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Laboratory evidence that dinotefuran, pyriproxyfen and permethrin combination abrogates *Leishmania infantum* transmissibility by sick dogs

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Abstract. Dogs are reservoir hosts of leishmaniasis caused by *Leishmania infantum* and transmitted by phlebotomine vectors. The effect of dinotefuran, pyriproxyfen and permethrin spot-on solution (Vectra®3D, Ceva Santé Animale, Libourne, France) on *Leishmania* transmissibility by naturally infected dogs via reared *Phlebotomus perniciosus*, was assessed. Dogs affected by leishmaniasis were submitted to xenodiagnosis and 6 infecting >10% of insects were treated topically on day 0. Antifeeding, insecticidal and anti-transmissibility effects were evaluated through xenodiagnoses performed on days 1, 7 and 28, using individual pre-treatment parameters as control. Feeding and mortality rates were assessed at 24 h, whereas promastigote infection, maturation and burden were assessed up to 96 h post blood meal (potentially infectious rate). On day 1, the anti-feeding efficacy was >95% in 4 dogs, insecticidal efficacy 100% in 4 dogs, and anti-transmissibility effect 100% in 6 dogs. Efficacy rates recorded on day 7 were very similar to day 1. On day 28, anti-feeding and insecticidal efficacy values were much broader, ranging 32.6–100% and 7.7–94.4%, respectively. Potentially infectious insects were recorded from two dogs, with sharp decrease in transmissibility rate as compared with pre-treatment condition. Altogether, Vectra®3D abrogated by >98% the potential *Leishmania* transmissibility by the examined pool of infected dogs over 1 month.

Key words. Leishmania infantum, sand flies, xenodiagnosis, transmissibility, affected dogs.

Introduction

Dogs are the only confirmed primary reservoir of zoonotic visceral leishmaniasis (Quinnell & Courtenay, 2009), a protozoan disease caused by *Leishmania infantum* in the Mediterranean, Middle East and Central Asia in the Old World, and in Latin American countries (World Health Organization, 2010; Dantas-Torres *et al.*, 2012). Outcomes of canine leishmaniasis (CanL) infection range from a subclinical condition to clinical stages characterized by increasing severity (Paltrinieri *et al.*, 2010; Solano-Gallego *et al.*, 2011). Earliest signs of progressive disease are lymph node enlargement and weight loss, followed by cutaneous abnormalities, renal alterations and ocular signs (Foglia Manzillo *et al.*, 2013). Although *L. infantum* infection can be acquired by non-vectorial modes such as venereal and congenital transmission, or via blood transfusion (EFSA Panel on Animal Health and Welfare, 2015), its main transmission route is by deposition into the host's skin of infective metacyclic promastigotes by the bite of infectious phlebotomine sand flies (Killick-Kendrick, 1999). All stages of canine infection can

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be potentially infectious to vectors; however, the duration and severity of CanL are associated with increased probability of transmission (Courtenay *et al.*, 2014; Gizzarelli *et al.*, 2021). The potential for *Leishmania* transmissibility by competent phlebotomine vectors is dependent on the capacity of multiplication, migration and maturation of flagellates to infective stages in the foregut about 1 week after an infected blood meal is ingested (Bates, 2007). Increased transmissibility may occur following reverse metacyclogenesis stimulated by a second ingestion of uninfected blood meal (Serafim *et al.*, 2018).

Phlebotomus perniciosus is the main competent vector of *L. infantum* throughout the western Mediterranean basin, including southern Europe (Portugal, Spain, southern France, Italy and northwest Croatia) and Maghreb (Morocco, Algeria and Tunisia) (European Centre for Disease Prevention and Control, 2020). Recently, this sand fly species was shown experimentally to have potential competence to transmit *Leishmania tropica* (Bongiorno *et al.*, 2019), a secondary agent of CanL in North Africa and Middle East (Baneth *et al.*, 2017). *Phlebotomus perniciosus* is also the main representative of the *Larroussius* subgenus, consisting of morphologically and biologically close-related species proven *L. infantum* vectors in endemic areas of central and eastern Mediterranean, such as *Phlebotomus neglectus*, *Phlebotomus perfiliewi* and *Phlebotomus tobbi* (Alten *et al.*, 2016).

