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Application of 10% imidacloprid/50% permethrin to prevent *Ehrlichia canis* exposure in dogs under natural conditions

Domenico Otranto [a,*], Paola Paradies [b], Gabriella Testini [a], Maria Stefania Latrofa [a], Stefania Weigl [a], Cinzia Cantacessi [a], Norbert Mencke [c], Donato de Caprariis [b], Antonio Parisi [d], Gioia Capelli [e], Dorothee Stanneck [c]

[a] *Department of Veterinary Public Health, Faculty of Veterinary Medicine, University of Bari, Strada Provinciale per Casamassima Km. 3, 70010 Valenzano (Bari), Italy*
[b] *Department of Animal Health and Welfare, Faculty of Veterinary Medicine, University of Bari, Strada Provinciale per Casamassima Km. 3, 70010 Valenzano (Bari), Italy*
[c] *Bayer Health Care AG, Animal Health Division Leverkusen, Germany*
[d] *Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata, Putignano (BA), Italy*
[e] *Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro (PD), Italy*

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Abstract

Canine monocytic ehrlichiosis (CME) caused by *Ehrlichia canis* is the most known canine tick-borne disease (TBD) spread throughout the world. Preventing tick bites is a priority to reduce the risk of TBDs and it was the aim of the present study to evaluate the efficacy of a combination of imidacloprid 10% and permethrin 50% (ImPer) (Advantix®; Bayer AG, Germany) in a spot-on formulation to control CME under field conditions. On January–March 2005, 845 dogs from two kennels in southern Italy (kennels of Bari (KB)- and Ginosa (KG)), with a history of tick infestation were initially tested by serology and PCR assay for *E. canis* infection. Data on *Leishmania infantum* infection were also available from a previous study carried out on the same dog population. One hundred twenty-six dogs (14.9%) presented anti-*E. canis* antibodies with a relative prevalence of 15.6% ($n = 65$ dogs in KB) and 14.2% ($n = 61$ dogs in KG). Five hundred thirty-five animals found negative both for *E. canis* and *L. infantum* infections were enrolled in three groups (Group A—treated with ImPer once a month; Group B—treated every 2 weeks; and Group C—untreated control animals) and monitored for *E. canis* infection by serology and PCR in November 2005 (first follow-up) and in March 2006 (second follow-up). The *E. canis* infection was serologically revealed, at the first and/or second follow-up, in 26 animals from Group C in KB and KG (mean incidence density rate (IDR), 13.24%) while in none of the animals from Group A (KB and KG) and only in one animal from Group B (IDR 1.13%) in KG. The final protection efficacy of ImPer ranged from 95.57% to 100% in Groups B and A. At PCR only 15 dogs from KG were positive for *Rickettsiales* only at the first follow-up and at the sequence analysis two (both in Group C) revealed 100% homology with *E. canis* sequences while 13 with *Anaplasma platys*. Four out of 13 *A. platys* PCR-positive dogs were also seropositive for *E. canis* at one or both follow-ups. ImPer, by virtue of its repellent and acaricidal activity against ticks, has been shown to be efficacious to prevent *E. canis* infection in treated dogs living under natural conditions in endemic areas.

Keywords: *Ehrlichia canis*; *Anaplasma platys*; Tick-borne diseases; *Rhipicephalus sanguineus*; Imidacloprid; Permethrin; Efficacy; Field trial

* Corresponding author. Tel.: +39 080 4679839; fax: +39 080 4679839.
E-mail address: d.otranto@veterinaria.uniba.it (D. Otranto).

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1. Introduction

Tick-borne diseases (TBDs), along with canine leishmaniosis (CanL), are among the most important canine vector-borne diseases (CVBDs) transmitted by arthropods (Sonenshine, 1991, 1993; Alvar et al., 1994; Lane and Crosskey, 1995). In particular, borreliosis, ehrlichiosis, rickettsiosis and viral encephalitis may be life threatening diseases in humans and dogs exposed to tick bites (reviewed by Shaw et al., 2001). TBDs are scattered in southern European countries although, over the last decade, the number of their reports (both autochthonous and imported) have been increasing through central and northern Europe (Trotz-Williams and Trees, 2003).

