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Efficacy of a combination of 10% imidacloprid/50% permethrin for the prevention of leishmaniasis in kennelled dogs in an endemic area

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Abstract

The efficacy of imidacloprid 10% and permethrin 50% (Advantix®; Bayer AG, Germany) in a spot-on formulation was evaluated in the field as a control measure to prevent canine leishmaniasis (CanL) in dogs in an endemic area of southern Italy. In February 2005, out of 845 dogs initially tested for CanL, 631 dogs which tested negative (315 from a kennel in Bari (KB) and 316 from a kennel in Ginosa (KG)) in a serological and a parasitological examination were allocated to one of three groups: Group A—treated with imidacloprid 10% and permethrin 50% once a month; Group B—treated every 2 weeks; and Group C—untreated control animals. All the dogs were examined serologically and parasitologically for CanL prior to the start of the study, in November 2005 (end of the sandfly season) and in March 2006 (end of the study). An initial CanL seroprevalence of 24.7% (209 dogs) was detected in KB and KG. In KB *Leishmania* infection, inferred by positivity in at least one of the three tests performed at the interim or final follow-up, was found in one animal from Group A and in nine from Group C. No positive animals were detected in Group B, thus giving a final protection efficacy of 88.9% in Group A and 100% in Group B. In KG *Leishmania* infection was identified in one animal from Groups A and B, respectively, and 11 from Group C (protection efficacy of 90.36% in Group A and 90.73% in Group B). The incidence density rates (IDRs) of infection in both Groups A and B at each kennel were statistically significantly lower than that registered in Group C (KB $p < 0.05$ and KG $p < 0.01$). The results clearly show that a combination of imidacloprid 10% and permethrin 50%, by virtue of its repellent activity against sandflies, is effective under both application regimes in preventing CanL in the field in endemic areas.
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Keywords: Canine leishmaniasis; Human leishmaniasis; *Leishmania infantum*; Control methods; Prevention; Sandfly; Dogs; Imidacloprid; Permethrin; Efficacy; *Phlebotomus* spp.

1. Introduction

Leishmaniasis, affecting dogs and humans, is a group of vector-borne diseases transmitted by more than

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40 species of phlebotomine sandflies in the Old World and 30 species of *Lutzomyia* in the Americas (Alexander and Maroli, 2003). Canine leishmaniasis (CanL) due to *Leishmania infantum* is among the most important protozoal diseases of dogs worldwide. The relevance of CanL has increased over the last decade also in terms of its zoonotic potential, which is enhanced by emerging immunosuppressive conditions (Fernandez-Guerrero et al., 2004). CanL is characterized by a wide range of clinical signs from severe and often fatal to asymptomatic forms in more than 50% of animals, mainly in endemic areas (Brandonisio et al., 1992; Dantas-Torres et al., 2006). Transmission of *Leishmania* spp. occurs when phlebotomine sandfly vectors feed on both symptomatic and asymptomatic infected dogs (Molina et al., 1994), thus making both these groups of animals significant for the transmission of human and canine leishmaniasis. *L. infantum* is widely distributed in many Mediterranean countries (reviewed in Gradoni, 2001). In particular, stable endemic foci have been reported in dogs from central and southern Italy, including the islands of Sicily and Sardinia (Brandonisio et al., 1992; Bongiorno et al., 2003; Paradies et al., 2006). In Italy, the prevalence of the disease ranges from 1.7 to 48.4% of dogs (reviewed in Gradoni, 2001) and new foci have been also reported in north-eastern regions (Capelli et al., 2004). In a recent survey, an annual incidence rate of 9.52% was calculated in an endemic area of southern Italy and, more specifically, a figure as high as 13.1% was reported in kennel dogs (Paradies et al., 2006).

