Geometric) Mean	Arithmetic (Geometric) Mean	Percentage efficacy (based on geometric means)	ANOVA *p*-Value
Day 2	47.3 (47.1)	0.8 (0.6)	98.2 (98.7)	< 0.0001
Day 8	41.7 (41.6)	0.0 (0.0)	100.0 (100.0)	< 0.0001
Day 15	33.7 (32.3)	3.8 (1.7)	88.6 (94.7)	< 0.0001
Day 22	41.3 (40.0)	8.5 (2.7)	79.4 (93.4)	0.0005
Day 29	49.8 (49.7)	12.3 (5.0)	75.3 (89.8)	0.001

on geo mean), 100% (100%), 88.6% (94.7%), 79.4% (93.4%), and 75.3% (89.8%), on days 2, 8, 15, 22 and 29, respectively (Table 4).

Discussion and conclusion

The study design classically used to assess repellency of insecticides after 1h of exposure of flying insects was used in this particular study to assess insecticidal activity after feeding [16]. Being systemic, afoxolaner binds to plasma proteins [15], and no repellent activity related to volatile molecules on the skin surface was expected. The number of fed mosquitoes observed at 1h was not different between the control and treated dogs (> 90% in both groups), thus confirming the absence of a repellent effect, which is assessed by the anti-feeding effect. No differences in the number of live and dead mosquitoes were observed at 1h collection, indicating that there was no immediate killing effect.

The insecticidal activity was high 24 hours after each exposure challenge, indicating that the *A. aegypti* mosquitoes ingested a lethal dose of afoxolaner during their blood meal. The blood meal of female *A. aegypti* takes only a few minutes (< 5 min) and the volume ingested is about 4-5 µL [9,11,17,18]. The study design based on 1 hour exposure to dogs allowed more than 90% of mosquitoes to feed and it is expected that the maximum proportion was reached in such a period. After a single administration at Day 0, the quantity of afoxolaner present in 4-5 µL of dog blood was enough to kill > 75% (> 89% in geometric mean) of the fed female mosquitoes throughout an entire month. There are currently no data on specific mosquito species sensitivity and these results should be confirmed in other important species such as *Culex pipiens* or *A. albopictus*.

Given the lack of anti-feeding effect, it is not expected that afoxolaner treatment in dogs would have a direct impact on the transmission of pathogenic agents by *A. aegypti* during the blood meal. Nevertheless, *A. aegypti* females need a blood meal every 2-3 days, and 48 hours is needed for oviposition [9,11,18]. This species is also unlikely to disperse and has restricted flight capacity, estimated to be less than 500 m [9,11]. The behaviour tends to be indoor. These biological aspects are in favour of a rapid decrease of the mosquito population biting treated dogs. A female would die after its blood meal and would not bite a second time; neither would the insect be able to lay eggs before dying. This hypothesis would need to be demonstrated under a simulated household environment, but the results of this study are in favour of an indirect protective effect in households where afoxolaner-treated dogs are living. Killing *Aedes* females before a new blood meal would reduce the rate of transmission of vector-borne pathogens, like heartworm in dogs.

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Conflict of interest

This clinical study was funded by Merial, now part of Boehringer Ingelheim Animal Health, 29 Avenue Tony Garnier, 69007 Lyon of which Frédéric Beugnet, Lénaïg Halos and Wilfried Lebon are employees.

ClinVet, of which Julian Liebenberg and Josephus Fourie are employees, is an independent South African Contract Research Organisation contracted to conduct the study.

All authors voluntarily publish this article and have no personal interest in these studies other than publishing the scientific findings that they have been involved in generating via planning, initiating, monitoring and conducting the investigations and analysing the results.

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RESEARCH ARTICLE OPEN ACCESS

Assessment of the insecticidal activity of oral afoxolaner against *Phlebotomus perniciosus* in dogs

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Abstract – Twelve healthy dogs were included in this laboratory efficacy study. Six dogs were randomly allocated based on body weight to an untreated control group and six to an afoxolaner (NexGard®) treated group. In the treatment group, afoxolaner was administered orally on Day 0 in accordance with label instructions. On Days 1, 14 and 28, each dog was exposed to 60 unfed female and 10 male *Phlebotomus perniciosus* sandflies for 1 h. At the end of each exposure period, sandflies were counted and assessed for viability and feeding status. There was no statistical difference in mortality (0.0–5.4%), nor in feeding proportion (61.6–78%) between the control and the treated groups at all 1-h post-exposure assessments. After collection, live fed and unfed sandflies were kept for viability assessments at 48 and 72 h post-exposure. In the untreated control group, the average percentages of live, fed, female sandflies after exposure, on Days 1, 14 and 28, ranged from 51% to 74% at 48 h and from 46% to 57% at 72 h, demonstrating model robustness over the 28 days of the study. Significantly fewer live fed sandflies were recorded for the afoxolaner treated group ($p < 0.01$). The insecticidal efficacy was 100%, 95.9% and 75.2% at 48 h post Days 1, 14 and 28 exposures, respectively, and 100%, 100% and 86.3% at 72 h post Days 1, 14, and 28 exposures, respectively. A single administration of oral afoxolaner (NexGard®) to dogs significantly killed *P. perniciosus* sandflies 48 and 72 h after blood feeding for 1 month.

Key words: *Phlebotomus perniciosus*, Sandfly, Insecticide, Afoxolaner, NexGard®, Dog.

Résumé – Évaluation de l'activité insecticide de l'afoxolaner par voie orale contre *Phlebotomus perniciosus* chez le chien. Douze chiens en bonne santé ont été inclus dans cette étude d'efficacité en laboratoire. Six chiens ont été répartis au hasard en fonction de leur poids corporel dans un groupe témoin non traité et six dans un groupe traité par afoxolaner (NexGard®), administré par voie orale le jour 0 conformément aux instructions de l'étiquette. Les jours 1, 14 et 28, chaque chien a été exposé à 60 femelles à jeun et 10 mâles de *Phlebotomus perniciosus* pendant une heure. À la fin de chaque période d'exposition, les phlébotomes ont été évalués en termes de viabilité et de statut alimentaire. Il n'y avait pas de différence statistique dans la mortalité (0,0 à 5,4 %), ni dans le taux d'engorgement (61,6 à 78 %) entre le groupe témoin et le groupe traité lors de toutes les évaluations après une heure. Après la collecte, les phlébotomes vivants gorgés et non gorgés ont été conservés aux fins d'évaluation de la viabilité 48 et 72 heures après l'exposition. Dans le groupe témoin non traité, le pourcentage moyen de phlébotomes femelles gorgées et vivantes après l'exposition aux jours 1, 14 et 28 variait de 51 à 74 % à 48 heures et de 46 à 57 % à 72 heures, démontrant la robustesse du modèle au cours des 28 jours de l'étude. Un nombre significativement moins important de phlébotomes gorgés vivants ont été enregistrés dans le groupe traité par afoxolaner ($p < 0,01$). L'efficacité insecticide était de 100 %, 95,9 % et 75,2 % 48 heures après les expositions des jours 1, 14 et 28, respectivement, et 100 %, 100 % et 86,3 % à 72 heures après les expositions des jours 1, 14 et 28, respectivement. Une seule administration d'afoxolaner (NexGard®) par voie orale à un chien tue de manière significative les phlébotomes *P. perniciosus* 48 heures et 72 heures après la prise de sang pendant un mois.

