

1                                    **Toward Diagnosing *Leishmania Infantum* Infection in**  
2                                    **Asymptomatic Dogs in Endemic Area**

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4                                    **Running title:** Diagnosis of canine leishmaniasis in asymptomatic dogs

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6                                    D. Otranto,<sup>1\*</sup> P. Paradies,<sup>2</sup> D. de Caprariis,<sup>2</sup> D. Stanneck,<sup>3</sup> G. Testini,<sup>1</sup> F. Grimm,<sup>4</sup> P.  
7                                    Deplazes,<sup>4</sup> and G. Capelli<sup>5</sup>

8  
9                                    <sup>1</sup>Department of Veterinary Public Health and Animal Sciences, Faculty of Veterinary  
10                                    Medicine of Bari, Italy

11                                    <sup>2</sup>Department of Animal Health and Welfare, Faculty of Veterinary Medicine of Bari, Italy

12                                    <sup>3</sup>Vet practitioner, Koln, Germany

13                                    <sup>4</sup>Institute of Parasitology, University of Zurich, Switzerland

14                                    <sup>5</sup>Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro (PD), Italy

15  
16                                    **\*Corresponding author**

17                                    Mailing address: Dipartimento di Sanità Pubblica e Zootecnia, Facoltà di Medicina  
18                                    Veterinaria

19                                    S.p. per Casamassima Km, 3, 70010,Valenzano, Bari (Italy).

20                                    Phone/Fax: +39 080-4679839

21                                    e-mail: [d.otranto@veterinaria.uniba.it](mailto:d.otranto@veterinaria.uniba.it)

22 **ABSTRACT**

23 The most frequently used diagnostic methods were compared in a longitudinal survey on  
24 *Leishmania infantum* infected asymptomatic dogs from an endemic area of Italy. On February  
25 -March 2005, 845 asymptomatic dogs were tested by IFAT, dipstick -DS- and ELISA tests for  
26 *L. infantum* and by IFAT for *Ehrlichia canis*. Dogs seronegative to *L. infantum* were further  
27 parasitologically evaluated by microscopical examination of lymph node and PCR on skin  
28 sample. 204 animals both serologically and parasitologically negative to *L. infantum* at the  
29 first sampling were enrolled in the trial and further examined for canine leishmaniasis (CanL)  
30 and for canine monocytic ehrlichiosis (CME) in November 2005 (i.e. the end of the first  
31 sandfly season), in March 2006 and 2007 (one and two year follow-ups respectively). At the  
32 initial screening the overall seroprevalence rates for *L. infantum* were 9.5% by IFAT, 17.1%  
33 by ELISA, 9.8% by DS and for *E. canis* 15%. Concordance between IFAT and DS was  
34 almost equal, whereas concordance with ELISA was lower. The results of the annual  
35 incidence of *Leishmania* infection were variable depending by the test employed with the  
36 highest values registered at the PCR (i.e. 5.7% and 11.5%) followed by the ELISA, IFAT and  
37 DS. Throughout the two years of observation, 55 animals (i.e. 26.9%) became positive for *L.*  
38 *infantum* at one or more diagnostical test at different follow-ups with 12.7% showing clinical  
39 signs related to CanL infection while the remaining 87.3% being asymptomatic. A  
40 diagnostical scheme to asses the infected status of *L. infantum* in asymptomatic dogs is here  
41 suggested.

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44 **Key words:** *Leishmania infantum*, leishmaniasis, asymptomatic dogs, endemic area,  
45 diagnosis, PCR, IFAT, ELISA, dipstick.

46

## 47 1. INTRODUCTION

48 Canine leishmaniasis (CanL) due to *Leishmania infantum* is transmitted by different species  
49 of *Phlebotomus* and it is considered one of the most important canine protozoal diseases of  
50 zoonotic concern (2). *L. infantum* is widely distributed in many Mediterranean countries and  
51 in Italy stable endemic foci have been reported in dogs from central and southern areas (4, 5,  
52 30, 32) with high percentages (up to 53.1%) of serologically positive animals (5). More  
53 recently, infections by *L. infantum* have spread through northern Italian regions (19). In  
54 central Europe CanL is a well known and emerging travel associated disease and occasional  
55 focal autochthonous transmission of *Leishmania* has been suspected (16). In dogs, infections  
56 may cause severe clinical forms or may remain asymptomatic for a long time (5, 7, 17). Many  
57 clinical features of CanL (e.g. lethargy, weight loss, anorexia, epistaxys, lymphadenospleno-  
58 megaly) may be similar to those of other diseases including Canine Monocytic Ehrlichiosis  
59 (CME) (13). Indeed, along with CanL, CME by *Ehrlichia canis* is an important canine vector  
60 borne disease (CVBD) in several countries of the Mediterranean basin (40) transmitted by the  
61 brown dog tick *Rhipicephalus sanguineus*. In endemic areas, CanL may represent a veterinary  
62 and public health issue mainly due to the high percentage of asymptomatic animals (up to  
63 85%) in endemic areas (7) which may serve, similarly to symptomatic ones, as reservoirs for  
64 the vector borne transmission of *Leishmania* spp. to receptive animals and humans (22, 24).  
65 Thus, the reliable identification of *Leishmania* infected asymptomatic reservoir animals is  
66 crucial for any successful control strategy.