Canine monocytic ehrlichiosis (CME) caused by *Ehrlichia canis* Rickettsiae is a cosmopolitan TBD transmitted (trans-stadially) by *Rhipicephalus sanguineus*. This tick species, known as ''Kennel tick'' or ''Brown Dog tick'', is the most commonly retrieved in subtropical and tropical regions around the globe. The Brown Dog tick serves as a vector for a wide range of organisms pathogenic to dogs. These are *Rickettsia conorii*, *Anaplasma platys* and *Hepatozoon canis* as reviewed by Shaw et al. (2001). CME is characterized by a wide range of clinical signs including lethargy, weight loss, anorexia, pyrexia, lymphadeno- and splenomegaly while the most commonly retrieved haematological abnormalities are thrombocytopenia and anaemia (reviewed in Harrus et al., 1997). *E. canis* infection is worldwide distributed in tight relationship with the presence of its vector. Endemic areas for CME have been identified in several countries of the Mediterranean basin with the highest prevalence of 87.5% recorded in a population of kennelled dogs from Corsica (Trotz-Williams and Trees, 2003). Italy is an endemic area for CME as the seroprevalence percentage ranges from 15.5% (Buonavoglia et al., 1995) to 22.6% (Capuano et al., 2002) in southern regions. To the best of our knowledge, no information is available about the annual incidence rate for the CME in dog populations living in endemic areas.

To prevent the transmission of TBDs, prophylactic protection against ticks is needed. Among others, a combination of imidacloprid 10% and permethrin 50% (ImPer) has been developed (Advantix®; Bayer AG, Germany) in a spot-on formulation to provide treatment of and prophylaxis against ticks, fleas, mosquitoes and phlebotomine sand flies (Mencke et al., 2003). The efficacy of this combination against ticks has been experimentally demonstrated by using different approaches under laboratory conditions (Epe et al., 2003; Mehlhorn et al., 2003; Young et al., 2003). Recently, the efficacy of ImPer to prevent the infestation by *R. sanguineus* in dogs living in a heavily tick infested area of southern Italy has been demonstrated under field conditions showing a protection against adults and immature stages up to 98.43% at day + 28 post-treatment (Otranto et al., 2005).

Studies on the prevention of TBDs in dogs are rare. In a laboratory study using ImPer it was demonstrated that transmission of *Borrelia burgdorferi* (s.s.) and *Anaplasma phagocytophilum* from naturally infected *Ixodes scapularis* ticks to dogs could be prevented (Spencer et al., 2003; Blagburn et al., 2004, 2005). Elfassy et al. (2001) studied the preventive effects of an amitraz impregnated collar against *I. scapularis*. In a single multi-center study the efficacy of fipronil to prevent *E. canis* infection has been investigated by seroconversion in 248 dogs over one season of tick exposure in Africa (Davoust et al., 2003). No information is available about the efficacy of ImPer to prevent CME under natural conditions while it has been recently demonstrated that it has a high efficacy to prevent CanL (Otranto et al., 2007).

Thus, it was the aim of the present work to evaluate under field conditions the efficacy of the 10% (w/v) imidacloprid/50% (w/v) permethrin topical spot-on solution (ImPer) as a control measure to prevent CME in dogs from an endemic area of southern Italy.

2. Materials and methods

2.1. Study design and procedures

The trial was conducted from February 2005 to April 2006 on dogs living in Apulia region, southern Italy (latitude: 42° and 39° North, longitude 15° and 18° East) following the same design and procedure of a previous study aimed to evaluate the efficacy of ImPer to prevent CanL in endemic areas (Otranto et al., 2007). In dogs from that area, heavy tick infestations by *R. sanguineus* have been previously reported (Otranto et al., 2005). In particular, in this area dog infestations by adult Brown Dog ticks peak from April to October (Manilla, 1998) and a seroprevalence for *E. canis* of 15.5% was previously reported (Buonavoglia et al., 1995).