Introduction

Canine leishmaniosis (CL) is an infectious disease due to the proliferation of the protozoan flagellate parasite *Leishmania infantum* in cells of the reticulo-endothelial system (i.e., monocyte cell line) [7]. This parasite is mainly transmitted by the bite of phlebotomine sandflies (*Phlebotomus* in the Old World, i.e., Africa, Asia, Europe; and *Lutzomyia* in the Americas) [11, 15, 16, 26]. *Leishmania* protozoans can also be transmitted, rarely, by blood transfusion, or vertically from mothers to their puppies [28].

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Although dogs constitute the main reservoir, *L. infantum* can also infect many other mammals like lagomorphs, rodents, foxes, cats, horses, and humans [15, 16, 26, 28]. CL is a major zoonosis and human cases are reported in endemic areas where the prevalence of CL in dogs is high [16, 28]. Two hundred cases in Italy and approximately 25 autochthonous cases in France are reported yearly [27, 30].

Canine leishmaniosis is endemic in more than 70 countries. It is highly endemic in countries around the Mediterranean basin, but also West Africa, Southern Asia, and Central and South America. In endemic countries, the seroprevalence in dogs can vary greatly from a few percentage points to more than 50%. Distribution can be highly heterogeneous between endemic foci (i.e., high seroprevalence in dogs, multiple clinical cases) and ectopic or new foci (i.e., low prevalence, few clinical cases and no or very few vectors) [15, 16, 26, 27].

Recent surveys have demonstrated a gradual spread to previously non-infected areas [10, 26, 27, 30]. Several authors have described new outbreaks from Southern or Central Italy to Northern Italy, such as Tuscany, Marche and Emilia-Romagna; in France with a spread to the West and Northwest [10]; as well as Catalonia, in northeastern Spain [1, 23], and Galicia, in northern Spain [25]. Canine leishmaniosis is becoming endemic in the Balkans and Romania, with extension towards Central and Northern Europe [23].

Phlebotomus perniciosus is one of the major vectors of canine leishmaniosis in Southern Europe [19, 20]. Other *Phlebotomus* species are also involved in North Africa, Southeastern Europe and Central Asia, e.g. *Phlebotomus ariasi, P. perfiliewi, P. sergenti*. *P. perniciosus* is a ubiquitous sandfly living in urban and peri-urban areas and having crepuscular activity [20]. *P. perniciosus* is a proven vector of this protozoan in Algeria, France, Italy, Malta, Portugal, Spain, Greece, and Turkey, and is a suspected vector in Morocco and Tunisia [24]. Over the past decades, there has been an increase in sandfly geographical distribution and density, which can be attributed to climate and ecological changes, but also to increased tourism [23, 24, 26].

There are two essential strategies to limit the transmission of *L. infantum* to dogs and humans: (1) control of the canine reservoir by using insecticides with repellent activity to prevent sandfly bites, by treating infected dogs, and by vaccinating dogs in enzootic areas; (2) control and reduce the vector density by acting on sandflies and/or sandfly ecosystems [26, 30].

Female sandflies take their blood meal in a short time, approximately 4 min and are able to inoculate *Leishmania* during that time [2, 9]. Therefore, systemic insecticides will not prevent the infection of dogs during the sandfly bite. However, they may decrease the population of sandflies and avoid further bites and transmission to mammals because sandflies take 6–10 days to lay eggs before biting a new host again [13]. They also do not fly long distances (maximum 1 km) and usually stay concentrated around 200–500 m [19, 20].

Afoxolaner is a systemic insecticide and acaricide compound from the isoxazoline group. Afoxolaner acts by inhibition of a specific receptor on GABA-gated chloride ion channels, resulting in uncontrolled activity of the central nervous system and death of the arthropods [28]. After oral administration, afoxolaner is rapidly absorbed and is highly bound to plasma proteins, therefore acting through a systemic pathway on hematophagous arthropods [21]. Afoxolaner is available as a palatable chew [18] given orally at the minimum dose of 2.5 mg/kg (NexGard®, Boehringer Ingelheim Animal Health). It is indicated for the treatment and control of fleas and ticks in dogs [3, 4, 12, 17], the treatment of *Demodex* and *Sarcoptes* [5, 6], and has been proven effective against *Otodectes* mites [8]. More recently, the insecticidal activity of afoxolaner against *Aedes aegypti* mosquitoes at 24 h post-exposure was demonstrated. It showed that *A. aegypti* mosquitoes ingested a lethal dose of afoxolaner during their blood meal [22]. Based on these results, we hypothesized that afoxolaner could also kill sandflies that feed on afoxolaner treated dogs [13].

Afoxolaner has no repellent properties. Nevertheless, we hypothesized that it could be a good candidate in the control of sandfly populations by killing those that would feed on treated dogs. Killing female sandflies before a new bite and preventing them from laying eggs would help to reduce the sandfly population in a restricted area.

Materials and methods

The design and conditions of this study were approved by animal welfare ethical committees of Boehringer-Ingelheim and ClinVet, and were performed in accordance with International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) Guideline 9, entitled Good Clinical Practice. The containment of the dogs also complied with the 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, and was approved by the Institutional Animal Care and Use Committee (IACUC). This study was a parallel group, blinded, randomized, negative controlled efficacy study. It was conducted with two groups of six dogs each.

A total of 12 healthy laboratory Beagle dogs (six males and six females) between 14 and 18 months-old and weighing between 7.9 and 13.6 kg were included in the study. Dogs were acclimatized to the study conditions for seven days and examined by a veterinarian. The 12 dogs were randomly allocated to two groups of six dogs (untreated control group and afoxolaner treated group) based on body weight. All dogs were observed daily from acclimation start to the end of the study for general health. None of the dogs participating in this study had been treated with any acaricide/insecticide compound within three months preceding Day 0. The animals were kept individually in cages with visual and auditory contact with conspecifics. Dogs from the same group were allowed to access an outdoor shared exercise area from Day 7 onwards. At least one toy was made available to each dog (replenished weekly). The dog cages were part of a semi-indoor animal unit with a natural photoperiod.