67 The definitive diagnosis of *Leishmania* infection in asymptomatic animals is troublesome  
68 since both serological and parasitological methods have inherent limitations (23). Indeed,  
69 serology may not be a good indicator of infection when used in cross sectional studies due to  
70 the varying time spanning between infection and seroconversion (i.e. from 3 months to 7  
71 years-[1]). Additionally, asymptomatic infected animals may remain seronegative as a

72 consequence of their individual immune response (3). Among the direct parasitological tests  
73 microscopical examination is a rapid and simple method, but has a low sensitivity,  
74 particularly in asymptomatic dogs, and thus is not recommended for mass screenings in  
75 endemic areas. *In vitro* culture techniques, although reliable and sensitive, are prone to  
76 microbiological contamination (12), especially if skin samples or samples collected under  
77 field conditions are used. Molecular tools have been developed to detect *Leishmania* DNA in  
78 putative dog reservoirs (33) and have been shown to be more sensitive than serology and  
79 culture techniques (10). Thus, although polymerase chain reactions (PCR) can be useful in  
80 detecting asymptomatic infected animals (10, 27) defining methodologies, protocol of  
81 amplifications, gene targets and tissue to be tested is matter of debate among scientists (6, 35).  
82 As a consequence, data currently available in literature about diagnosis of CanL in  
83 asymptomatic animals are controversial and a diagnostic “gold standard” is far from being  
84 clearly stated (23). Again, no longitudinal studies are available to investigate the serological  
85 and parasitological features in the course of first *L. infantum* infection in asymptomatic  
86 animals from an endemic area for CVBDs. Thus, it was the aim of the present study to  
87 compare the most frequently used diagnostic methods in a longitudinal survey on *L. infantum*  
88 infected asymptomatic dogs from endemic areas.

89

## 90 2. MATERIAL AND METHODS

### 91 2.1. Study design

92 From January 2005 to March 2007 samples were collected from dogs in the context of a  
93 previous field trial (29) with further sampling in March 2007 (see below). All animals were  
94 housed in two kennels of the Apulian region, southern Italy (latitude 42° and 39° North,  
95 longitude 15° and 18° East) where endemic CanL had been reported over the previous two  
96 years (30). Animals were kept (i.e. housing, food, temperature regulation and ventilation)

97 under their usual housing conditions (29). Animals were handled and sampled with the  
98 owners' consent and approval by the Ethical Committee of the Faculty of Veterinary  
99 Medicine of the University of Bari (Italy). Sex, age, weight and coat length were recorded for  
100 each dog.

101 Briefly, in January and February 2005 (i.e. in the absence of sandflies in the study area [18])  
102 about 2000 dogs were clinically evaluated. 845 dogs of both sexes and different ages that did  
103 not show any signs of dermatitis or lymphadeno-megaly were further tested by an  
104 immunofluorescence antibody test (IFAT), an immunochromatographic dipstick assay (DS)  
105 and an enzyme linked immunosorbent assay (ELISA) for the presence of specific anti-  
106 *Leishmania* antibodies and by IFAT for specific anti-*E. canis* antibodies. Dogs without  
107 detectable anti-*Leishmania* antibodies were further examined for the presence of amastigote  
108 stages of *Leishmania* parasites in stained lymph node smears and for *Leishmania*-DNA in  
109 dermal tissue samples by PCR (see below).