Dog protection from sand fly bites is universally considered to be the first-line approach to prevent CanL infections and transmission of zoonotic visceral leishmaniasis by infected dogs (WHO, 2010; Miró *et al.*, 2017). This can be achieved by the use of several topical formulations, such as collars, spot-ons or sprays, containing synthetic pyrethroids with proven anti-feeding (excito-repellent) and insecticidal effects against phlebotomine sand flies (Maroli *et al.*, 2010; Fondati *et al.*, 2018). After being applied to the dog's skin, their active ingredients spread over the entire surface of the body and the hair coat. Although discouraged by manufacturers and regulatory authorities, a combination of devices is commonly used by dog's owners, being the association of collar plus spot-on the most frequently employed (Zini *et al.*, 2020). Preventive measures against sand flies are typically adopted to protect healthy dogs from *L. infantum* infections, less so to avoid parasite spreading by CanL sick dogs, for which anti-leishmanial therapies may lead to decreased infectiousness to vectors, but have only temporary efficacy (Gradoni *et al.*, 1987; Miró *et al.*, 2011). Analogously, laboratory studies evaluating topical pyrethroid formulations against sand flies make use of naïve purpose-bred Beagles as per consensual international guidelines (Otranto *et al.*, 2021). Clinical trials of dinotefuran, pyriproxyfen and permethrin spot-on solution (Vectra®3D, Ceva Santé Animale) against *P. perniciosus*, have been found to confer elevated protection from sand-fly bites for 1 month in uninfected dogs under controlled conditions (Liénard *et al.*, 2013; Varloud *et al.*, 2015). The present study aimed to assess the effect of the spot-on formulation on the potential *L. infantum* transmissibility by infected dogs via the vector *P. perniciosus*. Animals naturally affected by clinical CanL were enrolled and xenodiagnosis assays performed over 1 month using reared sand flies.

Materials and methods

The study was part of a larger investigation on clinical characteristics of CanL associated with infectiousness to competent phlebotomine sand flies. Procedures of animals' enrolment and xenodiagnosis test have been reported in detail by Gizzarelli *et al.* (2021). Briefly, naturally infected dogs were identified based on clinical examination and multiple diagnostic assays, including immunofluorescence antibody test (IFAT) on sera (IFAT slides were provided by National Reference Centre for Leishmaniosis-CReNaL; anti-dog IgG used was by Sigma–Aldrich, St. Louis, MO, U.S.A.) and loop-mediated isothermal amplification (LAMP) on blood, fine-needle aspiration of lymph nodes and conjunctival swabs (Maurelli *et al.*, 2020). The study was conducted at the Regional Centre for Monitoring Parasitic Diseases (CREMOPAR), Eboli (SA), Italy, where dogs were housed and handled in accordance with the Animal Welfare and Good Clinical Practice guidelines (VICHGL9, 2000; Directive 2010/63/UE; National Legislative Decree 26/2014). Dog's owners signed an informed consent. Dogs were housed individually, observed daily for general health conditions and acclimatized for at least 5 days before pre-treatment test.

Reared sand flies were from a *P. perniciosus* colony maintained at the facilities of Istituto Superiore di Sanità (ISS), Rome, since June 2012 at standard rearing conditions (Lawyer *et al.*, 2017) and certified for pathogen-free status. An average of 98 (range 85–141) unfed females ageing 3–9 days, plus about 10% males to promote feeding behaviour, were allowed to feed for 90 min on caged dogs in pre-treatment tests performed 1–4 weeks before enrolment, and on days 1, 7 and 28 after treatment of enrolled dogs. Because of the chronic progression of CanL disease, it was assumed that *Leishmania* infectiousness to vectors did not change much in each dog during 5–8 weeks, so that each animal served as its own control by comparison with pre-treatment xenodiagnosis results. Only dogs infecting >10% of sand flies and with stable renal function (IRIS stage I, without or with mild proteinuria) were included in the study and treated with Vectra®3D on day 0. The product was applied as per label in a line-on. At the end of the study, the dogs were offered a treatment against CanL and were followed-up by the veterinary clinician team.