Briefly, dogs included in the trial were housed in two kennels from the above area, namely Bari (KB) (latitude: 41°5′ North, longitude: 16°5′ East) and Ginosa (KG) (latitude: 40°3′ North, longitude: 16°4′ East). The field study was carried out as a negative-controlled trial to test ImPer spot-on for the prevention

of *E. canis* infection. Three different groups were formed in both kennels as follows: Group A—dogs treated with ImPer at day 0 and every 28 ± 2 days; Group B—dogs treated with ImPer at day 0 and every 14 ± 2 days; Group C—untreated control dogs. Dog exclusion criteria were the same as used in the previous work, namely if under 7 weeks of age, with a history of apparent reactions to a component of the test or control product, presented with skin lesions at the application site or with pre-existing medical conditions and if treated in the previous 6 weeks with any insecticide/repellent applied to the animal or the environment (Otranto et al., 2007). The positivity to *Leishmania infantum* was also included among the exclusion criteria, since leishmaniosis may cause both cellular and antibody immune-suppression impairing the specific anti-*E. canis* antibody production (Roura, 2007). Suitable dogs were individually identified by using microchips and individual data (i.e. sex, age, weight and hair coat length) were recorded. Due to kennel organization and to avoid treatment transfer from one animal to another, dogs living in the same cage had to be placed in the same group and they were not allowed to walk outside.

The study has been carried out according to the principles of Good Clinical Practice (VICH GL9 (GCP), 2000). From early April (day 0) to November 2005 (at the end of fall season) treatment administrations were performed as scheduled for Groups A and B in both kennels. From January to March 2005, before starting the study, 845 mixed breed animals of different sex and age were tested serologically (to detect anti-*E. canis* antibodies) and by PCR on whole blood (to detect *Rickettsiales* pathogens) (see laboratory procedures section). A screening to detect *L. infantum* infection has also been performed (Otranto et al., 2007). Only animals serological and PCR negative for *E. canis* and *L. infantum* were enrolled in the trial (Table 1). Animals included in the study have been further tested to detect *E. canis* infection serologically and by PCR on November 2005 at the beginning of winter (first follow-up) and on March 2006 (final follow-up). The compound was administered topically as spot-on. At each treatment time, clinical observations were registered on the individual forms (available from the authors). The use of any other product with efficacy against arthropods on the study animals or in their environment was not permitted.

2.2. Blood collection and diagnostic procedures

Blood samples were collected from brachial or jugular veins, allowed to clot at room temperature and centrifuged (1700 × *g* for 10 min). Sera were separated and stored at −20 °C along with whole blood until testing.

2.2.1. IFAT procedure

IFAT was performed by using a commercial kit (Canine Ehrlichiosis FA Substrate Slide, VMRD, Pullmann, WA, USA, Lot P060228-002032908) using slides containing fixed *E. canis* in DH82 cells. The parasitized cells were exposed to sera diluted (1:50) in phosphate buffer solution (PBS, pH 7.2) in a moist chamber and, after washing, to fluoresceinated rabbit anti-dog IgG (rabbit anti-dog IgG; Sigma–Aldrich Chemie, Germany, Lot 125K4752) diluted 1:60; both incubations were at 37 °C for 30 min. Slides were observed under a fluorescence microscope at 100× to 250× and samples were scored positive when they produced cytoplasmic inclusion bodies fluorescence. The positive cut-off adopted was at a dilution of 1:50 and all positive sera were titred. For each test *E. canis* positive (Lot. P041109-001) and negative (Lot. P060623-001) canine sera were included as controls.