110 In March 2005, 204 animals (i.e. 102 for each kennel) which were both serologically and  
111 parasitologically negative for *L. infantum* were enrolled in a longitudinal follow up trial,  
112 irrespective of the presence of anti-*E. canis* antibodies. All animals remained untreated with  
113 any ecto-parasiticide through the study. They were examined serologically and  
114 parasitologically for CanL and CME in November 2005 (i.e. the end of the first sandfly  
115 season), in March 2006 (one year follow-up) and in March 2007 (two years follow-up).  
116 Specifically, serological tests for the presence of anti-*Leishmania* antibodies (IFAT, ELISA,  
117 DS) and anti-*E. canis* antibodies (IFAT) as well as PCR for the detection of *Leishmania* DNA  
118 in dermal tissue samples were performed at all follow up times. Additionally, lymph node  
119 smears were examined microscopically in March 2006 and 2007 for the presence of  
120 amastigote *Leishmania* stages. Clinical examinations were performed monthly and clinical

121 signs (i.e. lethargy, weight loss, anorexia, alopecia, dermatitis, conjunctivitis, epistaxys,  
122 onychogryphosis, lymphadenomegaly, etc.) were recorded.

## 123 2.2. Diagnostic procedures

### 124 2.2.1. Serological tests

125 Three different serological tests were used to reveal specific anti-*Leishmania* IgG antibodies.

126 **IFAT.** The test was performed using promastigotes of *L. infantum* zymodeme MON1 as  
127 antigen. The cells were exposed to sera diluted (1:80) in phosphate buffered saline (PBS) in a  
128 moist chamber, and then to fluorescinated rabbit anti-dog IgG serum diluted 1:40 (Rabbit  
129 anti-dog IgG; Sigma - Aldrich Chemie, Germany, Lot 125K4752) both at 37°C for 30 min.  
130 Samples were scored positive when they produced a clear cytoplasmatic or membrane  
131 fluorescence with promastigotes using a cut-off dilution of 1:80. Positive sera were titrated till  
132 they gave negative results.

133 **ELISA.** The assay was performed by using water soluble proteins of promastigote forms of *L.*  
134 *infantum* (zymodeme MON1) as antigens and goat anti-dog IgG antibodies (gamma chain  
135 specific) conjugated to alkaline phosphatase (Kirkegaard and Perry Lab, Inc., Gaithersburg,  
136 MD) as detection antibodies. Samples were considered positive if the absorbance at 405nm  
137 ( $A_{405}$ ) was above the arithmetic mean plus 3 SD of the  $A_{405}$  values of 48 *Leishmania* control  
138 dogs originating from a *L. infantum*, *E. canis* and *Babesia canis* free area of Southern  
139 Switzerland (21). Results were expressed in a system of arbitrary antibody units (AU) where  
140 0 AU correspond to the threshold value and 100 AU to the positive standard serum.

141 **DS.** A commercially available DS test (*Leishmania* RapydTest®; DiaSys Europe Ltd.,  
142 Wokingam, UK), based on the rk39 antigen and validated for dogs (28) was performed  
143 according to the manufacturer's instructions (Product Code: 1603; Lot CF 1079 and  
144 GK1062).

145

146 *E. canis* specific antibodies were detected by IFAT by using slides containing fixed *E. canis*  
147 in DH82 cells (Canine Ehrlichiosis FA Substrate Slide, VMRD, Pullmann, Washington, USA,  
148 Lot P060228-002032908). The parasitized cells were exposed to sera diluted (1:50) in  
149 phosphate buffered saline (pH 7.2) in a moist chamber and, after washing, to fluoresceinated  
150 rabbit anti-dog IgG (Rabbit anti-dog IgG; Sigma - Aldrich Chemie, Germany, Lot 125K4752)  
151 diluted 1:60; both incubations were done at 37°C for 30 min. Samples scored positive when  
152 they produced cytoplasmic inclusion bodies fluorescence at a dilution of 1:50 or higher. All  
153 positive sera were further titrated until the reaction became negative.

#### 154 2.2.2. Parasitological diagnosis of *CanL*

155 Tissue from the popliteal lymph nodes was sampled using a non-aspiration technique (20).  
156 Lymph node smears were stained using Diff Quick Stain (Medical Team Srl, Italy, Lot  
157 100510) and microscopically examined for the presence of amastigote stages (1.5 - 2.0 x 2.5 -  
158 5 µm) of *Leishmania* parasites.

159 One skin sample weighing about 30 mg per animal and collection time was taken from the  
160 right shoulder region using a disposable ophthalmology scalpel after clipping the hair over an  
161 area of about 0.5 x 0.5 cm. Samples were stored at -20°C in Eppendorf tubes containing 1 ml  
162 of PBS.