Xenodiagnosis assays were performed in individual large exposure cages as described by Gizzarelli *et al.* (2021). Two hours before exposure to the vector, dogs received Adaptil Express Calming tablets (Ceva Santé Animale, 1 tablet/10 kg of body weight) and were allowed to acclimatize inside the exposure cage in the dark for 30 min before the release of sand flies. Cages were located in separate rooms, one for pre-treatment assays, the other for assays on treated animals to avoid contaminations. At the end of the test, live and dead sand flies were recovered with the help of a mouth aspirator and transferred into plastic transport pots equipped with a fine gauze holding a piece of cotton soaked with glucose-saturated solution. Immediately after collection, the specimens were transported by car (trip duration approximately 2.5 h) inside preheated insulated container at appropriate humidity conditions. The pots were maintained thereafter in the usual rearing conditions at the ISS facilities.

At 24 h from exposure, live or dead blood-fed, and live or dead unfed specimens, were recorded. All live blood-fed sand flies were individually transferred into glass vials and observed daily for blood digestion and mortality. Blood feeding rate (total no. of blood-fed specimens–total no. of recovered specimens), anti-feeding rate (total no. of unfed specimens–total no. of recovered specimens) and mortality rate (total no. of dead specimens–total no. of recovered specimens) were determined. At each post-treatment assessment, anti-feeding and insecticidal efficacies were calculated by the comparison with the respective rates recorded for the same dog in the pre-treatment assays, as follows: [100 × (anti-feeding rate in treated dog–anti-feeding rate in untreated dog)/1 − anti-feeding rate in untreated dog] and [100 × (mortality rate in treated dog–mortality rate in untreated dog)/1 − mortality rate in untreated dog] (Liénard *et al.*, 2013).

At 96 h post blood meal, sand flies were dissected and microscopically examined to detect flagellate parasites in order to determine a promastigote infection rate (total no. of promastigote-positive specimens/total no. of dissected sand flies), being a rough measure of dog's infectiousness. Promastigote burden was also evaluated, scored as light (<100 parasites/gut), moderate (100–500 parasites/gut), heavy (500–1000 parasites/gut) or very heavy (>1000 parasites/gut) (Sádlová *et al.*, 2003). The 96 h period is necessary for the parasite to develop from the amastigote stage (ingested through the blood meal) to promastigotes in active multiplication, stage maturation and migration towards the foregut after blood digestion, before they transform into the metacyclic infective stage (Bates, 2007). The potential transmissibility rate of *L. infantum* by xenodiagnosed dogs (total no. of potentially infectious specimens–total no. of females used in the challenge) was thus determined by a combination of the two entomological parameters, the microscopy evidence for foregut migration of promastigotes, and their 'heavy' or 'very heavy' burden. Hence, an anti-transmissibility effect conferred by treatment could be calculated by the comparison of this parameter from the same dog in pre-treatment tests as follows:

[100 × (potential transmissibility rate in treated dog–potential transmissibility rate in untreated dog)/1 − potential transmissibility rate in untreated dog].

The non-parametric test of Kruskall Wallis was used to test entomological parameters from dogs at different days from treatment vs. pre-treatment condition, using SPSS Statistics v.23 (IBM, Armonk, NY, U.S.A.) and significance level of $P < 0.05$.