2.2.2. Molecular procedures

The genomic DNA was extracted from 300 μl of whole blood using a commercial kit (Genomic DNA Purification Kit Gentra Systems, MN, USA, Lot. GS19788) slightly modifying the manufacture's instructions as follows. Briefly, the cell lysis was obtained by adding 900 μl RBC lysis solution and by centrifugation for 1 min (13,000–16,000 × *g*). The pellet has been resuspended with 300 μl cell lysis solution and RNase treatment carried out by adding 1.5 μl RNase solution (37 °C for 15 min). After 7 min on ice, 100 μl protein precipitation solution were added and samples centrifuged (7 min at 13,000–16,000 × *g*). DNA was precipitated in 300 μl 100% isopropanol and centrifuged (3 min at 13,000–16,000 × *g*) before washing it in ethanol (3 min at 13,000–16,000 × *g*). Finally, the DNA was hydrated adding 100 μl DNA hydration solution at a concentration of 100 μg/ml and incubated (overnight at room temperature) before samples were processed. The amplification of *Ehrlichia* spp. DNA was performed using a genus-specific set of primers for the 16S rRNA gene (Parola et al., 2000). The genomic DNA (4 μl) was added to the PCR reaction mix (46 μl) containing 1× HotMaster Buffer (Eppendorf, Germany), 0.2 mM of each dNTPs, 50 pmol of each primer, 1 U of HotMaster Taq DNA polymerase (Eppendorf). The amplification program consisted of an initial denaturation step at 94 °C for 2 min, 30 cycles of 94 °C, 56 °C and 72 °C, each of 30 s with a final extension step at 72 °C for

Table 1
Composition and homogenicity of animals in the three groups enrolled in the trial divided according to variables related to the population sample

Kennel KB, $n = 262$	Group A, $n = 89$	Group B, $n = 83$	Group C, $n = 90$	Group homogeneity
Age (months)				
<24	41 (43.2%)	26 (27.4%)	28 (29.5%)	$\chi^2 = 6.235$, $P = 0.182$
24–48	23 (31.1%)	23 (31.1%)	28 (37.8%)	
48	25 (26.9%)	34 (36.65%)	34 (36.6%)	
Sex				
Females	52 (32.7%)	49 (30.8%)	58 (36.5%)	$\chi^2 = 0.818$, $P = 0.664$
Males	37 (35.9%)	34 (33.0%)	32 (31.1%)	
Weight (kg)				
<15	15 (31.3%)	11 (22.19%)	22 (45.8%)	$\chi^2 = 5.597$, $P = 0.231$
15–25	40(38.5%)	35 (33.7%)	29 (27.9%)	
>25	34 (30.9%)	37 (33.6%)	39 (35.5%)	
Hair coat length				
Short	34 (31.2%)	38 (34.9%)	37 (33.9%)	$\chi^2 = 5.904$, $P = 0.206$
Medium	34 (34.3%)	35 (35.4%)	30 (30.3%)	
Long	21 (38.9%)	10 (18.5%)	23 (42.6%)	
Kennel KG, $n = 273$	Group A, $n = 87$	Group B, $n = 89$	Group C, $n = 97$	Group homogeneity
Age (months)				
<24	34 (31.5%)	32 (29.6%)	42 (38.9%)	$\chi^2 = 2.475$, $P = 0.649$
24–48	16 (230.2%)	16 (30.2%)	21 (39.6%)	
48	37 (30.2%)	41 (30.2%)	34 (39.6%)	
Sex				
Females	51 (36.2%)	46 (32.6%)	44 (31.2%)	$\chi^2 = 3.229$, $P = 0.199$
Males	36 (27.3%)	43 (32.6%)	53(40.2%)	
Weight (kg)				
<15	28 (32.6%)	27 (31.4%)	31 (36.0%)	$\chi^2 = 1.125$, $P = 0.890$
15–25	26 (28.3%)	33 (35.9%)	33(35.9%)	
>25	33 (34.7%)	29 (30.5%)	33 (34.7%)	
Hair coat length				
Short	29 (35.4%)	30 (36.6%)	23 (28.0%)	$\chi^2 = 3.646$, $P = 0.456$
Medium	27 (27.6%)	31 (31.6%)	40 (40.8%)	
Long	31 (33.7%)	28 (29.3%)	34 (36.6%)	

Group A treated with imidacloprid 10%/permethrin 50% once a month, Group B treated with imidacloprid 10%/permethrin 50% twice a month, Group C untreated controls.