Nowadays, preventing sandfly bites is a priority to protect dogs from leishmaniasis and to reduce the risk of human infections too. Among the modern insecticides, organochlorides (DDT and BHC) have been employed in the environment against phlebotominae and have been shown to be effective in reducing sandfly populations in Brazil (Nery-Guimaraes and Bustamante, 1954) and India (reviewed in Lane, 1991). Nevertheless, disadvantages including high toxicity and harmful side effects for both animals and humans, resistance and their potential for environmental pollution have progressively limited their usage (reviewed in Rogan and Chen, 2005). Again, the main constraint in employing insecticides in the environment for sandfly control is the fact that their natural resting or breeding sites are difficult to find and often not accessible to treatment (Alexander and Maroli, 2003). Consequently, pharmacological research has been steered towards the development of chemical compounds for use on dogs as an effective measure in controlling CanL in endemic areas. In particular, the impact of mass use of deltamethrin-impregnated dog collars on the incidence of CanL has been evaluated (Maroli et al., 2001; Mazloumi Gavgani et al., 2002; Reithinger et al., 2004; Foglia Manzillo et al., 2006).

Recently, a combination of imidacloprid 10% and permethrin 50% has been developed (Advantix®; Bayer AG, Germany) in a spot-on dermal or topical formulation in order to provide treatment for, and prophylaxis against, ticks, fleas, mosquitoes and phlebotomine sandflies (Mencke et al., 2003). The repellent and insecticidal activity of imidacloprid 10% and permethrin 50% against bites from *Phlebotomus papatasi*, *Phlebotomus perniciosus* and *Lutzomyia longipalpis* has been demonstrated experimentally, and it has therefore been speculated that it could be effective in protecting dogs against CanL (Mencke et al., 2005). Nevertheless, no data are available under natural conditions on the repellent activity of imidacloprid 10% and permethrin 50% against sandflies, nor on its efficacy in preventing CanL.

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

2. Materials and methods

2.1. Study area

The trial was conducted from February 2005 to April 2006 on dogs living in the Apulian region, southern Italy (latitude 42° and 39° North, longitude 15° and 18° East). The dogs were housed in two kennels where endemic CanL had been reported over the previous 2 years (Paradies et al., 2006). Specifically, the kennel in Bari (KB) (latitude 41° 5′ North, longitude 16° 5′ East) had about 1100 animals and an estimated CanL prevalence of 20%, while the kennel in Ginosa (KG) (latitude 40° 3′ North, longitude 16° 4′ East) housed about 900 animals with an estimated CanL prevalence of about 17% (D. Otranto, unpublished results).

2.1.1. Study design and procedures

A negative-controlled, partially blinded (laboratory work) field study was carried out to test imidacloprid 10% and permethrin 50% spot-on for the prevention of CanL in endemic areas in two different application regimes (i.e. once or twice a month). The study was conducted according to the principles of Good Clinical Practice (VICH GL9 (GCP), 2000 http://www.emea.eu.int/pdfs/vet/vich/059598en.pdf) in the

guideline for the testing and evaluation of the efficacy of antiparasitic substances for the treatment and prevention of tick and flea infestation in dogs and cats (EMEA/CVMP/005/00, 2000 http://www.emea.eu.int/pdfs/vet/ewp/000500en.pdfl) and the guideline on Statistical Principles for Veterinary Clinical Trials (CVMP/816/00, 2000 www.emea.eu.int/pdfs/vet/ewp/081600en.pdf). Three different groups were formed in both kennels as follows: Group A—dogs treated with imidacloprid 10% and permethrin 50% on day 0 and every 28 $\pm$ 2 days; Group B—dogs treated with imidacloprid 10% and permethrin 50% on day 0 and every 14 $\pm$ 2 days; Group C—untreated control dogs.

Dogs were preventively excluded if they were under 7 weeks of age, had a history of apparent reactions to a component of the test product, presented with skin lesions at the application site or had pre-existing medical conditions and had been treated in the previous 6 weeks with any insecticide/repellent applied to the animal or the environment.

From January to February 2005, before the study started and in the absence of sandflies in the study area (Maroli et al., 1994a), 845 animals of both sexes and different ages and breeds were serologically tested to detect anti-*Leishmania* antibodies (see laboratory procedures section). The specificity of the rK39 dipstick was 100% while the sensitivity was 97.06% (Otranto et al., 2005b).

Potentially suitable dogs were individually identified using microchips and photography. Data (sex, estimated age, weight and coat length) were recorded on a separate individual form for each dog.