Results

Among potentially eligible dogs diagnosed as *Leishmania*-positive, six were enrolled because their clinical condition was compatible with inclusion criteria while showing vector infectiousness rates >10% at the xenodiagnosis test. Five dogs were males and only one was pure breed. Ages ranged from 1 to 7 years and body weight 7 to 19 kg; all dogs presented elevated anti-*Leishmania* antibody titres (≥1:2560) at the IFAT assay, a known prognostic marker for the increased probability of infectiousness (Courtenay *et al.*, 2002) (Table 1). In addition, LAMP resulted positive for lymph nodes matrix for all the six dogs, while five of them were positive also for conjunctival swab and one also for blood samples (Table 1). Clinical signs, each being assigned a score specific for this study (Gizzarelli *et al.*, 2021), were present in all dogs but of different types and severity. For example, cutaneous alterations ranged from single focal alopecia (score 1, dogs 1 and 3) to generalized dermatitis and ulcers (score 7, dog 5). A number of parasitological and clinical parameters were considered (IFAT value; number of LAMP-positive tissues; cutaneous signs; other non-cutaneous signs; haematological, renal and hepatic laboratory markers) whose scores were added up to contribute to an 'overall disease severity' score ranging from 6 (dog 3) to 14 (dog 5) (Table 1).

Table 2 reports on four entomological parameters recorded at the pre-treatment xenodiagnosis test. *Phlebotomus perniciosus* feeding rates ranged from 39.3% in dog 3 to 90.7% in dog 6. Mortality rates were generally elevated considering the untreated condition of dogs, from 9.1% (dog 4) to 29.1% (dog 5); this much probably reflecting a susceptibility of the reared insects submitted to unusual experimental conditions. Promastigote-infection rates were also elevated, in a range from 25.5% (dog 4) to 78.9% (dog 2); as expected, potentially infectious females were less than the positive ones – indicating a proportion of sand flies in which the parasite failed to multiply and develop further – and so were the rates, which ranged between 19.6% and 63.6%. The ratio 'infectious rate' over 'positive rate' varied from 0.6 (dog 2) to 0.9 (dog 6), but this did not differ significantly between dogs.

Table 1. Breed, age, sex and disease severity of the enrolled dogs.

Dog number	Sex	Age (years)	Weight (kg)	Breed	Immunofluorescence antibody test titre	Loop-mediated isothermal amplification*	Clinical scores		
							Cutaneous signs	Laboratory abnormalities	Overall disease severity
1	M	4	19	Épagneul Breton	1:2560	Positive	1	2	8
2	M	1	10	Mongrel	1:5120	Positive	4	2	10
3	M	2	7	Mongrel	1:10 240	Positive	1	1	6
4	M	6	19	Mongrel	1:5120	Positive	4	3	12
5	F	7	15	Mongrel	1:5120	Positive	7	2	14
6	M	3	7	Mongrel	1:2560	Positive	3	2	10

*Positivity for at least one matrix examined (blood, lymph nodes and conjunctival swabs).
Clinical scores were from Gizzarelli *et al.* (2021). Note that other parameters not included in the table contributed to the 'overall disease severity' score.

Table 2. Results of the pre-treatment xenodiagnosis test on the enrolled dogs using *Phlebotomus perniciosus* females.

Dog number	Weeks before treatment	No. of females recovered	Entomological parameters (%)			
			Feeding rate	Mortality rate	Promastigote infection rate	Potentially infectious rate
1	2	99	75.8	15.2	72.7	63.6
2	2	63	61.9	20.6	78.9	50.0
3	1	89	39.3	23.6	50.0	36.4
4	3	77	84.4	9.1	25.5	19.6
5	3	141	78.0	29.1	70.0	60.0
6	4	108	90.7	23.1	58.3	52.8

Entomological parameters use the number of recovered females as denominator.

Table 3. Entomological efficacy parameters in post-treatment xenodiagnosis tests performed on days 1, 7 and 28 using *Phlebotomus perniciosus* females.

