CVI Accepts, published online ahead of print on 7 January 2009
Clin. Vaccine Immunol. doi:10.1128/CVI.00268-08

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

Running title: Diagnosis of canine leishmaniasis in asymptomatic dogs

D. Otranto,[1*] P. Paradies,[2] D. de Caprariis,[2] D. Stanneck,[3] G. Testini,[1] F. Grimm,[4] P. Deplazes,[4] and G. Capelli[5]

[1]Department of Veterinary Public Health and Animal Sciences, Faculty of Veterinary Medicine of Bari, Italy

[2]Department of Animal Health and Welfare, Faculty of Veterinary Medicine of Bari, Italy

[3]Vet practitioner, Koln, Germany

[4]Institute of Parasitology, University of Zurich, Switzerland

[5]Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro (PD), Italy

***Corresponding author**

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

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

Phone/Fax: +39 080-4679839

e-mail: d.otranto@veterinaria.uniba.it

ABSTRACT

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

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

1. INTRODUCTION

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

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

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

2. MATERIAL AND METHODS

2.1. Study design

From January 2005 to March 2007 samples were collected from dogs in the context of a previous field trial (29) with further sampling in March 2007 (see below). All animals were housed in two kennels of the Apulian region, southern Italy (latitude 42° and 39° North, longitude 15° and 18° East) where endemic CanL had been reported over the previous two 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

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

2.2. Diagnostic procedures

2.2.1. Serological tests

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

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

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

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

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 $MgCl_2$, 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 (χ^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, Table2).

3.2 Follow up

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

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

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

Throughout the two years of observation, 55 animals (i.e. 26.9%) were tested positive for *L. infantum* in at least one of the diagnostic tests (Table 5). In particular, 28 animals were positive only at one of the parasitological tests (i.e. PCR and/or microscopy, group A) while 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

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

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

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

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

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

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

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

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

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

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

on skin samples has to be considered exposed, neither serological nor molecular tests may predict if it will remain long-lasting asymptomatic, it will clear the infection or exhibit progressive leishmaniasis. Accordingly, three animals which resulted positive for *Leishmania* only at the first screening, at the second converted to negative more likely as an affect of a clearing of infection. A similar picture was previously recorded in asymptomatic animals positive at nested PCR on bone marrow that converted into negative at the follow up (27).

Out of 55 animals positive at least at one test performed for *L. infantum* (see Table 5) only seven (12.7%) showed clinical signs related to CanL infection on March 2007 while the remaining 48 (87.3%) were all asymptomatic. These data nicely fit with percentage of asymptomatic dogs recorded in Brazil (i.e. 85.3% [7]). Although the clinical appearance of symptoms cannot be ruled out in the follow-up of *L. infantum* infected animals, the results of the present survey confirm the high prevalence of asymptomatic dogs in endemic area and pinpoint their potential role in spreading the disease to receptive hosts. Infected asymptomatic animals contribute in maintaining the endemicity of the disease by transmitting *Leishmania* spp. to new hosts (dogs or humans) via sandfly bites (22). Under the above circumstances, monitoring asymptomatic infected animals may be of relevance not only for the epidemiological evaluation of CanL in endemic areas but also to control the infection in human and dog populations by preventing sandfly bites both on infected and uninfected hosts.

The data presented clearly shows that there is no gold standard to detect *Leishmania* infections in asymptomatic dogs and highlights the difficulty to propose a clear diagnostic scheme to assess the infection status of asymptomatic dogs. Therefore, more than one test should be used for the diagnosis of CanL in endemic areas.

Acknowledgments

The authors wish to thank Norbert Mencke Bayer HealthCare (Germany) for partially supporting this research.

REFERENCES

1. **Baneth, G.,** 2006. Leishmaniases. In: Greene, C.E. (Ed.), Infectious Diseases of the Dog and Cat, 3rd edition. Saunders-Elsevier, St. Louis, Missouri, pp. 685-698.
2. **Baneth, G., A. F. Koutinas, L. Solano-Gallego, P. Bourdeau, and L. Ferrer.** 2008. Canine leishmaniosis - new concepts and insights on an expanding zoonosis: part one. Trends Parasitol. **24**:324-30.
3. **Barbiéri, C. L.** 2006. Immunology of canine leishmaniasis. Parasite Immunol. **28:**329–337.
4. **Bongiorno, G., A. Habluetzel, C. Khoury, and M. Maroli.** 2003. Host preferences of phlebotomine sandflies at a hypoendemic focus of canine leishmaniasis in central Italy. Acta Trop. **88:**109-116.
5. **Brandonisio, O., G. Carelli, L. Ceci, B. Consenti, A. Fasanella, and V. Puccini** 1992. Canine leishmaniasis in the Gargano Promontory (Apulia South Italy). Eur. J. Epidemiol. **8:**273-276.
6. **Cortes, S., N. Rolao, J. Ramada, and L. Campino.** 2004. PCR as a rapid and sensitive tool in the diagnosis of human and canine leishmaniasis using *Leishmania donovani* s.l. -specific kinetoplastid primers. Trans. R. Soc. Trop. Med. Hyg. **98:**12-17.
7. **Dantas-Torres, F., E. M. Felino de Brito, and P. S. Brandão-Filho,** 2006. Seroepidemiological survey on canine leishmaniasis among dogs from an urban area of Brazil. Vet. Parasitol. **140:**54-60.

