Five-month serological monitoring to assess the effectiveness of permethrin/fipronil (Frontline Tri-Act®) spot-on in reducing the transmission of Leishmania infantum in dogs

Elias Papadopoulos a, Athanasios Angelou a, Anastasia Diakou a, Lenaïg Halos b, Frederic Beugnet b,*

a Laboratory of Parasitology and Parasitic Diseases, School of Veterinary Medicine, Thessaloniki, Greece
b Merial SAS, 29 Av Tony Garnier, 69007 Lyon, France

A B S T R A C T

Canine vector-borne diseases are highly endemic in European countries of the Mediterranean basin. The use of repellent insecticides aids to reduce the risk of pathogen transmission. The primary objective of this 5-month serological field study conducted in a highly endemic area of canine leishmaniosis in Greece was to comparatively assess the effectiveness of a topical formulation of fipronil/permethrin (Frontline Tri-Act®/Frontect®) and of a deltamethrin collar (Scalibor®) for the prevention of Leishmania infantum transmission. Initially, 72 clinically healthy owned dogs were sampled for inclusion. Ten out of the 72 (13.8%) were seropositive according to the Snap Leish® ELISA test and removed. Ultimately, a total of 56 dogs were followed from May to October 2015 with monthly blood samples and serological tests based on Snap Leish® and Snap 4Dx® ELISA tests. Twenty-five dogs were treated with deltamethrin collar (Scalibor®) (Group 1) and 31 were treated monthly with the topical fipronil/permethrin formulation (Group 2). In group 1, three dogs out of 25 (12%) seroconverted for Leishmania whereas none of the dogs (0/31) seroconverted in group 2. In addition, this trial provided means to serological assess the transmission of other vector-borne pathogens. Regarding Anaplasma spp., one dog in each group was seropositive at the beginning of the study and no dogs became positive during the study. For Ehrlichia canis, 9/25 (36%) dogs in group 1 and 5/31 (16.1%) in group 2 were seropositive at inclusion time. Two out of 16 (11.8%) and 3/26 (11.5%) dogs became positive for E. canis after 3 months in groups 1 and 2, respectively. No Borrelia burgdorferi sensu lato seropositive dog was found during the study. In regard to Dirofilaria immitis, 7/25 (28%) dogs and 12/31 (38.7%) were antigen-positive in groups 1 and 2, respectively, at inclusion. There was no significant difference between groups 1 and 2 in regard to new seroconversions during the trial. This study, conducted in a highly endemic area for vector-borne pathogen transmission, confirmed that the use of deltamethrin and permethrin is a suitable approach to reduce the risk of L. infantum infection by sandflies.

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1. Introduction

Canine vector-borne diseases (CVBDs) are caused by a wide range of pathogens, including viruses, bacteria, protozoa, and helminths, which are transmitted by a variety of arthropods, such as ticks, mosquitoes, phlebotomine sandflies, and fleas (Mencke, 2013). Many CVBDs can be life-threatening for dogs and can represent a major zoonotic concern, like leishmaniosis (Otranto and Dantas-Torres, 2013), European countries of the Mediterranean basin (e.g., from East to West Greece, Italy, France, Spain, Portugal) are highly endemic, compared to the rest of Europe (René-Martellet et al., 2015; Maia and Cardoso, 2015).

However, data suggest that gradually several CVBDs (i.e. leishmaniosis, monocytic ehrlichiosis, granulocytic anaplasmosis, and dirofilariasis) are expanding to Northern countries (Beugnet and Marié, 2009). These epidemiological changes may be related to dogs travelling to endemic areas for holidays and adoptions from animal shelters, as well as climatic changes more favourable to vectors such as Rhipicephalus sanguineus sensu lato ticks, sandflies and invasive mosquitoes such as Aedes albopictus (Beugnet and Marié, 2009, Beugnet and Chalvet-Monfray, 2013).