Dog number	No. of females used in cage/ recovered	Anti-feeding efficacy (%)	Insecticidal efficacy (%)	Potential transmissibility rate (%)	Anti-transmissibility effect (%)
		Day 1 evaluation at 24 h		Day 1 evaluation at 96 h	
1	90/82	98.3	100.0	0.0	100.0
2	90/75	80.6	89.2	0.0	100.0
3	90/90	100.0	75.9	0.0	100.0
4	85/83	100.0	100.0	0.0	100.0
5	110/105	84.1	100.0	0.0	100.0
6	90/89	98.8	100.0	0.0	100.0
		Day 7 evaluation at 24 h		Day 7 evaluation at 96 h	
1	90/88	100.0	69.6	0.0	100.0
2	90/84	78.8	18.0	0.0	100.0
3	97/97	100.0	83.6	0.0	100.0
4	90/77	95.4	100.0	0.0	100.0
5	122/122	79.0	100.0	0.0	100.0
6	91/91	98.8	100.0	0.0	100.0
		Day 28 evaluation at 24 h		Day 28 evaluation at 96 h	
1	90/78	100.0	62.5	0.0	100.0
2	90/82	64.5	7.7	4.4	78.9
3	90/60	57.6	44.5	0.0	100.0
4	140/137	32.6	65.9	0.7	93.6
5	91/91	71.8	94.4	0.0	100.0
6	100/96	87.4	88.1	0.0	100.0

Parameter calculations include either the total number of females used in the cage, or the number of recovered females.

Post-treatment changes recorded in entomological parameters were used to calculate the efficacy of Vectra®3D treatment starting from day 1 and to day 28 (Table 3). On day 1 the onset of anti-feeding efficacy ranged from 80.6% (dog 2) to 100% (dogs 3 and 4), being >95% in 4 animals. Insecticidal efficacy was in the range from 75.9% (dog 3) to 100% in 4 animals (dogs 1, 4–6). No blood-fed females were found alive at 24 h for any dog, therefore, the entomological parameters associated with *Leishmania* infections at 96 h were set to zero and the anti-transmissibility efficacy resulted 100% for all dogs.

Day 7 assessments resulted in efficacy rates similar to day 1, showing an anti-feeding effect ranging from 78.8% (dog 2) to 100% (dogs 1 and 3), again being >95% in 4 dogs. Insecticidal efficacy was unexpectedly low in dog 2 (18.0%) but it was 100% in 3 animals (dogs 4–6). Only a few blood-fed females survived at 24 h (dog 2) but none at 96 h for any dog, therefore, the anti-transmissibility efficacy of treatment was again 100% for all animals.

On day 28 the anti-feeding efficacy was 100% in one animal (dog 1), whereas it was found as low as 32.6% in dog 4. Insecticidal efficacy was highest in dog 5 (94.4%) and confirmed, from day 7 result, to be the lowest in dog 2 (7.7%). Only sand flies, which had a blood meal on 2/6 animals (dogs 2 and 4) were found to be potentially infectious, but the anti-transmissibility efficacy for them was as high as 78.9% and 93.6%, respectively. Altogether, the tested spot-on abrogated by >98% the *Leishmania* transmissibility by the examined pool of infected dogs over 1 month.

Discussion

To the best of our knowledge, this is the first laboratory study aiming to assess the effect of a topical insecticide treatment against the transmission of *L. infantum* by infectious sick dogs via phlebotomine vectors. A main difference with clinical trials

of topical formulations against sand flies carried out according to consensual guidelines (Otranto *et al.*, 2021) lies in the composition of the canine population. In a standard laboratory study, groups of treated and control purpose-bred dogs are tested in parallel and are homogeneous in breed, sex, weight and naïve status, as well as in the attractiveness to sand fly bites as determined by preliminary challenges. Such parallel testing allows to reduce confounding effects for feeding and mortality rates calculation that may be associated with different rearing batches, transport and handling of sand flies, and authorizes the calculation of arithmetic means of values from all dogs in each treatment arm and hence provides a statistically robust comparison of effects between arms. Because of the present study design, the sand fly exposure of owned, untreated (healthy or *Leishmania* sick?) dogs in parallel with Vectra®3D-treated dogs at each of the 3 post-treatment individual assessments, was considered unfeasible for both practical and ethical considerations. Hence, due to the large variations of dog's characteristics, which also included type and severity of CanL signs, any entomological parameter was managed and analysed individually in comparisons between pre- and post-treatment conditions of each dog. The only efficacy parameter, which was assumed *a posteriori* to be homogeneous among the dogs, and hence treated as a mean, was the anti-transmissibility effect conferred by treatment in all post-treatment days evaluated: this was 100% in 16/18 determinations over 1 month (Table 3), for an average of 98.5%.