In March 2005, seronegative dogs were further tested by lymph-node smear examination and PCR on dermal tissue to diagnose CanL infection (see laboratory procedures section). Only animals which were both serologically and parasitologically negative were enrolled in the trial (Table 1). Overall, animals included in the study were examined serologically and parasitologically for CanL three times, i.e. immediately prior to the start of the study (February–March 2005), in November 2005 at the end of the sandfly season (interim test), and in March 2006 (final test). Specifically, serology, PCR on dermal tissue and lymph-node smear examination were performed at the start and end of the study, while in November lymph-node smear examination was not performed. From early April (day 0) to November 2005 (at the end of the sandfly season) treatment was administered as scheduled for Groups A and B in both kennels, KB and KG. The compound was administered topically as spot-on to the skin of the dogs by parting the hair with two fingers at the top of the back

Table 1
Incidence density rates of leishmaniasis in dogs from treated (A and B) and control (C) groups in kennels KB and KG

Dogs enrolled	Sampling time	No. of dogs in the cohort			No. of new cases[a]			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 05	105	101	109	–	–	–	–	–	–	–	–	–
Follow-up 1	November 05	93	99	102	0	0	9	69.75	73.5	76.5	0.00	0.00	11.76
Follow-up 2	March 06	88	94	90	1	0	0	29.92	24.08	22.83	3.34	0.00	0.00
Total KB					1	0	9	99.67	97.58	99.33	1.00[*] (0.95–1.90)	0.00[*]	9.06[*] (8.26–11.01)
Kennel KG													
Baseline	March 05	104	103	109	–	–	–	–	–	–	–	–	–
Follow-up 1	November 05	99	103	106	0	0	7	74.25	77.25	79.5	0.00	0.00	8.81
Follow-up 2	March 06	97	100	99	1	1	4	24.58	25.05	25.25	4.07	3.96	15.84
Total KG					1	1	11	98.83	102.75	104.75	1.01[**] (0.92–1.92)	0.98[**] (0.97–1.94)	10.50[**] (10.1–11.93)
KB + KG													
Baseline	March 05	209	204	218	–	–	–	–	–	–	–	–	–
Follow-up 1	November 05	192	202	208	0	0	16	144	97.58	156	0.00	0.00	10.26
Follow-up 2	March 06	185	194	189	2	1	4	54.50	49.13	48.08	3.67	2.04	8.32
Total					2	1	20	198.50	146.71	204.08	1.01[**] (0.96–1.44)	0.68[**] (0.49–0.88)	9.80[**] (9.17–10.55)

[a] New cases = dogs positive either serologically or parasitologically.
[*] Difference between treated and control group for $p < 0.05$.
[**] Difference between treated and control group for $p < 0.01$.

in shoulder region and applying the total contents of the pipette to the skin according to the manufacturer's instructions on the basis of the animal body-weight ranges.

The laboratory staff conducting the serological and parasitological tests was blinded concerning the dog treatments. It was decided not to blind the kennel staff (as additional bias-reducing factor) in order to reduce the risk of contaminating the untreated animals during handling by the kennel staff.

2.1.2. Animal management and care

Animals were kept (i.e. housing, food, temperature regulation and ventilation) under their usual housing conditions before, during and after the study. At each treatment time, clinical observations were recorded on the individual forms. Animals were removed from the study only if their general health deteriorated, if they died, or if they left the kennel because they had been adopted by private homes. Single files were filled in for concurrent treatments used during the experimental period. Adverse events were individually registered in accordance with the VICH GL9 (GCP) rules. The use of any other product with efficacy against insects on the study animals or in their environment was not allowed during the sandfly season.

2.1.3. Environmental examination procedures

From April to November 2005, the sandfly population density was evaluated using castor oil-coated sticky traps (30 cm × 21 cm) for 24-h periods. Sandflies were collected twice a month until the first appearance then weekly until the disappearance of the last insect, and a final sampling was carried out 2 weeks later. Sandflies were counted and identified at species level according to Maroli et al. (1994b). In the same period, the mean temperature and relative humidity values were recorded daily by two meteorological stations.

2.2. Diagnostic procedures

2.2.1. Sampling procedures

Blood samples were collected from the brachial or jugular vein, allowed to clot at room temperature and centrifuged at 1700 × *g* for 10 min. Serum was separated and stored at −20 °C until tested. Tissue from the popliteal lymph nodes was sampled using a non-aspiration technique (Menard and Papageorges, 1997). Skin samples were collected from the right shoulder region using a disposable ophthalmology scalpel after clipping the hair over an area of about 0.5 cm × 0.5 cm × 0.6 cm. They were stored at −20 °C in individual Eppendorf containers with 1 ml of phosphate buffer saline (PBS) solution until molecular processing took place.