5 min. A positive control containing genomic *E. canis* DNA and a negative control without DNA were included in all the assays. Amplification products (345 bp) were visualised on 2% (w/v) agarose gel (Ambion), stained with ethidium bromide (10 mg/ml), using a 100-bp DNA ladder as a marker and then photographed using the Gel Doc 2000-Gel Documentation. The PCR products were purified with the Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA). Then, the DNA was used as template for direct sequencing. The sequences were assembled using Bionumerics software package Version 4.1 and compared to cognate sequences in the genetic databases using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and FASTA (http://www.ebi.ac.uk/fasta33) web-based programs.

2.3. Statistical analysis

2.3.1. Sample size and group allocation

The minimum sample size ($n = 77$) was calculated (Thrusfield, 2000; software WinEpiscope 2.0) for a cohort study for each group (A, B and C) in each kennel following these assumptions: maximum expected non-exposed diseased animals (i.e. treated animals) = 5%; minimal relative risk (RR) to be detected significant RR = 4.5; power = 90%; level of confidence = 95%. Since a certain number of dogs must be expected to be lost to follow-up, more than 80 instead of 77 dogs were enrolled in each group (Table 1).

The group allocation and randomisation has been conducted independently in each kennel following the

same procedure. Due to kennel organization, dogs living in the same cage had to be placed in the same group. Cages were numbered, and three series of casual numbers (1/3 of the total) were generated by computer. At each random selection the homogeneity of the three groups in relation to dog epidemiological data (i.e. sex, age, weight and hair coat length) was evaluated using the chi-square test and number generation was stopped when no more significant differences were detected (Table 1).

2.3.2. Incidence calculation and efficacy assessment

To overcome the problem of dogs lost to follow-up during the study, the incidence of infection was studied by means of incidence density rate (IDR) (Moreira et al., 2004). IDRs were calculated for each follow-up as the number of positive dogs, either serologically or parasitologically (i.e. new infections) divided by the number of dog-years of follow-up (i.e. the number of years between the previous and the successive assessment for each dog at risk for *Ehrlichia* infection). Dogs tested once (e.g. lost, dead) did not contribute anytime to the incidence calculation. Differences between incidence rates in Groups A, B and C were calculated using Yates corrected chi-square test. Statistical calculations were performed by using an Excel sheet and with the statistical packages SPSS and WinEpiscope 2.0.

The ImPer repellent efficacy was expressed in terms of percentage of protection against *Ehrlichia* infection calculated at the end of the tick season both for dogs treated once and for dogs treated twice a month using the following formula: % of protection = (% of animals positive in control group – % of animals positive in treated group/% of animals positive in control group) × 100.

3. Results

Out of 845 dogs preliminarily tested for serology on January–March 2005 (i.e. 416 from KB and 429 from KG) 126 (14.9%) presented anti-*E. canis* antibodies with a relative prevalence of 15.6% ($n = 65$) in KB and 14.2% ($n = 61$) in KG. One hundred eighty-four animals (88 in KB and 96 in KG) have been excluded because they were positive only to *L. infantum* while *E. canis–L. infantum* co-infections were detected in 25 (2.9%) dogs. Finally, 535 animals were enrolled in the three groups and they were homogenous ($p < 0.05$) from the epidemiological point of view for individual dog characteristics (i.e. sex, age, weight and hair coat length) (Table 1).

The seroprevalence of positive dogs not included in the study, but which were still kept in the same cages were 34% (A), 56% (B) and 37% (C) in KB and were 31.7% (A), 32% (B) and 43% (C) in KG. The IDRs for each group and each kennel in the two successive follow-ups are reported in Table 2.