Greece is an endemic area for many tick-borne pathogens in dogs, especially Ehrlichia canis and Anaplasma platys, which can be related to the prevalence of dog infestation by R. sanguineus s.l. ticks. Greece is also a stable endemic focus of canine leishmaniosis (CanL), with an estimated mean seroprevalence of 22% in dogs (Ntais et al., 2013). Nevertheless, according to several surveys, in particular areas of high
In most cases of CVBDs, vector transmission remains the primary mode of infection. Therefore, the use of effective, persistent and repellent insecticides helps to protect dogs from contact with vectors and, in this way, to reduce the risk of pathogen transmission (Dantas-Torres and Otranto, 2016; Mencke, 2013; Otranto and Wall, 2008). The use of pyrethroids with repellent properties in impregnated collars and spot-on formulations has been demonstrated to be a suitable approach to reduce the risk of CVBD infection in dogs, in both experimental infection challenge studies and in field studies (Beugnet and Franc, 2012; Dantas-Torres and Otranto, 2016; Mencke, 2013). Prevention of Babesia canis transmission by Dermacentor reticulatus ticks has been demonstrated in experimental models based on dog exposure to infected ticks, in dogs treated with spot on formulations [fipronil/amitraz/(S)-methoprene (Certifect®) (Jongejan et al., 2011); imidacloprid/permethrin (Advantix®) (Fourie et al., 2013b); fipronil/permethrin (Frontline Tri-Act®/Frontect® or Effitix®) (Jongejan et al., 2015; Navarro et al., 2015)], collars [amitraz collar (Preventic®) (Last et al., 2007)], or oral products [afoxolaner (NexGard®) (Beugnet et al., 2014); fluralaner (Bravecto®) (Taenzler et al., 2015)]. Similar studies have demonstrated the ability of spot-on formulations [fipronil (Frontline®); fipronil/amitraz/(S)-methoprene (Certifect®); imidacloprid/permethrin (Advantix®); fipronil/permethrin (Frontline Tri-Act®/Frontect®)], or collars [imidacloprid/flumethrin (Seresto®)] to prevent the transmission of E. canis by R. sanguineus s.l. ticks in treated dogs (Davoust et al., 2003; Fourie et al., 2013a; Fourie et al., 2013c; Otranto et al., 2008; Stanneck and Fourie, 2013).

To study the prevention of Leishmania transmission, no experimental model is available and large field studies, conducted with privately owned or kennel dogs, are the only way to evaluate product efficacy. A reduction of transmission has been demonstrated in dogs treated with spot-on formulations [permethrin (Exspot®) (Giffoni et al., 2002); imidacloprid/permethrin (Advantix®) (Otranto et al., 2007, 2010, 2013; Podaliri Vulpiani et al., 2009)] or with collars [deltamethrin (Scalibor®) (Foglia Manzillo et al., 2006, Maroli et al., 2001)].

Sandflies are active at dusk and dawn during the warmer months of the year, particularly in areas with high relative humidity. As a result, long term protection is needed throughout the transmission period (Otranto and Dantas-Torres, 2013; Dantas-Torres and Otranto, 2016). Applying preventive measures is essential not only for local dogs living in endemic areas, but also for the ones coming from non-endemic regions, in order to lower the risk of disease transmission and dissemination.

The World Association for the Advancement of Veterinary Parasitology (WAAVP) included in the recent guidelines the fact that specific claims regarding the prevention or reduction of tick-borne pathogen transmission risks are now possible (Marchiondo et al., 2013). The permethrin/fipronil combination in a spot-on formulation (Frontline Tri-Act® - Frontect®) has demonstrated both repellent activity and sustained speed of kill against fleas and ticks (Dumont et al., 2015b), as well as repellent and insecticidal activity against mosquitoes, sandflies and stable flies (Dumont et al., 2015a; Fankhauser et al., 2015a; Fankhauser et al., 2015b). It was then hypothesized that a monthly use would provide protection against the transmission of some canine vector-borne agents transmission.

The primary objective of this 5 month field study was to assess the effectiveness of a fipronil/permethrin topical formulation (Frontline Tri-Act®) when administered every 28 days in dogs, against the transmission of Leishmania infantum in a Greek hyper-endemic area. The secondary objective was to assess the prevalence of several vector-borne pathogens in dogs including E. canis, Anaplasma spp., Borrelia burgdorferi s.l. and Dirofilaria immitis. For ethical reasons,
the study protocol included a positive control with a deltamethrin collar (Scalibor®, Merck).

2. Materials and methods

2.1. Study location

The study was conducted from April to October 2015 in the area of Xanthi, Northern Greece, around the village of Toxotes, by the Nestos river (41.0870° N, 24.7830° E), known to be hyper-endemic for canine leishmaniosis (Fig. 1). Gallidis et al. (2016) reported a seroprevalence of Leishmania infection of 48% in dogs in this region. Due to high humidity, abundant domestic and wildlife host populations, as well as the presence of many stray or semi-stray dogs living without any anti-parasitic protection, this area is very suitable for vectors like R. sanguineus s.l. ticks and sandflies.