2.2.2. Immunochromatographic dipstick test

The dipstick test (Leishmaniasis RapydTest®) is a qualitative membrane-based immune assay for the detection of anti-*Leishmania* antibodies in serum or plasma prepared for the diagnosis of human leishmaniasis and validated for dogs (Otranto et al., 2005b). The membrane is pre-coated with rK39 antigen at the test line and with anti-protein A antibodies (protein A colloidal gold conjugate) at the control line. The test was performed according to the manufacturer's instructions (Product Code: 1603; Lot CF1079 and DF1060). The results were considered positive if two distinct red lines appeared (one in the test region and another in the control region), negative when no red or pink lines appeared in the test region, and invalid if the control line failed to appear.

2.2.3. Direct parasitological observation of lymph-node smears

The lymph-node smears were prepared and stained using Diff Quick Stain (Medical Team Srl, Italy, Lot 100510). The cytological examination was considered positive if amastigotes (1.5–2.0 μm × 2.5–5 μm) were present.

2.2.4. Molecular procedures

After disruption in liquid nitrogen and pestling, genomic DNA was extracted from about 50 mg of skin samples using a commercial kit (Genomic DNA Purification Kit, Gentra Systems, MN, USA). A *L. infantum* kinetoplastid minicircle DNA was amplified using MC1 (5′GTTAGCCGATGGTGGTCTTG3′) and MC2 (5′CACCCATTTTTCCGATTTTG3′) primers (Cortes et al., 2004). The genomic DNA (4 μl) was added to the PCR reaction mix (46 μl) containing 2.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 8.3 and 50 mM KCl, 250 μM of each dNTP, 50 pmol of each primer and 1.25 U of Ampli *Taq* Gold (Applied Biosystems). Optimal conditions for PCR amplification were standardised as follows: initial denaturation at 94 °C for 12 min, 30 cycles consisting of denaturation at 94 °C for 30 s, annealing at 60 °C for 20 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. A positive control containing genomic *L. infantum* DNA and a negative control without DNA were included in all the assays. Amplification products (∼447 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 system (BioRad).

2.3. Statistical analysis

2.3.1. Sample size

The minimum sample size ($n = 83$) was calculated (free software WinEpiscope 2.0) for a cohort study for each group (A–C) in each kennel using the following assumptions: maximum expected non-exposed diseased animals (i.e. treated animals) = 5%; minimum relative risk (RR) to be detected significant RR = 4; power = 85% and confidence level = 95%.

Since a certain number of dogs must be expected to be lost to follow-up during this long study period, especially in the kennel situation, more than 100 instead of 83 dogs were enrolled in each group (Table 1).

2.3.2. Group allocation

Group allocation and randomisation were conducted independently in each kennel following the same procedure. Due to kennel organisation, dogs living in the same cage had to be placed in the same group. Cages were numbered, and three series of random numbers (each 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 coat) was evaluated using the chi-squared test and number generation was stopped when no more significant differences were detected.

2.3.3. Incidence calculation and efficacy assessment

To overcome the problem of dogs lost to follow-up during the study, the incidence of infection was studied using the incidence density rate (IDR) (Moreira et al., 2004). IDRs were calculated at 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 current assessment for each dog at risk for *Leishmania* infection). Dogs tested once (e.g. lost, dead) did not contribute at any time to the incidence calculation. Differences between incidence rates in Groups A–C were calculated using Yates' corrected χ^2 test. Statistical calculations were performed with an Excel spreadsheet and the statistical packages SPSS and WinEpiscope 2.0.

The repellent efficacy was expressed in terms of percentage protection against *Leishmania* infection calculated at the end of the sandfly season, both for dogs treated once and for dogs treated twice a month, using the following formula:

$$\%\,\text{protection} = (\%\,\text{of animals positive in control group} - \%\,\text{of animals positive in treated group})/(\%\,\text{of animals positive in control group}) \times 100$$

Dogs were considered positive if they tested positive either serologically or parasitologically.