On Day 0, NexGard® (2.27% w/w afoxolaner chewable tablets) was administered orally to all dogs assigned to the

Table 1. Arithmetic means (±95% confidence interval [CI], $\alpha = 0.05$) for female sandflies status at 1, 48, and 72 h post-exposure.

Group	Hours post-exposure	Days of exposure and status of collected female sandflies											
		Day 1				Day 14				Day 28			
		Fed live	Fed dead	Unfed live	Unfed dead	Fed live	Fed dead	Unfed live	Unfed dead	Fed live	Fed dead	Unfed live	Unfed dead
Control	1 h	37.8 (±8.5)	0 (na)	13.8 (±6.9)	0.2 (±0.3)	26.7 (±7.3)	1.0 (±0.9)	6.7 (±3.3)	1.5 (±1.9)	35.8 (±13.6)	0.5 (±0.7)	15.0 (±9.0)	1.5 (±1.4)
	48 h	24.5 (±5.2)	13.3 (±7.0)	12.0 (±6.1)	2.0 (±1.2)	15.0 (±7.3)	12.7 (±2.3)	4.7 (±3.1)	3.5 (±2.5)	26.3 (±9.4)	10.0 (±5.4)	11.3 (±7.7)	5.2 (±4.7)
	72 h	20.7 (±5.1)	17.2 (±7.9)	10.3 (±5.3)	3.7 (±1.9)	14.0 (±6.6)	13.7 (±2.2)	3.8 (±2.5)	4.3 (±2.2)	20.5 (±6.6)	15.8 (±9.0)	9.5 (±5.8)	7.0 (±5.4)
Afoxolaner	1 h	38.2 (±5.0)	0 (na)	9.5 (±4.9)	1.8 (±2.3)	26.5 (±7.0)	1.2 (±1.2)	8.8 (±5.8)	3.3 (±1.6)	34.7 (±12.0)	0.7 (±0.4)	16.3 (±10.4)	4.8 (±2.9)
	48 h	0 (na)	38.2 (±5.0)	5.0 (±3.5)	5.3 (±3.9)	0.7 (±0.7)	27.0 (±5.9)	6.0 (±5.6)	6.2 (±2.7)	7.7 (±10.6)	27.7 (±11.8)	10.7 (±7.8)	10.5 (±3.6)
	72 h	0 (na)	38.2 (±5.0)	4.0 (±2.4)	6.3 (±4.7)	0 (na)	27.7 (±6.3)	5.3 (±4.4)	6.8 (±3.0)	4.0 (±7.1)	31.3 (±10.6)	8.0 (±5.0)	13.2 (±5.4)

na, not applicable.

treated group in accordance with European label instructions. On Day 1, 14 and 28, dogs were exposed to 60 unfed females and 10 males *P. perniciosus*, in a dark room, in a dark room for 60 min (±5 min). The sandfly numbers varied for each dog and each challenge, but the exact counts were performed at collection time 1 h after each exposure. For the sandfly exposure, each dog was sedated using medetomidine (Domitor®, Zoetis) and the head of the dog was placed into a sandfly proof net (dimensions: 40 cm × 40 cm × 40 cm). Although males do not take blood meals, their presence improves female engorgement [28]. At the end of each 1-h exposure period, sandflies were smoothly vacuumed from the enclosure, categorized (male/female), counted and assessed for viability status (live/dead). As classically performed for insects and acarians, moribund sandflies were counted as live, which is more restrictive in the assessment of effectiveness [22]. Sandflies were also categorized as either fed or unfed, separated, and transferred to containers. All live fed female sandflies were then kept and incubated in vials at approximately 25 °C and >60% relative humidity to perform further viability assessments (live, dead) at 48 and 72 h after each exposure.

A laboratory-bred strain of *P. perniciosus* originating from Italy was used for the exposures [29]. Sandflies were unfed and aged from 3 to 10 days on the day of challenge.

The efficacy of afoxolaner against *P. perniciosus* was calculated using the total number of live fed female sandflies at 1, 48 and 72 h after each exposure, according to the formula below:

$$\text{Insecticidal efficacy}\,(\%)\,\text{against sandflies} = 100 \times (P_c - P_t)/P_c,$$

where P_c = Arithmetic mean number of the proportion* of live fed female sandflies in the control group; P_t = Arithmetic mean number of the proportion* of live fed female sandflies in the treated group;

$$*\text{Proportion of live fed sandflies per animal} = [(\text{Live fed sandflies}/(\text{Live} + \text{Dead})\,\text{fed sandflies})].$$

In addition, feeding proportion (at each 1-h post-exposure) and mortality % (1, 48 and 72 h post-exposure) were calculated for each control dog, according to the formulas below:

$$\text{Feeding proportion}\,[\%] = [(\text{Total fed sandflies}/\text{Total collected sandflies}) \times 100],$$

$$\text{Mortality}\,[\%] = [(\text{Total dead fed sandflies}/\text{Total collected fed sandflies}) \times 100].$$

The groups were compared using a Wilcoxon Sum Rank Test. SAS Version 9.3 TS Level 1M2 was used for the statistical analyses.

Results

No adverse event was recorded after treatment or during the study duration.

The live or dead status, as well as the engorgement status, of the sandflies was assessed at all time-points (Table 1). The mortality observed in the control group on Days 1, 14 and 28 after the 1-h exposure ranged from 0.0% to 3.3% (Table 2) and the feeding proportion ranged from 66% to 77% (Table 3), indicating that the sandfly strain was vigorous and that the feeding model worked. The mortality observed in the treated group on Days 1, 14 and 28 after the 1-h exposure ranged from 0.0% to 5.4% (Table 2) and the feeding proportion ranged from 61.6% to 78% (Table 3). There was no statistical difference in the mortality rate, nor in the feeding proportion between the control and the treated groups at all 1-h post-exposure assessments for all time-points.

In the control group, sandfly mortality was 33.3%, 49.3% and 26.3% at 48 h and 43.5%, 52.5% and 39.5% at 72 h post-exposure on Days 1, 14 and 28, respectively. In the treated group, 100% of the fed sandflies were dead at the 48 h assessment on Day 1, 100% at 72 h on Day 14 and 91.7% at 72 h on Day 28 (Table 2).

Significantly fewer live fed sandflies were recorded for the afoxolaner treated group compared to the negative control group, both at 48 and 72 h assessments, after each exposure on Days 1, 14 and 28 ($p < 0.01$) (Table 4).

The afoxolaner insecticidal efficacy against *P. perniciosus* was 100%, 95.9% and 75.2% at 48 h post day 1, 14 and 28 challenges, and 100%, 100% and 86.3% at 72 h post day 1, 14, and 28 challenges (Table 4).