163 After disruption in liquid nitrogen and pestling (i.e. two thaw- freezing cycles), genomic DNA  
164 was extracted from about 30 mg of skin samples using a commercial kit (Genomic DNA  
165 Purification Kit, Gentra Systems, Minnesota, USA). A *L. infantum* kinetoplastid minicircle  
166 DNA fragment was amplified using the MC1/MC2 primer pair (6). Genomic DNA solution (4  
167 µl) was added to the PCR reaction mix (46 µl) containing 2.5 mM MgCl<sub>2</sub>, 10 mM Tris-HCl,  
168 pH 8.3 and 50 mM KCl, 250 µM of each dNTP, 50 pmol of each primer and 1.25 U of Ampli  
169 *Taq* Gold (Applied Biosystems, Milan, Italy). Optimal conditions for PCR amplification were  
170 standardised as follows: initial denaturation at 94°C for 12 min, 30 cycles consisting of

171 denaturation at 94°C for 30 s, annealing at 60°C for 20 s, extension at 72°C for 30 s, and a  
172 final extension at 72°C for 5 min. A positive control containing genomic *L. infantum* DNA  
173 and a negative control without DNA were included in all the assays. Amplification products  
174 (~447 bp) were visualised on 2% w/v agarose gels (Ambion, Milan, Italy) upon staining with  
175 ethidium bromide.

176

## 177 *2.3 Statistical analysis*

### 178 *2.3.1 Sample size*

179 The minimum sample size (n=128, i.e. 64 for each kennel) to estimate incidence was  
180 calculated, using the software WinEpiscope 2.0 (39), following these assumptions: dog's  
181 population for each kennel (n =800), expected incidence 11% (29), maximum error accepted  
182 5% and confidence level (95%). Since a certain number of dogs must be expected to be lost to  
183 follow-up during a long study period, especially in the kennel situation, more than 100 instead  
184 of 64 dogs were enrolled in each group.

### 185 *2.3.2 Incidence calculation and test performance*

186 Crude incidence was calculated at each sampling date and for each test as the proportion of  
187 positive dogs in comparison to susceptible population. For each calculation dogs previously  
188 positive at the same test were excluded and did not contribute any longer to the incidence  
189 calculation, although they were tested each time. Yearly incidence and apparent recovery  
190 rates (% of dogs positive and resulted negative one year later at the same test) were calculated  
191 at March 2006/2007 and March 2007, respectively, regardless the results of November 2005.

192 Concordance among serological tests performed in the initial screening (March 2005) on  
193 asymptomatic dogs was evaluated by k statistics (39). Kappa values were ranked as low  
194 (0.2<k<0.4), moderate (0.4<k<0.6), good (0.6<k<0.8), or excellent (k>0.8) as suggested by  
195 Everitt (8). At the follow up examinations (November 2005, March 2006 and March 2007),



196 relative sensitivity ( $SE_{rel}$ ) and relative specificity ( $SP_{rel}$ ) of the serological tests were assessed  
197 on the basis of the PCR results obtained at the same sampling time. Se and Sp were calculated  
198 according to Greiner and Gardner (11).

199 Association between PCR results and ELISA AU was screened by one-way analysis of  
200 variance (ANOVA) after converting the AU in log (data+1) to normalize the AU distribution;  
201 association between PCR results and IFAT titer was screened by chi-square test after  
202 categorization of the titer.

203 Statistical calculations were performed with the statistical package SPSS, version 13.0 for  
204 Windows, and WinEpiscope 2.0 (available at: <http://www.clive.ed.ac.uk/winepiscope/>).

205

### 206 3. RESULTS

#### 207 3.1 Initial screening

208 The results of the serological tests carried out at the initial screening of 845 dogs are  
209 summarized in Table 1. Overall seroprevalence rates for anti-*L. infantum* antibodies were  
210 17.3% (positive reaction in one or more tests) and for anti-*E. canis* antibodies 15% (data not  
211 shown). The prevalence of anti-*E. canis* antibodies was similar regardless of the serological  
212 status for *Leishmania* antibodies when assessed by IFAT or DS (ranging from 14.1% to  
213 17.7%), but was significantly higher ( $\chi^2$  test;  $p < 0.01$ ) in ELISA -positive (26.6%) than in  
214 ELISA -negative dogs (12.4%; Table 1).

215 Parasites were detected microscopically in stained lymph node smears of 3 (i.e. 0.7%) out of  
216 453 dogs. The above three dogs were positive at serological tests. Concordance between  
217 IFAT and DS was almost perfect (kappa = 0.965, rating “excellent”), with only five discordant  
218 cases, whereas concordance with ELISA was lower (54 discordant cases, kappa = 0.71, rating  
219 “good”). Test agreement between ELISA and DS was also rated “good” (63 discordant cases,  
220 kappa = 0.678, Table 2).