2.2. Study design

This controlled study included 2 treatment groups of dogs (Table 1). Dogs in group 1 were treated with Scalibor® (4% w/w deltamethrin collar, Merck/MSD) in order to protect dogs against ticks, repel sandflies and mosquitoes, and to reduce the risk of vector-borne diseases for 5 months. Dogs in group 2 were treated topically with Frontline Tri-Act®/Frontect® spot-on formulation. All product applications were repeated every 3 months (May 2015).

Enrollment decision: negative SNAP leish test + clinical scoring = 0 (i.e. normal Blood Count, no fever, no cachexia, and no evocative dermatological signs of leishmaniosis) from negative dogs. From seropositive dogs were checked to avoid analysing blood samples from negative dogs.

Exclusion applied to dogs who did not meet the above inclusion criteria, or that had been treated with ectoparasiticides within 30 days prior to Day 0. Dogs that were debilitated, suffering from disease or injury, fractious, or presenting abnormalities at the application site (neck area), and dogs judged unsuitable for inclusion in the study, based on the investigator's opinion, were also excluded.

After inclusion, animals that became sick, were injured, or became unsuitable to remain in the study, in the investigator's opinion, had to be removed.

Initially 72 clinically healthy owned dogs were sampled for inclusion. Ten out of the 72 (13.8%) were seropositive according to the SNAP Leish® test and removed. Finally, 60 healthy owned dogs seronegative for Leishmania were included in the study, 28 in group 1 (treated with deltamethrin collar) and 32 in group 2 (treated with fipronil/permethrin spot-on). Following removal of three and one cases for various reasons, a total of 25 and 31 dogs were followed until the completion of the study (5 months) and retained for analysis, with a mean age of 3 [1.5 to 7.5] and 3.4 [1 to 9] years in Groups 1 and 2, respectively.

Dogs were managed similarly and with due regard for their well-being. General health observations had to be conducted once daily by the owners, who had to contact their respective veterinarians in case of any adverse events or health problem. Dogs could receive medications and vaccinations for routine disease control prior to study initiation, and during the conduct of the study if necessary, with the exception of other ectoparasiticides than those of the study. Other medications that would not interfere with the study objective could be administered during the study.

2.3. Treatment administration

Dogs were weighed and treated according to the European labelling instructions of each product. Dogs living together in one household had to receive the same treatment, and were therefore allocated by pair. Group 1 dogs were treated once with a Scalibor® collar. In case of collar removal or loss, another collar was re-applied the same day on the dog. Group 2 dogs were treated every 28 days (± 2 days) for 5 months with Frontline Tri-Act®/Frontect® spot-on formulation. All product applications were performed by a trained veterinarian. All dogs were observed hourly for 4 h ± 15 min after treatment administration.

2.4. Diagnosis and clinical follow-up of the dogs

Blood samples were collected from the brachial or jugular veins of dogs, and serum separated and stored at −20 °C until tested. Serum samples were stored frozen at the study site and shipped on ice to the laboratory for analysis. Samples were identified by date of collection and animal ID type of sample as below. Treatment groups stayed blinded for the laboratory.

The latest blood samples were checked first and then all samples from seropositive dogs were checked to avoid analysing blood samples from negative dogs.

Table 1

<table>
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<tr>
<th>Study day(s)</th>
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| Enrollment day and first treatment day = Day 0 | - Clinical exam. Weigh dogs for clinical and allocation purposes  
- Blood collection for analyses: Cell Blood Counts (CBC), Serology  
- Serology:  
  - L. infantum (SNAP Leish, IDEXX)  
  - Ehrlichia canis, Anaplasma spp., Borrelia burgdorferi s.l. and Dirofilaria immitis antigen (SNAP 4Dx, IDEXX)  
- Enrollment decision: negative SNAP leish test + clinical scoring = 0 (i.e. normal Blood Count, no fever, no cachexia, no evocative dermatological signs of leishmaniosis)  
- Allocation to treatment group 1 or 2  
- Treatment of group 2 dogs with Frontline Tri-Act®/Frontect® based on their weigh (monthly Treatment)  
- Treatment of group 1 dog with a Scalibor collar.  
Clinical exam: body temperature, dermatological examination, weight evaluation.  
- Blood collection for serology:  
  - L. infantum (SNAP ELISA)  
  - E. canis, Anaplasma spp., and D. immitis antigen (SNAP 4Dx IDEXX)  
- Treatment of group 2 dogs with Frontline Tri-Act®/Frontect® (monthly Treatment)  
- Clinical exam  
- Blood collection for CBC, Serology  
- Serology:  
  - L. infantum (SNAP)  
  - E. canis, Anaplasma spp., B. burgdorferi s.l. and D. immitis antigen (SNAP 4Dx IDEXX). |