Table 2. Mortality % of fed female sandflies at 1, 48, and 72 h post-exposure.

Group	Day	Hours post-exposure		
		1 h	48 h	72 h
		Mortality (%)	Mortality (%)	Mortality (%)
Control	D1	0.0	33.3	43.5
	D14	3.3	49.3	52.5
	D28	0.9	26.3	39.5
Afoxolaner	D1	0.0	100.0	100.0
	D14	5.4	97.9	100.0
	D28	2.6	81.8	91.7

Mortality [%] = [(Total dead fed sandflies/Total collected fed sandflies) × 100].

Table 3. Feeding proportion of female sandflies at 1 h post-exposure.

Group	Day	Feeding proportion (%)
Control	D1	72.8
	D14	77.0
	D28	66.0
Afoxolaner	D1	78.0
	D14	69.6
	D28	61.6

Feeding proportion [%] = [(Total fed sandflies/Total collected sandflies) × 100].

Table 4. Average proportions of live fed sandflies and insecticidal efficacy at 1, 48 and 72 h post-exposure.

Day	Hours post-exposure	Average proportion of live fed sandflies ± 95% CI		Insecticidal efficacy (%)	*p*-value**
		Control group	Afoxolaner group		
1	1 h	100*	100*	–	
	48 h	66.7 ± 13.6	0*	100%	$p < 0.0027$
	72 h	56.5 ± 13.1	0*	100%	$p < 0.0027$
14	1 h	96.7 ± 2.5	94.6 ± 6.0	–	
	48 h	50.7 ± 213.6	2.1 ± 1.9	95.9%	$p < 0.0047$
	72 h	47.5 ± 311.8	0*	100%	$p < 0.0047$
28	1 h	99.1 ± 1.2	97.4 ± 2.1	–	
	48 h	73.7 ± 6.6	18.2 ± 21.2	75.2%	$p < 0.0129$
	72 h	60.5 ± 12.2	8.3 ± 14.4	86.3%	$p < 0.0043$

Proportion of live fed sandflies per animal [%] = [(Live fed sandflies/Total fed sandflies) × 100].

Insecticidal efficacy (%) against sandflies = 100 × $(P_c - P_t)/P_c$, where P_c = Arithmetic mean number of the proportion of live fed female sandflies in the control group; P_t = Arithmetic mean number of the proportion of live fed female sandflies in the treated group.

* For all samples showing 0 or 100% live sandflies, there is no 95% CI.

** *p*-value based on a Wilcoxon sum rank test on live fed female counts between groups at each time-point.

Discussion and conclusions

The study demonstrated the insecticidal activity, thus the mortality of female sandflies, after a blood meal on afoxolaner treated dogs. Female sandflies take their blood meal in a short time, approximately 4 min [9], and they ingest 4–5 µL of blood [2, 11]. Proteins provided by the blood meal are necessary for egg production [19, 20]. *P. perniciosus* eggs are laid 6–10 days after a blood meal, and before the next blood meal [29]. With a limited volume of blood and therefore a low quantity of afoxolaner ingested, we did not expect an immediate killing effect, but did expect the death within several hours or days after feeding, so before the females lay eggs or bite a second time. *Leishmania* promastigotes need 7 to 10 days to become infective in the female sandfly [11, 19]. Therefore treating dogs having canine leishmaniosis would prevent transmission to other dogs. *Phlebotomus* sandflies do not fly long distances (maximum 1 km) and stay usually concentrated around 200–500 m. Therefore, the biological features are in favor of the possibility of obtaining a decrease in the sandfly population biting afoxolaner treated dogs, thereby possibly reducing the rate of *Leishmania* transmission in endemic areas [13].

In this study, the observed mortality of sandflies in the control group at 1 h post exposure was low (less than 3.3% after each challenge) and the feeding proportion was higher than 66%, demonstrating the relevance of the experimental model.

A single administration of afoxolaner was enough to kill 100% of sandflies within 48 h of exposure on Day 1, 96% and 100% of sandflies within 48 h and 72 h of exposure on Day 14; and 75.0 and 86.4% of sandflies within 48 h and 72 h of exposure on Day 28, respectively. Recently another isoxazoline, fluralaner, also demonstrated efficacy against *Phlebotomus papatasi* sandflies after oral administration to dogs [14]. The authors reached a similar conclusion on the beneficial activity of systemic isoxazolines for the control of vector populations.

Isoxazolines, like afoxolaner or fluralaner, do not provide prevention of *Leishmania* transmission as the female sandflies bite before dying. Therefore, preventative measures like repellent application or vaccination are still needed for individual dog protection. Nevertheless, the insecticidal activity of systemically active isoxazolines against the vector is an additional beneficial measure to decrease vector density and decrease risk at the population level.

Competing interest

The work reported herein was funded by Boehringer-Ingelheim Animal Health. Wilfried Lebon and Frédéric Beugnet are current employees of Boehringer-Ingelheim. Nadège Perier and Nesrine Aouiche are veterinarians, finishing a Master 2 degree in Science at Lyon University. Leon Meyer and Noua Lekouch are employees of the CRO ClinVet.

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RESEARCH ARTICLE OPEN ACCESS

Insecticidal efficacy of afoxolaner against bedbugs, *Cimex lectularius*, when administered orally to dogs

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Abstract – The objective of this experimental study was to assess the insecticidal efficacy of afoxolaner (NexGard®) against bedbugs (*Cimex lectularius*) on dogs. For each challenge, 20 bedbugs were placed in two chambers positioned in contact to the dog's skin for 15 min, after which live fed parasites were counted and incubated for survival evaluations. On Day 0, 7 dogs assigned to the treated group were administered afoxolaner orally at the registered dose. All 14 dogs were challenged on Days 1, 7, 14, 21 and 28, and the collected live fed *C. lectularius* incubated for 72 h (Day 1), and 72 h and 96 h (Days 7, 14, 21 and 28) for survival evaluation. The percent feeding in the control group ranged from 95% to 98.6% and the percent of live fed bedbugs at 96 h ranged from 99.3% to 100% in the control group, demonstrating the viability of the strain and their capacity to feed on dogs. Significantly fewer live fed bedbugs were counted in the treated group, compared to the control group, at all time-points. The reduction of live fed *C. lectularius* in the afoxolaner group was 41.4% at 72 h after the Day 1 challenge, and 77.2%, 82.7%, 85.0% and 63.5% at 96 h after the Days 7, 14, 21 and 28 challenges, respectively. It is hypothesized that monthly treatment of dogs with afoxolaner could help in preventing a bed bug population from installing in a household if bedbugs bite dogs in the presence of humans.