2.3.4. Assessment of safety

To assess potential adverse events related to treatment and their frequency after treatment with the investigational veterinary product, suspected adverse drug reactions (SADR) were monitored at each follow-up for the treatment group. Any abnormal clinical signs were reported on a SADR form. The percentage of animals showing SADR compared to the total number treated was calculated using the following formula:

$$\%\,\text{of SADR} = (\text{number of animals with SADR}/\text{total number of animals treated}) \times 100.$$

3. Results

Out of 845 dogs initially tested for serology in January—February 2005 (i.e. 416 from KB and 429 from KG), 209 animals (24.7%) presented anti-*Leishmania* antibodies with a relative prevalence of 23.3% ($n = 97$) in KB and 26.1% ($n = 112$) in KG. Five dogs were excluded from the trial before the first treatment. In particular, in KB two seronegative dogs were positive at the cytological examination and two died before the trial started, while in KG one seronegative dog was positive at PCR. The remaining dogs enrolled in the three groups proved to be homogeneous ($P < 0.05$) from an epidemiological point of view for individual dog characteristics (i.e. sex, age, weight and coat length). The prevalence of seropositive dogs which were not included in the study but which were still kept in the same cages in the three groups was 22% (A), 16.5% (B) and 18.5% (C) in kennel KB and 24% (A), 28.5% (B) and 20.3% (C) in kennel KG. The IDRs for each group and each kennel in the two successive follow-ups are reported in Table 1.

Table 2
Results at serology, cytological examination of lymph-node smears and at PCR on skin samples of each positive dog from the two kennels at the middle and final follow up

Positive dogs	November 2005		March 2006		
	Serology	PCR	Serology	Cytology	PCR
KB					
Group A					
Dog 1	−	−	+	+	+
Group C					
Dog 1	−	+	+	+	+
Dog 2	−	+	−	−	+
Dog 3	−	+	−	−	+
Dog 4	−	+	−	−	+
Dog 5	−	+	−	−	+
Dog 6	+	+	+	−	−
Dog 7	−	+	−	−	−
Dog 8	−	+	−	−	−
Dog 9	−	+	−	−	+
KG					
Group A					
Dog 1	−	−	+	+	+
Group B					
Dog 1	−	−	+	+	−
Group C					
Dog 1	−	−	+	+	+
Dog 2	−	+	+	−	+
Dog 3	−	+	−	−	+
Dog 4	−	+	−	−	−
Dog 5	−	−	+	−	−
Dog 6	−	−	+	−	−
Dog 7	−	+	+	−	+
Dog 8	−	+	−	−	+
Dog 9	−	+	−	−	−
Dog 10	−	−	+	+	−
Dog 11	−	+	−	−	−

In KB the *Leishmania* infection, inferred by positivity in at least one of the three tests performed (i.e. serology, PCR and microscopic detection) at the interim or final follow-up, was identified in one animal from Group A and in nine from Group C. No positive animals were detected in Group B. In KB, the final protection efficacy of the repellent was 88.9% in Group A and 100% in Group B. In KG, *Leishmania* infection, inferred as above, was revealed in 1 animal from Group A, 1 from Group B and 11 from Group C, giving a protection efficacy of 90.36% in Group A and 90.73% in Group B. Results at the three different tests of each positive dog from the two kennels are reported in Table 2. No adverse systemic or topical reactions were registered in any treated dogs (Groups A and B). Forty-eight dogs (7.6%) were lost during the study period. In particular 35 dogs (11%) from KB and 13 dogs (4.1%) from KG kennel.

Table 3
Number and percentage of sandfly species captured and identified in KB and KG, divided according to sex

Sandflies	Number and percentage of sandflies		Total
	Females	Males	
Phlebotomus perniciosus	50 (24.9%)	151 (75.1%)	201 (58.6%)
Phlebotomus neglectus		1	1 (0.3%)
Sergentomya minuta	31 (23.7%)	100 (76.3%)	131 (38.2%)
Phlebotomus spp.	10		10 (2.9%)
Total			343

Sandflies were first captured at the very beginning of June 2005 in KB and KG, while the last capture occurred in mid-October. The number and species of sandflies collected throughout the observation period are reported in Table 3. In particular, *Phlebotomus perniciosus* was the most common species identified (58.6%) with 104 specimens collected in KB and 97 in KG. The population of *P. perniciosus* peaked in August–September when the highest rate of humidity was recorded at both sites. The only specimen of *Phlebotomus neglectus* was collected in mid-June in KG.