221 3.2 Follow up

222 The 204 dogs negative for *Leishmania* in all diagnostic tests, thus enrolled into the  
223 longitudinal study, represented a population homogenous for sex, age, weight and hair length  
224 ( $\chi^2$  test,  $p>0.05$ ) and they included 28 dogs (i.e. 13.7%) positive for *E. canis* antibodies.  
225 Complete two years data sets (all tests, all sampling dates) were available for 173 dogs.

226 The incidences of CanL and CME calculated for each sampling date and diagnostic technique  
227 are summarized in Table 3. Test specific annual incidence rates of *Leishmania* infections,  
228 calculated for the periods of March 2005 to March 2006 and March 2006 to March 2007 were  
229 5.7% and 11.4% for PCR, 5% and 5.9% for ELISA, 2.6% and 6.4% for IFAT and 2.1% and  
230 0% for DS (Table 3).

231  $SE_{rel}$  and  $SP_{rel}$  of the serological tests determined on the basis of the skin PCR results showed  
232 considerable variations with respect to the diagnostic technique and the sampling date (Table  
233 4). In general, test  $SE_{rel}$  were low (less than 50%) but test  $SP_{rel}$  were high ranging from 92 to  
234 100%.  $SP_{rel}$  did not change with time, while  $SE_{rel}$  showed significant variations among tests  
235 and sampling time. In particular the maximum  $SE_{rel}$  for all tests was recorded in March 2006  
236 and the lowest after one year in March 2007. The  $SE_{rel}$  of the ELISA was significantly higher  
237 than that of IFAT and DS for the November 2005 and March 2006 sampling dates. Out of five  
238 dogs classified doubtful at DS test (data not shown), three were negative at all the other  
239 *Leishmania* and *Ehrlichia* diagnostic tests at each follow-up while the remaining two were  
240 clearly positive for anti-*E. canis* antibodies (antibody titres ranging from 1:200 to 1:1.600).

241 Throughout the two years of observation, 55 animals (i.e. 26.9%) were tested positive for *L.*  
242 *infantum* in at least one of the diagnostic tests (Table 5). In particular, 28 animals were  
243 positive only at one of the parasitological tests (i.e. PCR and/or microscopy, group A) while  
244 18 were only serologically positive at one or more tests (group B). Only 9 dogs were both

245 parasitologically and serologically positive (group C). Out of the 18 serologically positive  
246 animals of group B, 9 were positive at the ELISA only.

247 After the first sandfly season in November 2005, 25 animals were positive at one or more  
248 tests. Among them, 11 dogs were positive at the PCR only, 11 at one of the serological tests  
249 only (ELISA =9; IFAT =2) and 3 were both, parasitologically and serologically positive. The  
250 remaining 30 animals become positive at least one of the parasitological and/or serological  
251 tests at the following sampling times (i.e. on March 2006 or March 2007) (Table 5).

252 At the final follow-up date (March 2007), only seven animals (4%) out of the remaining 173  
253 showed symptoms of leishmaniasis (i.e. dermatitis, lymphadenopathy, conjunctivitis, skin  
254 ulcers) (Table 5). Two among them belonged to group A and B, respectively, while the  
255 remaining five animals belonged to group C. The 32 dogs ELISA positive throughout the  
256 observation period showed AU levels ranging from 0.08 to 96.79. Only 5 of these dogs were  
257 PCR positive and were significantly associated with higher ELISA titers ( $p<0.05$ ). In  
258 particular 4 PCR positives showed above 7.5 AU in ELISA, while only 1 was 0.73. Out of 22  
259 IFAT positive dogs, 7 were also PCR positive and showed no statistical association with  
260 titers. In particular 4 PCR positive were IFAT positive at 1:80, 1 at 1:160 and 2 at 1:320.

261

#### 262 4. DISCUSSION

263 The results of the present work clearly imply that diagnosis of early CanL and identification  
264 of asymptomatic carrier animals in an endemic area might be a complex task. Indeed, at the  
265 initial screening while the seropositivity to *L. infantum* was similar at IFAT and DS (i.e. about  
266 9.5%) it was higher when inferred by ELISA (i.e. ~17%). Both, IFAT and DS are less  
267 sensitive than ELISA for the detection of specific antibodies in asymptomatic dogs without  
268 other infections and kept outside of an endemic area (21). In the present study focusing on  
269 early infections in animals living in an endemic area, ELISA proved to be the most sensitive

270 serological test on two occasions (November 2005 and March 2006) whereas no significant  
271 differences between the serological tests were noted in March 2007.