Key words: Dogs, Bedbugs, *Cimex lectularius*, Afoxolaner, Insecticidal activity.

Résumé – Efficacité insecticide de l'afoxolaner administré par voie orale à des chiens contre les punaises de lit, *Cimex lectularius*. L'objectif de cette étude expérimentale était de déterminer l'efficacité insecticide de l'afoxolaner (NexGard®) contre les punaises de lit (*Cimex lectularius*) chez les chiens. Pour chaque exposition, 20 punaises de lit ont été mises dans deux chambres placées en contact avec la peau des chiens pendant 15 minutes. Après cela, les parasites vivants et gorgés ont été comptés et incubés pour évaluer leur survie. Le jour 0, 7 chiens affectés au groupe traité ont reçu de l'afoxolaner (NexGard) par voie orale à la dose commerciale. Les 14 chiens ont été exposés aux punaises aux jours 1, 7, 14, 21 et 28, et les *C. lectularius* vivants et gorgés, collectés, ont été incubés pendant 72 h (jour 1) et 72 et 96 h (jours 7, 14, 21 et 28) pour l'évaluation de la survie. Le pourcentage d'engorgement dans le groupe témoin variait de 95 % à 98,6 % et le pourcentage de ces punaises vivantes à 96 h variait de 99,3 à 100 %, démontrant la viabilité de la souche et la capacité à se nourrir des chiens. Le nombre de punaises vivantes était significativement plus faible dans le groupe traité, par rapport au groupe témoin, à chaque point de contrôle. La réduction de *C. lectularius* vivants dans le groupe afoxolaner était de 41,4 % à 72 h après l'exposition du jour 1, et respectivement de 77,2 %, 82,7 %, 85,0 %, et 63,5 % à 96 h après les expositions des jours 7, 14, 21, et 28. On peut donc faire l'hypothèse que le traitement mensuel des chiens avec de l'afoxolaner pourrait empêcher une population de punaises de lit de s'installer dans un foyer, si les punaises piquent les chiens en présence d'humains.

Introduction

Bedbugs are hematophagous arthropods. The discovery of specimens in tombs at Tell al-Armana, Egypt, suggests that these insects have been pestering humans for at least 3550 years [1, 14]. With social progress and insecticide development, bedbugs became uncommon in developed countries in the 1950s. Nevertheless, a clear bed bug resurgence has been observed since 2000 in many western countries [1, 12].

Bedbugs, *Cimex lectularius* Linnaeus, 1758 and *Cimex hemipterus* Johan Christian Fabricius, 1803, belong to the Hemiptera order of insects, in the Cimicidae family. They are related to Reduviidae, i.e. kissing bugs like *Triatoma* spp. and *Rhodnius* spp., the vectors of Chagas disease (*Trypanosoma*

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cruzi infection) in Central and South America. Unlike in the case of kissing bugs, no vector role is known for bedbugs [7]. They do, however, represent a major nuisance due to their bites inducing papules, erythema, pruritus, pain, but also psychologic fear [1, 20]. Typically, the bites frequently follow a line or curve [20].

Adult *C. lectularius* and *C. hemipterus* are reddish-brown, flat, wingless, oval insects (4–7 mm). Both males and females are hematophagous and can live for 12 months without feeding and even 1.5–2 years in colder environments. Under a constant temperature of 14–27 °C, eggs hatch 4–10 days after mating, yielding the nymphs, which are 1–3 mm long, and lighter than adults in color. Each of the 5 nymphal stages require a blood meal, which can last 10–20 min, to be able to moult to the next stage in about 3–7 days. Bedbugs fear light and are generally active in the dark [1, 14]. They hide in any small dark place, such as bedclothes, mattresses, springs, bed frames, cracks, crevices, sofa, carpets, and wallpaper. Bedbugs can travel long distances when being dispersed by human clothes, luggage, or furniture. Overcrowding and derelict living conditions may be factors that increase the bed bug burden in an area [1, 13].

Bed bug eradication from a contaminated site is a challenge due to their ability to hide and the difficulty to kill all insects in the environment. Insecticide resistance is also an increasing problem [6, 8]. Successful bed bug elimination relies mainly on good cooperation between the owner of the contaminated site and the pest manager for site assessment, thorough inspection, identification, and eradication [13]. In heavily contaminated environments, an "efficient search-and-destroy" operation must be imposed, starting by removing all bed linens and washing them at a temperature higher than 60 °C. Checking and dismantling all furniture is the next step to access all bedbug hiding places, to identify and destroy eggs, nymphs, and adults. It is always best to vacuum the affected area first to reduce the overall bed bug population. The use of persistent insecticides provides residual protection against survivors, but integrated pest management is key and we cannot only rely on the use of pesticides. In buildings, it may be advisable to treat adjoining apartments or rooms, even when no bedbugs were found during the inspection [1].

Bedbugs probably originated as ectoparasites of bats, infesting humans when they co-habitated in caves [1, 14]. As humans quickly domesticated dogs and cats in the prehistoric period, *Cimex* insects may also have been in contact with these mammals in ancient times. They thus may opportunistically feed on household pets like dogs and cats, which needs to be better assessed in the field [1, 14]. Therefore, it may be interesting to study the effectiveness of systemic insecticides like NexGard administered to dogs against bedbugs. The impact on bed bug populations would need to demonstrate that bedbugs bite dogs in the presence of humans who are considered to be the preferred hosts.

Isoxazolines represent the most recent chemical group of insecticides developed for use in domestic animals. Their mode of action is systemic, after oral administration, or through transcutaneous absorption [22]. To date, the only assessment of the insecticidal activity of a systemic insecticide against bedbugs was conducted using an *in vitro* design with a macrocyclic lactone, moxidectin [23]. No assessment has been conducted under real conditions with an *in vivo* design. Isoxazolines are highly bound to plasma proteins (i.e. 99%), have a long half-life (i.e. 9–14 days for oral afoxolaner), and provide high insecticidal activity against hematophagous parasites [16, 22]. Afoxolaner, formulated as a palatable chewable tablet kills existing and new infesting fleas and ticks for a month [10]. Additionally, it is effective against mites (i.e. *Sarcoptes scabiei*, *Demodex canis*, *Otodectes cynotis*) [2, 3, 5], and has demonstrated insecticidal activity against hematophagous flying insects (i.e. sandflies, *Phlebotomus perniciosus*, and mosquitoes, *Aedes aegypti*) through the treatment of dogs [17, 21]. Recently, an insecticidal effect of afoxolaner and fluralaner has also been demonstrated in treated dogs against kissing bugs (*Triatoma infestans*), the vector of Chagas disease [15, 18]. We therefore hypothesized that the blood of isoxazoline-treated dogs could be able to kill bedbugs. This study was conducted to determine the insecticidal efficacy of NexGard® (Boehringer Ingelheim Animal Health) against bedbugs (*C. lectularius*) feeding on treated dogs.