In KB the *E. canis* infection, at the middle or the final follow-up was serologically revealed in 13 animals from Group C (IDR = 13.08%) while none of the animals from Groups A and B was found positive thus resulting in a ImPer final protection efficacy of 100% in Groups A and B. Similarly, in KG the *E. canis* infection, at the middle or the final follow-up was serologically detected in one animal from Group B (IDR = 1.13%) and 13 animals from Group C (IDR = 13.40%) while none of the animals from Group A was found positive. The final protection efficacy of ImPer was of 100% in Group A and of 95.57% in Group B. Out of the 27 animals *E. canis*-seropositive at the first and/or at the

Table 2
Incidence density rates of ehrlichiosis in dogs from treated Groups A and B and control (C) group in kennels KB and KG

Dogs enrolled	Sampling time	No. of dogs in the cohort			No. of new cases^			Dog-years of follow-up			Incidence/100 dog-years (95% CI)		
		A	B	C	A	B	C	A	B	C	A	B	C
Kennel KB													
Baseline	March 2005	89	83	90	–	–	–	–	–	–	–	–	–
Follow-up 1	November 2005	82	81	85	0	0	10	61.50	62.08	76.50	0.00	0.00	13.07
Follow-up 2	March 2006	78	79	83	0	0	3	26.42	20.00	22.92	0.00	0.00	13.09
Total					0	0	13	87.92	82.08	99.42	0.00*	0.00*	13.08*(12.22–15.56)
Kennel KG													
Baseline	March 2005	87	89	97	–	–	–		–	–	–	–	–
Follow-up 1	November 2005	84	89	93	0	1	11	63.00	66.75	76.50	0.00	1.50	13.07
Follow-up 2	March 2006	84	86	80	0	0	2	28.33	21.83	20.50	0.00	0.00	14.63
Total					0	1	13	91.33	88.58	97.00	0.00**	1.13*	13.40*(11.22-17.35)

^ new cases = dogs positive serologically.
* Difference between treated and control group for $p < 0.01$.

second follow-up in both kennels, 22 animals were seropositive, at the first follow-up showing titres ≥1:100 till 1:1600 with the exception of only one animal (titre 1:50, see Table 3). At the second follow-up 26 dogs were positive showing titres ranging from 1:50 to 1:800 while one animal sample was not tested since not available. In particular five animals tested negative at the first follow-up seroconverted (Table 3).

In KG, two dogs (both in Group C) resulted PCR-positive for *E. canis* revealing 100% homology with the *E. canis* sequence available in current public databases (accession no. EF139458), while 13 dogs (i.e. one dog from Group A (IDR = 1.09%), three from Group B (IDR = 3.39%) and nine from Group C (IDR = 9.28%)) were positive for other *Rickettsiales*, namely *A. platys* (accession no. EF139459). Dogs positive at PCR for *E. canis* were serologically positive for this pathogen at both follow-ups. Four out of 13 *A. platys* PCR-positive dogs were also seropositive for *E. canis* at one or both follow-ups.

Table 3
Serological results of each *Ehrlichia canis* positive dog from the two kennels and antibody titres at the first (Follow-up 1, November 2005) and the second (Follow-up 2, March 2006) follow-ups. The animals positive at the PCR are also reported as footnotes. Serum from one dog was not available (N/A)

		Follow-up 1	Follow-up 2
KB			
Group C	Dog 1	Negative	1:50
	Dog 2	1:400	1:400
	Dog 3	1:100	1:200
	Dog 4	Negative	1:100
	Dog 5	Negative	1:100
	Dog 6	1:100	1:100
	Dog 7	1:100	1:100
	Dog 8	1:100	1:50
	Dog 9	1:100	1:100
	Dog 10	1:100	1:100
	Dog 11	1:100	1:100
	Dog 12	1:100	1:100
	Dog 13	1:200	N/A
KG			
Group B	Dog 1	1:1600	1:800
Group C	Dog 1[a]	1:100	1:200
	Dog 2	1:100	1:100
	Dog 3	1:400	1:200
	Dog 4[b]	1:200	1:400
	Dog 5	Negative	1:50
	Dog 6[b]	1:100	1:100
	Dog 7[b]	1:50	1:50
	Dog 8[b]	Negative	1:50
	Dog 9[a]	1:400	1:100
	Dog 10	1:100	1:100
	Dog 11	1:200	1:200
	Dog 12	1:100	1:100
	Dog 13	1:200	1:200

[a] *E. canis* PCR-positive dog.
[b] *A. platys* PCR-positive dog.