a Day 0 is not the same calendar day for all animals. All dogs were included in April and May 2015.
Natural infections with *L. infantum* were primarily studied, but the transmission of other vector-borne pathogens was also checked by serology as secondary criteria. The list of assessed pathogens included *E. canis, Anaplasma* spp., *D. immitis* and *B. burgdorferi* s.l.

The serology testing was performed by using SNAP – IDEXX tests:

- *Leishmania*: SNAP Leish® was performed at enrollment, and then on a monthly basis. This test has 99.2% specificity, which reduces the risk of false positive results, and a 96.3% sensitivity.
- *Ehrlichia/Anaplasma/Dirofilaria/Borrelia* serological status was assessed using a SNAP 4Dx® test performed monthly.

2.5. Data analysis
The primary variable was the proportion of dogs in each group that became seropositive for *L. infantum*. The proportion of animals that became seropositive in each group were compared using Fischer exact test. The level of significance was set at 5% on a two-sided test.

3. Results
No ticks were seen during the study period, neither by dog owners (daily inspection), nor during the monthly veterinary consultation. One collar was lost by a dog in August 2015, and replaced the same (daily inspection), nor during the monthly veterinary consultation. In regard to *Leishmania* infection status (Table 2): In group 1 (deltamethrin treated dogs), three dogs out of 25 (12%) seroconverted. One of them during the first month of the study, one after 2 months and the last one after 3 months. No dog (0/31) seroconverted in group 2 (fipronil/permethrin treated dogs) (p > 0.05).

Concerning tick-borne pathogens and *D. immitis* infection, some clinically healthy dogs were already infected at inclusion time and remained infected for the whole duration (5 months), while others became seropositive during the study period.

Regarding *Anaplasma* spp. (Table 2), one dog in each group was seropositive at the beginning of the study and remained seropositive during the following 5 months, but no new dog was found positive (no significant difference between groups).

For *E. canis* (Table 2), among healthy dogs, 9/25 (36%) in group 1 and 5/31 (16.1%) in group 2 were seropositive at inclusion time, and remained positive. Two out of 16 (11.8%) and 3/26 (11.5%) originally negative dogs became positive after 3 months in groups 1 and 2, respectively (no significant difference between groups, p > 0.05). After 5 months, a total of 19/56 (33.9%) dogs were *E. canis* seropositive.

No *B. burgdorferi* s.l. seropositive dog was found during the study, which is not surprising as *kodes* spp. ticks are not reported in this area.

In regard to *D. immitis*, the detection of female antigens showed 7/25 (28%) and 12/31 (38.7%) antigen positive dogs in groups 1 and 2, respectively, at inclusion. In October 2015, after 5 months, 5/18 (27.7%) new cases and 0/19 (0%) new antigen positive dogs were detected in groups 1 and 2, respectively (p < 0.05).

4. Discussion
The use of repellent molecules such as synthetic pyrethroids on dogs has become the most effective tool for prevention of *L. infantum* infection in these animals (Dantas-Torres and Otranto, 2016). Their mode of action, both irritating and neurotoxic effect causes arthropod disorientation and sudden abandonment of the host, which can be followed by the vector’s death if the contact with the treated animal coat was enough for it to get a lethal dose (Beugnet and Franc, 2012). The effect of synthetic pyrethroids in spot-on formulations or collars may last from 2 to 3 weeks to approximately 8 months depending on the formulations, the actives, and the targeted parasites (Beugnet and Franc, 2012). In the case of deltamethrin spot-on collar, the repellent effect is indicated as 5 months against sandflies and 6 months against mosquitoes. The acaridical effect against *Rhizophalus* ticks is labelled for 6 months. Concerning the topical formulation of fipronil/permethrin (Frontline Tri-Act®/Frontect®), experimental studies have demonstrated a repellent effect (measured as anti-feeding effect after 1 h of exposure) against mosquitoes and sandflies >80% for 4 to 5 weeks (Dumont et al., 2015a). Repellent and acaridical effect against *Rhizophalus* ticks is also demonstrated for 4 weeks (Dumont et al., 2015b).