Materials and methods

The study was approved by the Clinvet Institutional Animal Care and Use Committe (IACUC), and followed European Union Directive 2010/63/EU on the protection of animals used for scientific purposes [11]. It was conducted in accordance with the VICH GL9 guideline on Good Clinical Practices (https://www.ema.europa.eu/en/vich-gl9-good-clinical-practices).

The study was a blinded, randomized, negative-controlled, efficacy study. The design was inspired by the European Medicine Agency (EMA) and the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines for the testing and evaluation of the efficacy of antiparasitic substances against ectoparasites [9, 19]. The study included two groups of seven dogs each, selected from an initial group of 16 dogs. Group 1 dogs were sham-dosed (negative control), whereas group 2 dogs were treated orally once with afoxolaner (NexGard®, Boehringer Ingelheim Animal Health) at label dose. Sixteen dogs (males and females) were acclimatized to their cages from Day −7. On Day −6, an initial exposure to 20 *C. lectularius* was conducted to evaluate the susceptibility of each dog to experimental infestation, for random allocation of the dogs to the study groups, and to test the model. The two dogs with the lowest number of live fed bedbugs at 15 min post-exposure were removed.

Fourteen healthy dogs, 5 beagles and 9 mixed-breeds, 7 males and 7 females, weighing between 11.90 kg and 17.65 kg, >6 months, that had not been treated with a topical or systemic acaricide/insecticide over the 12 weeks preceding Day 0 were randomly allocated to groups 1 and 2 on the basis of *C. lectularius* live fed pre-treatment counts.

On Day 0, all dogs assigned to group 2 were administered a 68 mg NexGard chew, in order to deliver at least 2.5 mg/kg afoxolaner, in accordance with the label [10]. Dogs in group 1 were sham-dosed by bringing the animal to the table, opening the mouth and then returning the animal to its cage. Dogs were observed hourly for 4 h after NexGard/sham-dose

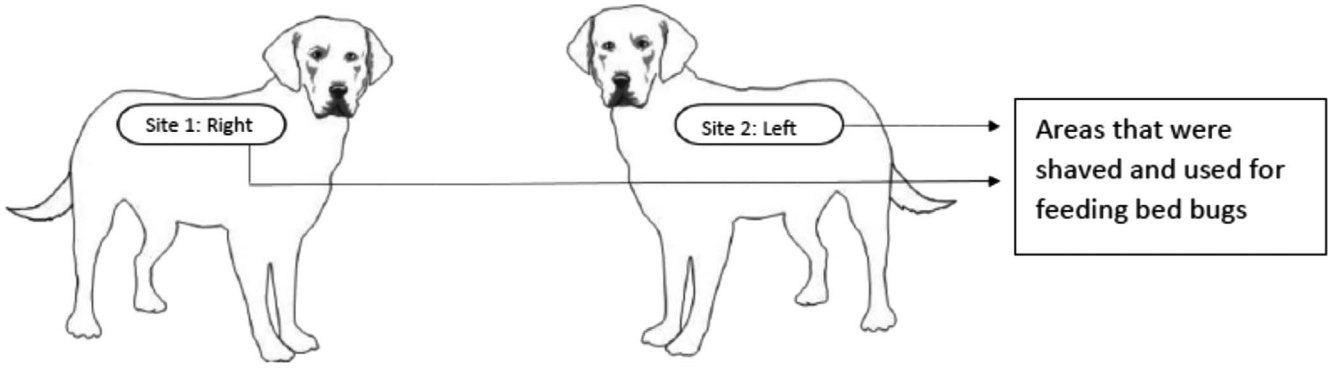

Figure 1. Areas of feeding of the bedbugs on dogs.

administration, and daily from Day −7 to Day 28 for general health and adverse reactions.

Dogs were exposed to 20 unfed, adult *C. lectularius* on Days 1, 7, 14, 21 and 28 to assess insecticidal activity. The bedbugs were fed on rabbits one week earlier, following the breeding process of the colony, including one blood meal per week. For each *C. lectularius* challenge, 20 unfed adult bedbugs were placed in chambers positioned in close contact to the dog's skin for at least 15 min, after which time live fed parasites were counted and incubated for survival evaluation. Feeding assessment on all bedbugs was performed immediately post-challenge by visual observation of the fed state of the bedbugs. The live fed bedbugs were then incubated in an insectarium and viability assessments were performed at 72 h (Day 1), and 72 h and 96 h (Days 7, 14, 21, 28) post-feeding. For each challenge, dogs were sedated with medetomidine (Domitor®, 100 µg/kg, 0.1 mL/kg intramuscular injection prior to exposure to the bedbugs, at the end of which, atipamezole (Antisedan®, 200 µg/kg, 0.04 mL/kg intramuscular injection) was used to reverse the sedation. The sedated dogs were placed in a dark room to facilitate feeding of the bedbugs. Two chambers, covered with an appropriate material (mesh netting) through which the bedbugs could feed, containing ten adults (5 males and 5 females) each, were held against the shaved areas of the dog, by applying light pressure to ensure sufficient contact with the dog's skin. The shaving is related to the mesh netting of the chambers which would flatten the hairs and form a barrier on unshaved dogs/rabbits. After each feeding phase, the dog's skin was examined for any abnormality. No adverse reactions to the feeding of the bedbugs were observed. Shaved areas for placement (i.e. Site 1 [right side of dog] and Site 2 [left side of dog]) of feeding chambers on each dog, were prepared, on Day −7, and then re-used for all subsequent exposures (Fig. 1).

The *C. lectularius* strain had originally been collected in human habitats in Bloemfontein, South Africa in 2017, and since then was maintained in an insectarium and fed weekly on rabbits using classical methods to breed bedbugs [4]. Adult unfed male and female bedbugs were used in the challenges.

The fed and live bedbugs were incubated in an insectarium with a controlled temperature (22.0 °C to 26.9 °C) and humidity (67.3–76.2% relative humidity) and assessed for live/dead status at 72 h and 96 h [4].

Following the WAAVP guideline on how the assess insecticidal efficacy, the primary efficacy criterion was comparison of the number of live fed bedbugs counted in the treated group compared to the negative control on the various assessment days [9, 19]. The difference in dead bed bug counts was considered a secondary criterion.