The final protection efficacy of ImPer against *A. platys* inferred by the molecular detection of the pathogen on the whole blood was of 88.2% in Group A ($p < 0.05$), 63.5% in Group B (not significant). A cumulative efficacy in animals from Groups A and B was of 82.5% which was a statistically significant difference compared to Group C ($p < 0.05$).

Almost all the 27 dogs seropositive for *E. canis* were housed in the same and/or in close cages in which positive animals were found at the beginning of the trial. In particular, out of the 14 dogs seropositive at the first and/or at the second follow-up in Group C in KG, eight were housed in three cages with a maximum number of four seropositive animals in the same cage. The above four animals were also positive at PCR for *A. platys* ($n = 3$) and *E. canis* ($n = 1$).

4. Discussion

The results show that the combination of 10% imidacloprid/50% permethrin (Advantix®; Bayer AG, Germany) in a spot-on formulation is highly efficacious to prevent infection by *E. canis* under natural conditions in endemic areas. In particular, by virtue of its repellent activity against ticks ImPer protects dogs from *R. sanguineus* bites under natural conditions of infestation (Otranto et al., 2005) and, consequently, from the transmission of *E. canis* as well as other TBDs. In fact, the IDR of *E. canis* infection registered in treated Groups (A and B) is significantly lower than that of Group C (in both KB and KG, $p < 0.01$) thus resulting in a high percentage of protection against CME infection (from 95.57% to 100%) in dogs treated once or twice a month. The two treatment regimes did not show any difference in the efficacy to prevent CME infection as already demonstrated for CanL (Otranto et al., 2007). This finding confirms laboratory records about the repellent and acaricidal efficacy of ImPer achieved when administrated every 28 days (Young et al., 2003).

The initial seroprevalence of CME registered in this study (14.9%) is in general agreement with previous data from the same region (15.5%, Buonavoglia et al., 1995) and it confirms that *E. canis* infection is endemic in southern Italy. Meanwhile, a cumulative incidence rate of 13.24% recorded in Group C (from both kennels) over only one season of tick activity indicates that the spreading of CME is tightly linked to the presence of *R.*

sanguineus vector as well as that of animals acting as carriers. Anti-*E. canis* IgG antibodies in endemic areas are indicative of exposure to the pathogen and persist in sub-clinical phase or in carrier animals (reviewed in Waner et al., 2001). As all the animals initially screened were asymptomatic, it is reasonable that seropositive dogs at the beginning of the trial (14.9% in March 2005) were carriers of the pathogen. This could also explain the occurrence of new cases of infection in dogs housed in the same or close cages to those of animals positive for CME at the first screening.

The finding by molecular detection only at the first follow-up of two *E. canis* positive animals (out of the 21 seropositive dogs) confirms that *E. canis* bacteriemia is transitory and that immunocompetent dogs may eliminate the parasite (Hibler and Greene, 1986). Consequently, PCR on blood may fail in the detection of *E. canis* as demonstrated in a study in which different diagnostic techniques (i.e. PCR, western blotting, IFAT and culture of parasite) have been compared (Iqbal and Rikihisa, 1994) and as it has been discussed by Seaman et al. (2004). This assessment is further supported by the finding of no dogs positive for *E. canis* at the PCR detection among the 25 seropositive animals at the second follow-up, thus more likely indicating that those dogs recovered from the infection.