272 The significantly higher percentage of positive ELISA results in dogs also positive for *E.*  
273 *canis* antibodies (Table 1), indicates some kind of interaction between the two tests. One  
274 possible explanation is a cross-reaction of ELISA test, resulting in false *Leishmania* positives,  
275 or it is also possible that *Leishmania* specific antibodies detectable only by the ELISA might  
276 be responsible for false positive reactions in the *Ehrlichia* IFAT.

277 Nonetheless, *E. canis* infection might induce immunosuppression and therefore potentially  
278 increase the susceptibility of infection to *L. infantum* (14). It is broadly accepted, that  
279 threshold levels of any serological test should be adapted to meet the local conditions. This is  
280 reflected by the finding that in our study 44.7% of sera positive only at ELISA at the initial  
281 sampling were only marginally above the cut-off level. The concordance between IFAT and  
282 DS (K=0.965) tests was high, with only few discordant cases (Table 2). However, IFAT is  
283 also a quantitative test which could provide useful information about the immune reactivity of  
284 *L. infantum* infected individuals. rK39 DS represents an alternative to currently available  
285 diagnostic tests, especially when used in mass screening surveys in which antibody titers are  
286 not required, and it is ideal for use under field conditions. Again, DS test was validated vs.  
287 IFAT using sera from dogs positive or negative at lymph node smear parasitological  
288 examination thus in animals with a visceral generalized form of infection (28). Accordingly,  
289 by comparing ELISA, IFAT and rK39 DS the latter test showed to be mainly helpful for  
290 confirming clinically suspected cases while it was not very sensitive in detecting  
291 asymptomatic infections (21). Consequently, in asymptomatic animals from endemic areas a  
292 negative serological DS test may result from sub-clinically infected animals as well a positive  
293 test from dogs co-infected by *E. canis* (28). Thus, all serological tests should be evaluated  
294 carefully according to the epidemiological context of the area and the aim of the investigation.

295 In addition, in asymptomatic animals a low humoral reactivity may lead to low antibody  
296 concentration and, ultimately, to borderline titers which may result in false negative or  
297 positive due to cross -reactivity (15).

298 Compared to spleen and bone marrow, popliteal lymph nodes have been shown to be the most  
299 suitable tissue for direct parasitological detection in *L. infantum* infected symptomatic animals  
300 (25). Conversely, in asymptomatic animals at initial screening cytology showed a very low  
301 percentage of positivity (i.e. 0.7%) and low Se when compared to serological techniques as  
302 already demonstrated in animals from endemic areas (34). Thus cytology on lymph node is  
303 not useful to diagnose *Leishmania* infection in asymptomatic animals since protozoa may  
304 remain at dermal site or may be present with a low parasitic load (15) as a consequence of an  
305 effective immune response (31). Similarly, out of the 55 animals newly infected at one or  
306 more follow-ups only 9 (i.e. 16.3%) were positive at the cytological examination which  
307 indicate that cytology should be not the first choice parasitological test in diagnosing *L.*  
308 *infantum* asymptomatic animals. Again, the difficulties in sample collection from not enlarged  
309 lymph nodes impair the use of cytology in asymptomatic animals. Interestingly, five out the 9  
310 animals positive at cytological examination showed clinical symptoms while other 3 became  
311 positive only at final follow-up (Table 5) thus being not possible to evaluate the appearance of  
312 clinical signs. Only one dog positive at cytology and PCR on March 2006 became negative at  
313 all tests in the last follow-up.

314 In the last decades, different protocols of PCR were shown to be sensitive and specific to  
315 diagnose CanL in a variety of animal tissues (i.e. bone marrow, lymph nodes, skin and blood-  
316 [9]). A number of studies investigated different gene markers and protocols for the diagnosis  
317 of CanL but results are in many cases discordant. PCR on skin samples (at any collection  
318 time) was positive in 34 (i.e. 61.8%) out of 55 infected animals. Although PCR on skin  
319 samples cannot be considered as a “gold standard” for detecting *L. infantum*, especially in

320 endemic areas (36) it most likely is the more sensitive method for diagnosing animal exposure  
321 to sandflies bites and/or infection.

322 Accordingly, the results of the annual incidence of *Leishmania* infection, calculated in March  
323 2006 and March 2007 were variable depending by the test employed with the highest values  
324 registered at the PCR (i.e. 5.7% and 11.4%) followed by the ELISA, IFAT and DS. However,  
325 by calculating the  $SE_{rel}$  and  $SP_{rel}$  of the serological tests on the basis of the results of the skin  
326 PCR, the  $SE_{rel}$  was always very low while the  $SP_{rel}$  was good (from 92 to 100%, Table 4). As  
327 a consequence, serological tests are not optimal to detect exposed or newly infected animals.  
328 Indeed, since dogs infected by *L. infantum* (especially if asymptomatic) might not seroconvert  
329 immediately after infection or they may develop an immune responsiveness oriented toward a  
330 self healing cellular immune reaction, serological test may lead to false-negative results.