The insecticidal efficacy against bedbugs was calculated at each assessment day according to the formula given below. Efficacy calculations were based on arithmetic mean values:

$$\text{Insecticidal efficacy}\,(\%)\,\text{against bedbugs} = 100 \times (M_c - M_t)/M_c,$$

where:

M_c = mean number of live, fed bedbugs in the control group at each assessment time point (72 h and 96 h after the 15 min feeding phase);
M_t = mean number of live, fed bedbugs in the treated group at each assessment time point (72 h and 96 h after the 15 min feeding phase).

Due to the lack of bed bug specific guidelines, the Committee for Medicinal Products for Veterinary Use (CVMP) guideline "Guideline for the testing and evaluation of the efficacy of anti-parasitic substances for the treatment and prevention of tick and flea infestation in dogs and cats" suggestion that at least six animals were to be used per group was followed.

The groups were compared using an ANOVA with a treatment effect on logarithmic transformed fed bed bug (count + 1) data. The level of significance of the formal tests was set at 5%. All tests were two-sided.

Results

No significant difference was recorded between the two groups regarding dog body weights ($p = 0.7128$) and live fed bed bug counts ($p = 0.8597$) during the assessment performed in the acclimation period. This indicated homogenous distribution between the two groups.

No abnormal signs were observed during the daily observations, during the specific post-administration observations. No adverse skin reactions were observed on the bedbugs feeding site.

Table 1. % Insecticidal and % mortality observed on bedbugs at 72 h and 96 h post 15 min feeding exposure to dogs (arithmetic means).

	Day 1 + 72 h	Day 7 + 72 h	Day 7 + 96 h	Day 14 + 72 h	Day 14 + 96 h	Day 21 + 72 h	Day 21 + 96 h	Day 28 + 72 h	Day 28 + 96 h
Control									
Mean number Live fed	19.00	19.40	19.40	19.10	19.00	19.00	19.00	19.70	19.60
Mean number Dead fed	0.10	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.10
Mean number Unfed	0.90	0.50	0.50	0.80	0.80	1.00	1.00	0.30	0.30
Treated									
Mean number Live fed	11.10	5.60	4.40	4.70	3.30	4.70	2.90	9.00	7.10
Mean number Dead fed	7.30	13.70	14.90	14.00	15.40	13.60	15.40	10.30	12.10
Mean number Unfed	1.50	0.70	0.70	1.30	1.30	1.70	1.70	0.70	0.70
% Insecticidal efficacy	**41.4**	71.13	**77.2**	75.4	**82.7**	75.2	**85.0**	54.3	**63.5**
p-value	0.0059	0.0059	0.0059	0.0059	0.0059	0.0059	0.0059	0.0059	0.0059
% Mortality treated fed	**39.7**	71.0	**77.2**	75.0	**82.3**	74.3	**84.1**	53.4	**63.0**
p-value	0.001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

p-value: One-way ANOVA with a treatment effect.
Mean numbers in arithmetic means.
Bold values indicate that it is the last time-point after each exposure day.

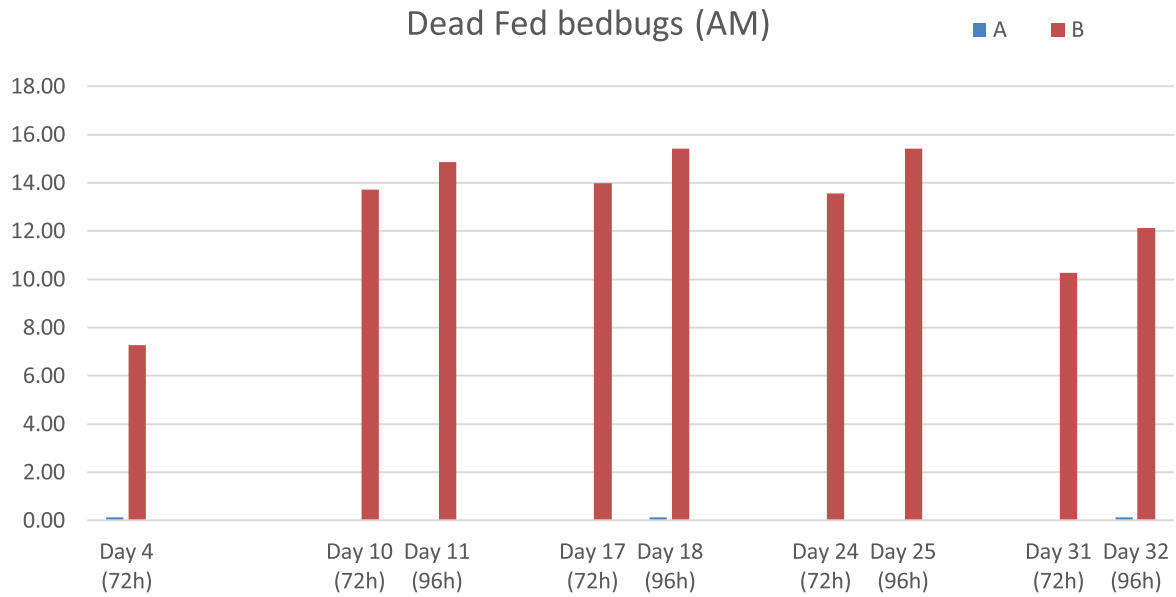

Figure 2. Number of dead fed bedbugs counted in the control group (A-blue) and the treated group (B-red).

Bed bug challenges and viability assessments

Percentage of feeding in control group 1 ranged from 95.0% to 98.6% at all assessment time points, demonstrating good host tropism for dogs (Supplementary Figs. 1 and 2). Percentage of live bedbugs observed at 72 h and 96 h in control group 1 ranged from 99.3% to 100%, demonstrating the very good viability of the bedbugs (Table 1, Fig. 2).

A significantly smaller number of live fed bedbugs was recorded for treated group 2, compared to negative control group 1 at all assessment time points ($p \leq 0.02$) and significantly more dead fed bugs were counted in the treated group (Table 1).

The insecticidal efficacy observed on Day 1 was 41.4% at 72 h after the feeding phase (no evaluation was performed at 96 h). On the subsequent infestations, afoxolaner showed efficacy ranging between 63.5% and 85.0% at 96 h (Fig. 3).