Interestingly, the finding of 13 dogs from KG positive with PCR assay for *A. platys* suggests that an outbreak of this infection occurred. No cross-reactivity between anti-*A. platys* antibodies and *E. canis* antigen has been reported (French and Harvey, 1983; reviewed in Waner et al., 2001). The relative prevalence of *A. platys* in dogs from Group C (9.67%) is higher than that recently reported in Sicily (i.e. 4%, de la Fuente et al., 2006). *A. platys* infection in dogs causes thrombocytopenia inducing mild clinical manifestations in countries of the Mediterranean basin (Sainz et al., 1999) where it has been found to co-infect kennel dogs with *E. canis* (Sparagano et al., 2003). The occurrence of co-infections by *E. canis* and *A. platys* may result from the fact that both rickettsiales are transmitted by *R. sanguineus* (Groves et al., 1975; Lewis et al., 1977; Sparagano et al., 2003). In the present study, out of the nine dogs from Group C found to be *A. platys* infected, four (44.4%) were co-infected with *E. canis*. The detection of *A. platys* at the first follow-up only indicates a likely exposure of dogs to the pathogen. The transient positivity of *A. platys* in blood has been previously demonstrated in animals experimentally infected which were negative at the real time TaqMan PCR at 17 and 28 days post-infection (Eddlestone et al., 2007). In our experience the employment of PCR based assay in studies aiming to reveal TBDs exposure on whole blood may not be indicative due to the transient presence of the pathogen which depends by several factors (e.g. aetiological agent, individual host characteristics and immune response, tick infestation and load of pathogen transmitted).

Previously no field studies have been conducted to evaluate the efficacy of ImPer against ticks to prevent CME under natural conditions. A single multi-center study tested the efficacy of fipronil to prevent CME in eight sites from two endemic areas in Africa on a total of 248 animals (55 treated and 193 untreated) (Davoust et al., 2003). In that study the final seroprevalence in treated dogs ranged from 2.7% to 5.5% while the mean value in all untreated control animals was 57% (Davoust et al., 2003). In our study, the mean IDR was 13.24% in untreated groups and ranged from 0% to 1.13% in treated animals. The two trials above differ not only for the number of animals tested in two kennels (i.e. 348 treated and 187 untreated in our study) but also because we selected animals (before the enrolment in the trial) for being negative both to *E. canis* infection (for clinical presentation, IFAT and PCR on whole blood) and *L. infantum* (by clinical presentation, serology, PCR on skin samples and limphonodal cytology; Otranto et al., 2007). Furthermore, the study of Davoust et al. (2003) might have been affected by the enrolment of few animals from eight sites (more likely characterized by different habitats influencing the distribution and density of *R. sanguineus* ticks) and by the absence of information on the occurrence of CanL co-infections causing possibly a down regulation of the dogs' immune system, and on CME seroprevalence at the beginning of the trial.

5. Conclusions

The evaluation of any acaricidal and/or insecticidal compound in the field adds value to the results achieved under laboratory conditions since it represents a ''natural situation''. However, there are many difficulties to perform such studies working under field conditions including monitoring a homogenous and significant sample of dogs living in the same environment thus under the same parasitic load, presence of a high-parasitic pressure/load to evaluate compound efficacy and selecting animals that have not been treated with other acaricides. Furthermore, in areas which are endemic for CVBDs as southern Italy is, the occurrence of co-infections deserves to be carefully considered. At the beginning of this trial it was recorded that about 3% of animals were seropositive for (and thus

co-infected by) *E. canis* and *L. infantum*. The decision to not enrol 184 animals positive for CanL in a negative-controlled trial to test ImPer for the prevention of ehrlichiosis has been based on the fact that leishmaniosis down regulates immune system (both cellular and antibody immunological responses) of infected animals thus possibly resulting in reactivations of sub-clinical or carrier status of CME or in an impairment in the specific anti-*E. canis* antibodies production. The above two conditions could result in false seropositive or false seronegative at the follow-ups.

The results of this trial clearly show that a regular treatment (i.e. both twice or once in a month) with ImPer is efficacious to prevent *E. canis* infections in dogs, by reducing tick infestations in treated animals. On the basis of our finding a regular use of ImPer on dogs is an effective prophylactic method to reduce the risk of CVBDs (including CanL, Otranto et al., 2007) in endemic areas where both animals and humans are at risk of infection.

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