331 From a parasitological standpoint, molecular detection of *L. infantum* on skin samples may  
332 indicate prior exposure to infected phlebotomine sand fly bites but also an active infection in  
333 resistant (i.e. immunocompetent) dogs, or both (38).

334 The above issue remains one of the crucial and debated aspect in diagnosing CanL in  
335 asymptomatic dogs. Indeed, a dog which has been bitten by one or more *L. infantum* infected  
336 sandflies should be considered an individual which has been “exposed” and so far which  
337 needs to be monitored in the follow-up. Similarly, in a previous study 37% of seronegative  
338 asymptomatic dogs from an endemic area were positive at the PCR on skin as a likely  
339 consequence of an effective cellular immunity (37). *L. infantum* promastigotes start  
340 developing into macrophage cells at the site of inoculation in its amastigote forms and the  
341 infection may spread resulting into a “systemic form” (26). At this stage an antibody immune  
342 responsiveness should occur according to the individual immune reactivity (3). The above  
343 infection pattern could explain the reason why at the first follow-up on November 2005, 10  
344 animals were positive only at the *Leishmania* PCR test. Although an animal positive at PCR

345 on skin samples has to be considered exposed, neither serological nor molecular tests may  
346 predict if it will remain long-lasting asymptomatic, it will clear the infection or exhibit  
347 progressive leishmaniasis. Accordingly, three animals which resulted positive for *Leishmania*  
348 only at the first screening, at the second converted to negative more likely as an affect of a  
349 clearing of infection. A similar picture was previously recorded in asymptomatic animals  
350 positive at nested PCR on bone marrow that converted into negative at the follow up (27).  
351 Out of 55 animals positive at least at one test performed for *L. infantum* (see Table 5) only  
352 seven (12.7%) showed clinical signs related to CanL infection on March 2007 while the  
353 remaining 48 (87.3%) were all asymptomatic. These data nicely fit with percentage of  
354 asymptomatic dogs recorded in Brazil (i.e. 85.3% [7]). Although the clinical appearance of  
355 symptoms cannot be ruled out in the follow-up of *L. infantum* infected animals, the results of  
356 the present survey confirm the high prevalence of asymptomatic dogs in endemic area and  
357 pinpoint their potential role in spreading the disease to receptive hosts. Infected asymptomatic  
358 animals contribute in maintaining the endemicity of the disease by transmitting *Leishmania*  
359 spp. to new hosts (dogs or humans) via sandfly bites (22). Under the above circumstances,  
360 monitoring asymptomatic infected animals may be of relevance not only for the  
361 epidemiological evaluation of CanL in endemic areas but also to control the infection in  
362 human and dog populations by preventing sandfly bites both on infected and uninfected hosts.  
363 The data presented clearly shows that there is no gold standard to detect *Leishmania*  
364 infections in asymptomatic dogs and highlights the difficulty to propose a clear diagnostic  
365 scheme to assess the infection status of asymptomatic dogs. Therefore, more than one test  
366 should be used for the diagnosis of CanL in endemic areas.

367

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371

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ACCEPTED

499 TABLE 1. Detection of anti *-Leishmania infantum* antibodies by different tests (i.e. IFAT,  
 500 ELISA, dipstick -DS) at the study starting point as well as anti *-Ehrlichia canis* antibodies in  
 501 dogs with (L-pos) or without (L-neg) detectable anti *-L. infantum* antibodies

Test	Anti <i>-Leishmania infantum</i> antibodies	Anti <i>-Ehrlichia canis</i> antibodies	
		L-pos dogs	L-neg dogs
IFAT	79/831 (9.5%)	14/79 (17.7%)	106/752 (14.1%)
ELISA	143/837 (17.1%)	38/143 (26.6%)*	86/694 (12.4%)*
DS	83/845 (9.8%)	14/83 (16.9%)	112/762 (14.7%)

502 \* p<0.001

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503 TABLE 2. Measure of agreement (kappa values) among results (expressed as negative –neg-  
 504 and positive -pos) of IFAT, ELISA, dipstick (DS) *Leishmania* tests calculated on 845 dogs at  
 505 the study starting point.  
 506