4. Discussion

The results clearly show that the combination of 10% imidacloprid/50% permethrin (Advantix®; Bayer AG, Germany) in a spot-on formulation is highly efficacious in preventing CanL under natural conditions in endemic areas by virtue of its repellent activity against sandflies. In particular, dogs treated with both application regimes (i.e. once or twice a month) showed a very high percentage of protection from sandfly bites in Group A (treated once a month), between 88.9 and 90.36%, and Group B (treated twice a month), between 90.73 and 100%. The IDRs registered in both Groups A and B were significantly lower than that registered in Group C in both kennels (KB $p < 0.05$ and KG $p < 0.01$).

Up to now no field studies have ever been conducted to evaluate the efficacy of imidacloprid 10% and permethrin 50% against sandflies under natural conditions. Currently, the results from two studies have been published reporting the efficacy of deltamethrin dog collars in a CanL focus in southern Italy (Vesuvius area, Campania region) (Maroli et al., 2001; Foglia Manzillo et al., 2006).

In the study published by Maroli et al. (2001), the impact of deltamethrin dog collars on the incidence of CanL was assessed in the intervention area and compared with the incidence in areas where no collars were fitted. A protection rate against CanL of 50 and 86% over two consecutive transmission seasons was recorded (Maroli et al., 2001). However, two factors might have affected the above trial, namely the vast area from which the dogs came (characterised by different microhabitats which might have influenced the distribution and density of sandflies) and secondly, the fact that the occurrence of sandfly biting was only inferred by the seroconversion rate to *L. infantum* immediately after the sandfly season. No parasitological methods were used in that particular study to diagnose CanL infection (Maroli et al., 2001). In a recently published field study, testing the efficacy of deltamethrin collars in kennelled dogs the protection of CanL was 72.3% in the first and 45.1% in the second transmission season, resulting in a cumulative rate of protection of 50.8% over both consecutive transmission seasons (Foglia Manzillo et al., 2006). Also in this study the *Leishmania* infection was diagnosed by serology (i.e. IFAT) and only dogs who tested positive were subjected to parasitological examinations on bone marrow and/or popliteal lymph node aspirate smears. Due to the selected IFAT as diagnostic tool, the detection of CanL infection is most likely to be underestimated. In fact, since the CanL incubation period in endemic areas ranges from 3 months to 7 years (Baneth, 2006), dogs infected by CanL (especially if asymptomatic) might not seroconvert immediately after infection, thus resulting in false-positive or false-negative subjects in treated and control groups (e.g. including dogs already infected at the start of the study). In fact, in our study, of 23 dogs which had a positive result in one of the three tests employed in November 2005 and/or March 2006, 16 (69%) were seronegative but positive on parasitological examination. It is remarkable that in endemic areas parasitological and/or serological positivity may not be related to the disease but only confirms that infection occurred. In fact, it has been reported that about 50% of animals are asymptomatic in southern Italy (Brandonisio et al., 1992) and this prevalence peaks at up to 85.3% in Brazil (Dantas-Torres et al., 2006). Accordingly, in the present study the parasitological results (inferred by the cytological examination and PCR on dermal samples) were not concordant at the two sampling times in six of the 16 dogs positive in November 2005 in the PCR assay but negative in the cytological and/or PCR assay in March 2006. This could be explained by a Th1 immune response that may have induced dogs to recover from the infection as previously suggested (Quinnell et al., 2001).

An important factor to be considered in trials for the evaluation of arthropod repellent activity under natural conditions is that animals should be subjected to the same parasitic burden irrespective of the treatment regime (Otranto et al., 2005a). Since very different sandfly microhabitats may be represented in multicentre studies, the random enrolment of cages (as a single unit of kennel dogs) in this study allowed us to use animals more likely to be subjected to a similar sandfly burden. Again, the decision to conduct the trial on kennel dogs in endemic areas, instead of using privately owned dogs, was encouraged by the fact that in the kennel situation the high density of dogs constitutes a ‘‘natural lure’’ for sandflies. In addition, working with a high number of kennel dogs reduces bias due to the lack of control over individual owners and different management practices (i.e. risk of undeclared ectoparasiticide treatment of animals and the environment, and different husbandry, e.g. overnight indoor housing during the trial). The percentage of lost animals (7.6%) in the present study was very low compared to that reported in the studies by Maroli et al. (2001) and Foglia Manzillo et al. (2006) who had problems linked to death of animals, animals moving house with their owners, and the owner’s refusal to have animals re-examined. All these constraints resulted in a high total percentage of dogs lost to follow-up (of about 50% in both studies).