Discussion

There are limited data on the infestation of dogs and cats by *C. lectularius* bedbugs. They are thought to bite pets, but the frequency remains unknown [1, 17]. The fact that bedbugs are maintained in the laboratory using weekly feeding on rabbits provides initial evidence that mammals other than humans may be functional hosts [4]. In this study, 18–20 of the bugs (>95% in all challenges) did take a blood meal in both groups at each challenge, demonstrating a high tropism for dogs, with no natural repellent effect that could have been related to skin thickness or dog odour. Dogs were shaved smoothly, avoiding any skin damage or irritation, on a small surface corresponding to the vials containing the bedbugs, exactly as performed on rabbits. The shaving was performed to ease the application of the vial, avoiding bedbug escape. We do not consider that bedbugs would have any difficulty

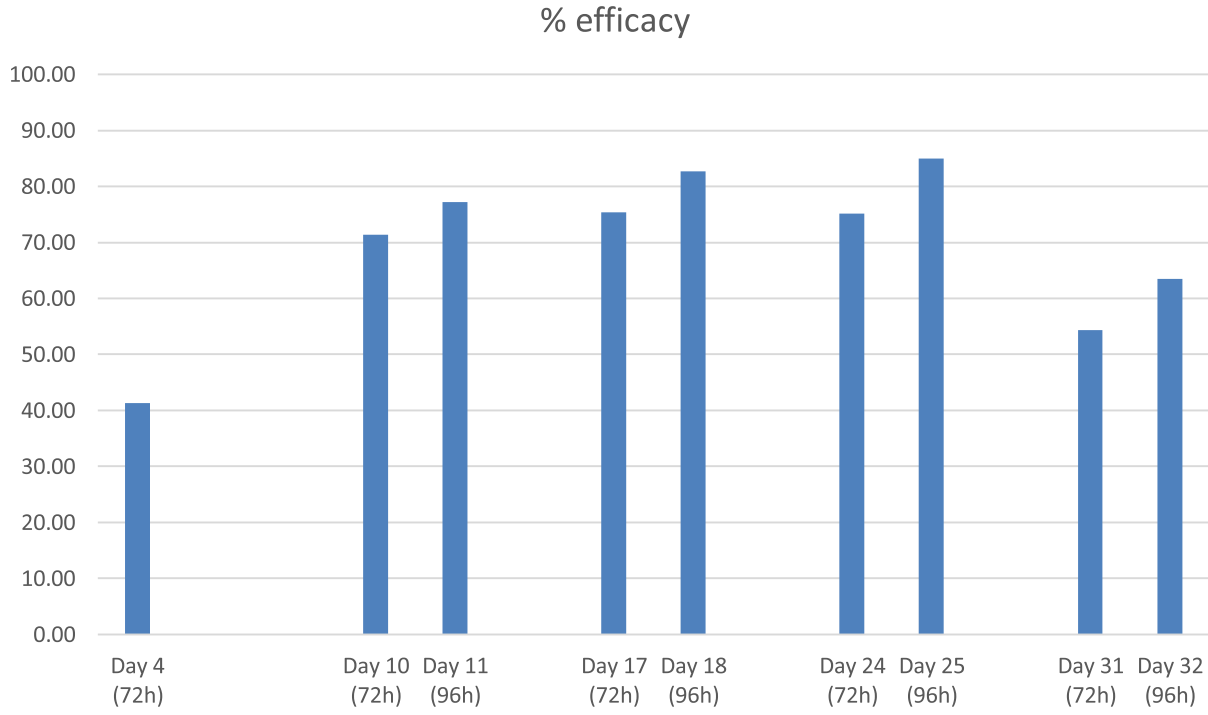

Figure 3. % NexGard efficacy against bedbug feeding on treated dogs.

moving past hair, exactly as ticks do. Morphologically bedbugs are dorsoventrally flat and in that sense close to the form of ticks. Under natural conditions, they may also have easy access to body parts that are not hairy like the abdomen, especially when the dogs sleep.

There was no bedbug feeding rate difference observed between the two groups. Afoxolaner is an insecticide acting through blood ingestion, therefore no repellent effect is expected against insects [16, 22]. The absence of a difference in feeding rates between control dogs and treated dogs had already been observed in insecticidal efficacy studies against mosquitoes and sandflies [17, 21].

Feeding on untreated dogs did not impair the viability of the bedbugs. The arithmetic mean of dead bedbugs in the untreated control group ranged between 0 and 0.1 at 96 h, corresponding to a maximum of 1 dead bed bug for 120 bedbugs used in each exposure (20 × 6 dogs).

Afoxolaner demonstrated significant insecticidal activity at all time-points. The lowest efficacy was observed after the Day 1 challenge, which may be related to the pharmacokinetic properties of the treatment and a lower plasma concentration of the active substance. Thereafter, afoxolaner efficacy was above 77.2% at 96 h for 3 weeks, with a peak at 85.0%, decreasing to 63.5% at the end of the month. The insecticidal activity was greater at 96 h than 72 h, indicating gradual death of the bedbugs. These observations are consistent with the known significant correlation between afoxolaner plasma concentrations and efficacy [16], and was already observed in the efficacy studies against mosquitoes and sandflies [17, 21]. This is not the case in flea and tick efficacy studies as the minimum dose of afoxolaner (i.e. 2.5 mg/kg) was determined to provide sustained efficacy >95% against fleas within 24 h and >90% against ticks within 48 h for a full month [10, 16, 22]. The actual mortality percentages could be higher than those observed at 96 h, if the bugs had been incubated for longer observations.

As bedbugs may take a blood meal each week or more frequently [1, 4, 20], it would have been interesting to study the efficacy after a second bite on the bedbugs that had survived the first one, but this was not originally planned in this study design.

Our study results are the first demonstration of insecticidal efficacy of an isoxazoline compound, afoxolaner, against bedbugs. This opens the door for new possibilities to control bed bug infestations by regularly treating dogs living in households. Nevertheless, further assessments need to be carried out, especially to verify that bedbugs take blood meals on dogs in the presence of humans. This could be performed through PCR techniques on collected bedbugs, identifying specific dog markers. The authors are working on this type of epidemiological survey and are collecting bedbugs in apartments and households where dogs and cats are present. Another next step could be to assess the capability of bedbugs to take a blood meal on cats, as they do on dogs, and to assess the efficacy of isoxazoline against bedbugs through treated cats. Finally, an additional important step will be to perform investigations to measure the effect of this potential new mode of control under real situations through epidemiological surveys.

Supplementary materials

Supplementary material is available at https://www.parasite-journal.org/10.1051/parasite/2021004/olm

Supplementary Figures 1 and 2. Experimental device with adult bedbug feeding on dogs.

Competing interest

The work reported herein was funded by Boehringer Ingelheim Animal Health. Wilfried Lebon, Nesrine Aouiche and Frédéric Beugnet are current employees of Boehringer Ingelheim. The other authors are employees of the Contract Research Organization Clinvet. NEXGARD® is a registered trademark of Boehringer Ingelheim. All other trademarks are the property of their respective owners. This document is provided for scientific purposes only. Any reference to a brand or trademark herein is for informational purposes only and is not intended for a commercial purpose or to dilute the rights of the respective owner(s) of the brand(s) and trademark(s).

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