Tests and results		Kappa value (rank) and significance	
	<b>DS neg</b>	<b>DS pos</b>	
<b>IFAT neg</b>	750	2	k=0.965 (“excellent”)
<b>IFAT pos</b>	3	76	p<0.001
	<b>ELISA neg</b>	<b>ELISA pos</b>	
<b>IFAT neg</b>	692	54	k=0.710 (“good”)
<b>IFAT pos</b>	0	79	p<0.001
	<b>DS neg</b>	<b>DS pos</b>	
<b>ELISA neg</b>	694	0	k=0.678 (“good”)
<b>ELISA pos</b>	63	80	p<0.001

507  
 508

509

510 TABLE 3. Prevalence at starting point and incidence rates (I) of *Leishmania infantum* and  
 511 *Ehrlichia canis* infection calculated for each diagnostic technique. Dogs previously positive at  
 512 the same test were excluded from calculation

513

	<i>Leishmania</i>			<i>Ehrlichia</i>	
	ELISA	IFAT	DS*	PCR	IFAT
	I (pos/N)	I (pos/N)	I (pos/N)	I (pos/N)	I (pos/N)
<b>Prevalence at starting point</b>					
Mar. 05	0% (0/204)	0% (0/204)	0% (0/204)	0% (0/204)	13.7% (28/204)
<b>Intermediate incidence determinations</b>					
Mar. 05 –Nov. 05	5.1% (9/178)	2.0% (4/196)	0% (0/196) 13 doubtful 2.2% (4/179)	7.2% (14/195)	20/168 (11.9%)
Nov. 05- Mar.06	2.0% (3/149)	1.1% (2/187)	4 doubtful	0.6% (1/178)	5/146 (3.4%)
<b>Yearly incidence rates</b>					
Mar. 05-06	5.0% (8/159)	2.6% (5/192)	2.1 (4/192)	5.7% (11/193)	13.8 (25/176)
Mar. 06-07	5.9% (9/153)	6.4% (11/173)	0% (0/168)	11.4% (19/166)	9.3% (13/139)
<b>Yearly recovery rates (neg'07/pos'06)</b>					
Mar. 06-07	33.3% (2/6)	25% (1/3)	all	60% (6/10)	25.6% (10/39)

514 \* doubtful results were considered negative



515 TABLE 4. Relatives sensitivity ( $SE_{rel}$ ) and relative specificity ( $SP_{rel}$ ) of IFAT, ELISA,  
 516 dipstick (DS) *Leishmania* serological tests compared to the PCR on skin in asymptomatic  
 517 dogs.

	November 2005		March 2006		March 2007	
	$SE_{rel}$	$SP_{rel}$	$SE_{rel}$	$Sp_{rel}$	$SE_{rel}$	$SP_{rel}$
<b>ELISA</b>	33% <sup>aAB</sup>	95%	50% <sup>bcAC</sup>	95.5%	9% <sup>BC</sup>	93%
<b>IFI</b>	14% <sup>aD</sup>	99%	27% <sup>bDE</sup>	99%	13% <sup>E</sup>	92%
<b>DS</b>	-	-	18% <sup>CF</sup>	99%	4.5% <sup>F</sup>	100%

518 Equal letters correspond to significant difference for  $p < 0.01$

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519 TABLE 5. Diagnostic follow up of the 55 dogs resulted positive for *Leishmania infantum*

520 infection at one or more tests

No. of dogs	November 05				March 06					March 07				
	IFAT	DS	ELISA	PCR	IFAT	DS	ELISA	Mic	PCR	IFAT	DS	ELISA	Mic	PCR
<b>Group A (28), parasitologically positive animals</b>														
15														+
4*				+					+					
3				+					+					+
3				+										
2													+	+
1								+	+					
<b>Group B (18), serologically positive animals</b>														
5													+	
2			+											
2			+				+							
3			+				+			+			+	
1										+				
1			+							+			+	
1							+							
1*			+							+				
1										+			+	
1	+				+	+				+			+	
<b>Group C (9), parasitologically and serologically positive animals</b>														
2*													+	+
1*				+	+			+	+				+	
1	+			+										
1													+	+
1	+			+	+	+			+	d	d	d	d	d
1*			+	+	+	+	+		+	+	+	+	+	+
1*	+				+	+				+			+	+
1*								+		+			+	

521  
 522 \*Single animals which showed symptoms of leishmaniasis (i.e. dermatitis,  
 523 lymphadenopathy, –conjunctivitis, skin ulcers) and/or hypergammaglobulinemia in each  
 524 line; d, dead animals.