Moreover, the spot-on formulation proved to be easy to apply and does not present the same constraints as collars which might be lost or broken due to the dog fighting or playing (Maroli et al., 2001; Foglia Manzillo et al., 2006). Interestingly, the high number of dogs infected with CanL or diseased (24.7%) which were not included in the trial but were living in the same cages as animals enrolled in the study groups (22% Group A, 16.5% Group B and 18.5% Group C in kennel KB and 24% Group A, 28.5% Group B and 20.3% Group C in kennel KG) ensured that the burden of CanL was maintained despite the relatively low density of sandflies registered during the 2005 sandfly season. These results highlight the importance of kennels in maintaining human and CanL foci in endemic areas and the likely risk for humans, particularly when kennels are located near human dwellings, as is the case with KB (about 1 km away from the city centre of Bari).

The seroprevalence registered at the start of the study (24.7%) is in keeping with that previously reported in kennel dogs in southern Italy and the incidence in untreated dogs (Group C) from the two kennels (9.06–10.50%) (Paradies et al., 2006). In the two kennels, the

sandfly season lasted from June to October with populations peaking in July—September depending on the temperature and relative humidity. In particular, *P. perniciosus* is the species most frequently identified (58.6%) followed by *Sergentomya minuta* (38.2%). The finding of one specimen of *P. neglectus* is sporadic because its habitat is characterised by hilly areas (about 200 m above sea level), as reported in the same region (Puccini et al., 1977).

The insecticidal and repellent efficacy of imidacloprid 10% and permethrin 50% against sandfly bites has been demonstrated experimentally by exposing dogs treated weekly and untreated control dogs to *P. papatasi* and *P. perniciosus* females and determining survival and feeding rates after a period of 4 weeks (Mencke et al., 2003; Mencke et al., 2005). Since *P. papatasi* and *P. perniciosus* are the species most frequently retrieved in the Mediterranean and Middle East regions, where *P. perniciosus* is in contrast to *P. papatasi* the major vector of *L. infantum*, prevention of bites should provide effective protection against CanL. This study confirms *P. perniciosus* as the most widespread species of sandfly in southern Italy (Maroli et al., 1994b) which, in turn, has also been reported as the species most sensitive to repellents (Fossati and Maroli, 1986). All these data are concordant in indicating that in areas where *P. perniciosus* is predominant, use of imidacloprid 10% and permethrin 50% might be very effective in the prevention of sandfly bites and thus of infection with CanL.

5. Conclusion

In recent years, measures to prevent CanL have included euthanasia of seropositive dogs (Alvar et al., 1994), insecticidal control of phlebotomine sandflies (reviewed in Alexander and Maroli, 2003) and vaccination strategies for humans and animals (Kubar and Fragaki, 2005). Euthanasia of infected dogs is an ethically unacceptable approach to much of society in many countries, while vaccination, although not even commercially available on a large scale, raises questions about the role vaccines might have on the Th1 and Th2 immune responses in naturally infected animals and humans (Kubar and Fragaki, 2005). Recently, a vaccine constituted by fucose–mannose-ligand enriched fraction of *L. donovani* showed 92–97% of protections against zoonotic visceral leishmaniasis (Saraiva et al., 2006) and it has been registered in Brazil for veterinary use against symptomatic CanL (Borja-Cabrera et al., 2002, 2004). Nevertheless, it has been demonstrated that vaccination with a multi-subunit recombinant *Leishmania* polyprotein is not effective in preventing CanL infection and disease progression in dogs under field conditions (Gradoni et al., 2005).

Thus, protection of dogs, the main reservoirs of *L. infantum*, against the sandfly vectors is the best option for controlling the spreading of CanL. Without any doubt, the highly effective repellent activity of the imidacloprid 10% and permethrin 50% combination against sandflies on dogs under natural conditions constitutes a promising new tool for preventing CanL in endemic areas, thus offering a hopeful perspective towards reducing the incidence of both canine and human leishmaniasis.

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