To estimate *Leishmania* transmissibility by our infected dogs, a 'potentially' infectious condition was determined microscopically in specimens up to 4 days after blood feeding. Morphological metacyclogenesis, the only available marker of 'actual' sand fly infectivity, may take place in the insect foregut starting from about 7 days after parasite ingestion along with host's blood (Bates, 2007). Monitoring gut infections over this long period, however, would be hardly achievable because laboratory-reared females of *P. perniciosus* are characterized by high mortality (around 90%) from day 5 post blood meal, immediately after laying eggs (Lawyer *et al.*, 2017). A proxy for the infectivity was therefore a compromise in order to have a sufficient number of live females to dissect after blood digestion. Another technical limitation associated with the fragility of sand flies, was the relatively elevated mortality of specimens released in cages with untreated dogs (Table 2). This was probably caused by handling and transport of the insects to the study site and back on the same day; on the other hand, sand flies from treated dogs were also exposed to the same experimental conditions, and the formula used for the insecticidal efficacy rate calculation reduced the background of mortality in controls.

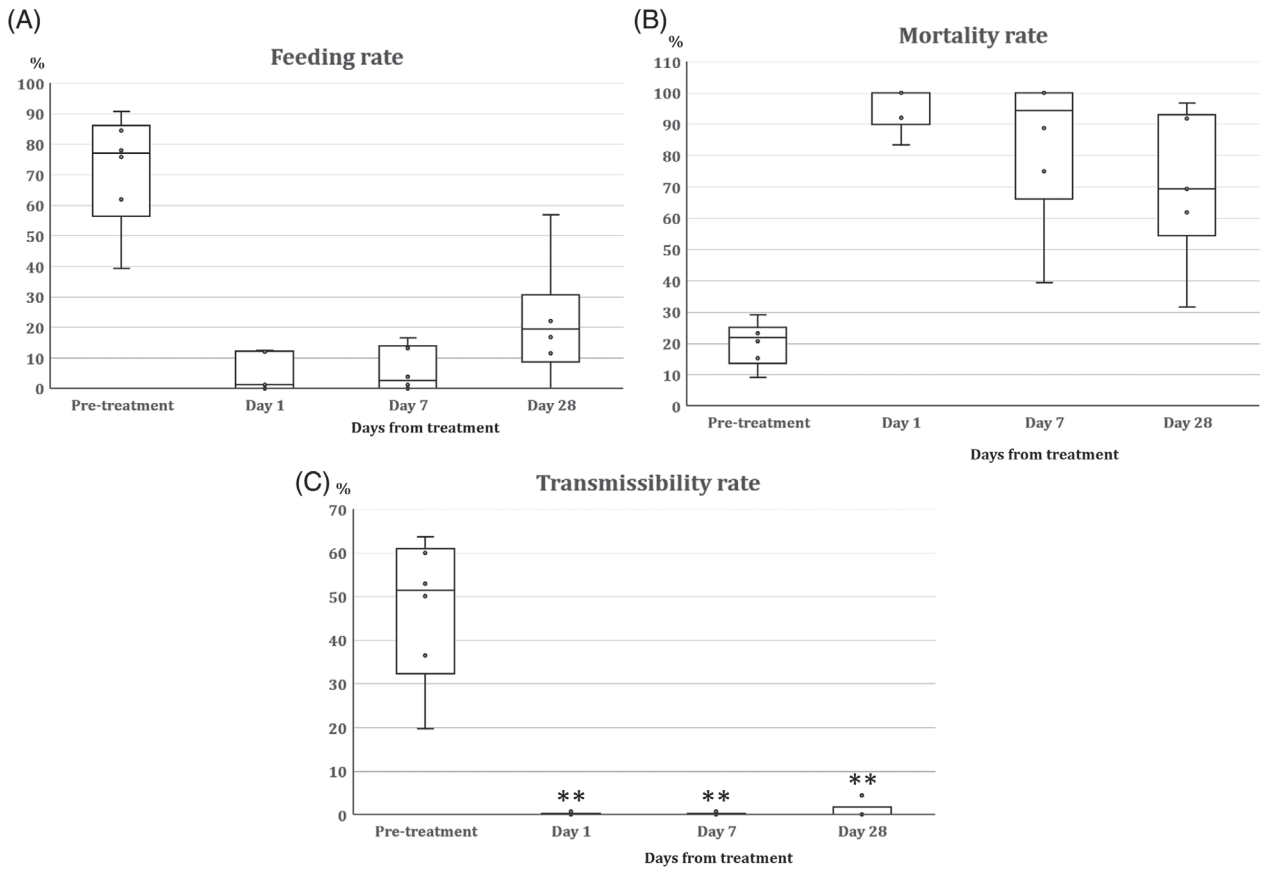

Fig. 1. Box-and-whisker plots showing the feeding (A), mortality (B) and transmissibility (C) rates recorded in 6 dogs before treatment and on days 1, 7 and 28 from treatment. Statistical differences from pre-treatment condition are indicated: $*P < 0.05$, $**P < 0.01$.

At the last assessment on day 28, dogs 2 and 4 were found to harbour potentially transmissible infections, although at much lower rate as compared with pre-treatment condition. Apparently, the cause could not be attributable to the promastigote-infection rates detected in sand flies, which fed on these animals before treatment: actually, dog 2 scored the highest one (78.9%) but dog 4 the lowest one (25.5%) (Table 1). Rather, a hypothesis could be that in the presence of cutaneous lesions including ulcerative ones – in fact, both animals had the second-highest score among the study dogs – the active ingredients of the product did not spread homogeneously over the body surface and the hair coat. Interestingly, on day 28 the lowest insecticidal efficacy (7.7%) was recorded in dog 2, and the lowest anti-feeding efficacy (32.6%) in dog 4.