It usually takes around 24 h for the active(s) to spread throughout the skin after a spot-on or a collar application. In order to achieve the highest level of protection, it is recommended to administer the first treatment before the sandfly, mosquito and/or tick season begins (Dantas-Torres and Otranto, 2016). The key point to maintain the ectoparasiticide efficacy is to apply the spot-on formulation every 4 weeks or to avoid the collar loss. In this study, the spot-on treatments were applied every 4 weeks by a trained veterinarian. One dog lost its deltamethrin collar after 4 months, which was replaced the same day.

The level of protection conferred against *Leishmania* infection should be estimated by taking into account the incidence of infection, which was actually not characterized during the course of this trial. However, this area was selected following recent leishmaniosis surveys including both healthy and sick owned dogs showing a seroprevalence ranging from 42 to 55%, for dogs aged 4 years old in average (Gallidis et al., 2016).

The study described here was based on seronegative dogs from a healthy population, aged 3 years old in average. It could thus be reasonably hypothesized that the transmission incidence was at least 50% during this one season long field trial. Comparing 0% of infection based on seroconversion to the 50% incidence hypothesis, this would result in a protection of 100% in the fipronil/permethrin treated group. For ethical reason, it is not possible to include any untreated dog. In addition to serology, PCR detection of *L. infantum* DNA may have been conducted in dogs (Francino et al., 2006). It will probably be added in further studies that the authors would like to conduct in the same area, but it was not done.
during this study for technical and logistical reasons. It is well established that PCR sensitivity is quite low when based on blood, and it was difficult to propose bone marrow or lymph node samplings to all these dogs. Some publications describe the interest of PCR from lachrymal secretion (de Almeida Ferreira et al., 2008; Pilati et al., 2009). The authors will further assess the possibility to use this technique based on cotton swab samples.

In any case, the repellent properties of pyrethroides decrease the rate of transmission but cannot provide a complete protection under natural dogs’ living conditions that usually include rain, wind, swimming activities.

For *C. canis*, 14/56 (25%) healthy dogs were seropositive at inclusion. This shows the endemic situation in regard to *R. sanguineus* s.l. tick infection and to the corresponding tick-borne diseases. No tick was seen on dogs during any of the monthly veterinary visits, nevertheless, 2/17 (11.8%) and 3/26 (11.5%) dogs became positive after 3 months in groups 1 and 2, respectively. The prevention of *E. canis* transmission remains difficult to obtain due to the rapid inoculation by infected ticks, in as little as 3 h after attachment (Fourie et al., 2013d); whereas the transmission of *Babesia* is slower, i.e. usually after 72 h. The confirmation of the protection against some tick-borne diseases has been studied in few field studies, as it is always more difficult to recruit privately-owned dogs in sufficient numbers based on the estimated incidences of the diseases, as well as to follow these dogs for several months (Otranto et al., 2008; Sainz et al., 2015).

Concerning the two *Anaplasma* positive dogs, it was most probably related to infection with *A. platis* that can be detected with the SNAP 4Dx test due to cross-reactivity with *A. phagocytophilum* (René-Martellet et al., 2015; Sainz et al., 2015). As for *Leishmania* and tick-borne pathogens, this area appeared to be also endemic for *D. immitis* transmission by mosquitoes, especially taking into account the age of dogs (average of 3 years old), with 33.9% (19/56) antigen positive dogs at inclusion time (Polizopoulou et al., 2000).

Assuming that the prepatent period of *Dirofilaria immitis* is close to 5 months, it is not possible to estimate a level of protection towards transmission with a 5-months study, ending in October, as infections occurring in July, August or September would have led to positive dogs in December, January, and February, respectively. Four new positive dogs were diagnosed in August, meaning that they probably had been infected in March, at least one month before the beginning of the study and thus the treatments. One dog was found positive in October 2015, corresponding to a possible May/June infection, that is to say 1 or 2 months after the first treatments. It would be of interest to have a full year follow up of the treated dogs, in order to be able to assess a reduction in the risk of *Dirofilaria* infective L3 larvae transmission by mosquitoes, using repellent products.

This study, conducted in a highly endemic area for vector-borne pathogen transmission, confirmed that the topical use of pyrethroids providing repellent activity is a suitable approach to reduce the risk of *L. infantum* infection by sandflies.

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**Conflict of interest**

This clinical study was funded by Merial S.A.S., 29 avenue Tony Garnier, 69007 Lyon of which Frédéric Beugnet and Lénai Halos are employees.