It has been assumed that the repellent and insecticidal effects on sand flies (Fig. 1A,B) are due to the activity combination of permethrin, an excito-repellent and moderate insecticidal pyrethroid, and dinotefuran, an insecticidal neonicotinoid (Liénard *et al.*, 2013). Dinotefuran was indeed shown to be highly toxic to phlebotomine sand flies (Qualls *et al.*, 2015). Moreover, a synergic activity was demonstrated between the two active ingredients against insects and is expected to provide an improved efficacy (Cartereau *et al.*, 2018). A claimed 3- or 4-week duration of elevated anti-feeding efficacy was reported by a number of commercial spot-on or a spray containing permethrin as active ingredient (reviewed by Fondati *et al.*, 2018). The anti-transmissibility activity recorded by Vectra®3D in our sick dogs was probably due to both effects, which were differently expressed among the dogs on day 28 (Fig. 1C). In already infected dogs, the first expected benefit of the product is to avoid the spread of the pathogen from the reservoir host. The product will also prevent the bites from potentially infectious sand flies, limiting new *Leishmania* challenge in those animals, which are already weakened (Foglia Manzillo *et al.*, 2006), or from other vector-borne diseases expected to worsen the clinical picture.

Among the available canine topical insecticides with proven efficacy against sand flies, the choice for a spot-on formulation vs. long-lasting deltamethrin-impregnated collars for leishmaniasis control (Miró *et al.*, 2017) should be based on a number of criteria. They include, among others, a required broader spectrum of activity against arthropod vectors, avoidance of abrupt loss of protection especially in dogs living in groups (e.g. collar loss or disruption in kennelled dogs), and fast onset of action – reached by Vectra®3D within the first 24 h of application, whereas collars require several days before appropriate levels of pyrethroid spread over the dog's body.

In conclusion, monthly Vectra®3D topical treatment of the canine population in *L. infantum* endemic areas, including healthy and infected animals, represents a reliable tool for the control of the zoonotic disease.

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Conflict of interest

MV and AC are employees of Ceva Santé Animale.

Author contributions

GB, AB, GO, LR, MV, AC and LG designed the study; GB, RB and CK performed mass sand fly rearing; AB, GB, LR, VFM, MG, MPM, DG, NEHBF and LG performed the experiments on dogs; LG drafted the manuscript; all authors reviewed the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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