8. **Everitt, B.S.,** 1989. Statistical Methods for Medical Investigations. Oxford University Press, New York/Edward Arnold, London.
9. **Gomes, A. H., I. M. Ferreira, M. L. Lima, E. A. Cunha, A. S. Garcia, M. F. Araújo, and V. L. Pereira-Chioccola**. 2007. PCR identification of *Leishmania* in diagnosis and control of canine Leishmaniasis. Vet. Parasitol. **144:**234-241.
10. **Gomes, A. H., I. M. Armelin, S. Z. Menon, and V. L. Pereira-Chioccola**. 2008. *Leishmania* (V.) *braziliensis*: Detection by PCR in biopsies from patients with cutaneous leishmaniasis. Exp. Parasitol. in press.
11. **Greiner, M**. & **Gardner, I.A.**. 2000. Epidemiologic issue in the validation of veterinary diagnostic tests. Prev. Vet. Med., **45**:3-22.
12. **Gutierrez, Y., G. H. Salinas, G. Palma, L. B. Valderrama, C. V. Santrich, and N. G. Saravia.** 1991**.** Correlation between histopathology, immune response, clinical presentation, and evolution in *Leishmania braziliensis* infection. Am. J.Trop. Med. Hyg. **45**:281-289.
13. **Harrus, S., I. Aroch, E. Lavy, and H. Bark.** 1997. Clinical manifestations of infectious canine cyclic thrombocytopenia. Vet. Rec. **141:**2247-2250.
14. **Harrus, S., T., Waner, D., Friedmann-Morvinski, Z., Fishman, H., Bark, A., Harmelin.** 2003. Down-regulation of MHC class II receptors of DH82 cells, following infection with *Ehrlichia canis*. Vet. Immunol. Immunopathol. **96**:239-243.
15. **Iniesta, L., S. Fernández-Barredo, B. Bulle, M. T. Gómez, R. Piarroux, M. Gállego, J. M. Alunda, and M. Portús.** 2002. Diagnostic techniques to detect cryptic leishmaniasis in dogs. Clin. Diagn. Lab. Immunol. **9**:1137-1141.
16. **Koehler, K., M. Stechele, U. Hetzel, M. Domingo, G. Schönian, H. Zahner, and E. Burkhardt.** 2002. Cutaneous leishmaniosis in a horse in southern Germany caused by *Leishmania infantum*. Vet. Parasitol. **109**:9-17.

17. **Lanotte, G., J. A. Rioux, J. Periers, and Y. Vollhardt.** 1979. Ecologie des leishmanioses dans le sud de la France. 10. Les formes évolutives de la leishmaniose viscérale canine. Ann. Parasitol. Hum. Commun. **54**:277-295.

18. **Maroli, M., F. Bigliocchi, and C. Khoury.** 1994. Sandflies in Italy: observations on their distribution and methods for control. Parassitologia **36:**251-264.

19. **Maroli, M., L. Rossi, R. Baldelli, G. Capelli, E. Ferroglio, C. Genchi, M. Gramiccia, M. Mortarino, M. Pietrobelli, and L. Gradoni.** 2008. The northward spread of leishmaniasis in Italy: evidence from retrospective andongoing studies on the canine reservoir and phlebotomine vectors. Trop. Med. Int. Health **13**:256-264.

20. **Menard, M., and M. Papageorges**. 1997. Fine-needle biopsies: how to increase diagnostic yield. Comp. Cont. Ed. Pract. Vet. **19**:738–740.

21. **Mettler, M**., **F**. **Grimm, G**. **Capelli, H**. **Camp, and P**. **Deplazes.** 2005. Evaluation of enzyme-linked immunosorbent assays, an immunofluorescent-antibody test, and two rapid tests (immunochromatographic-dipstick and gel tests) for serological diagnosis of symptomatic and asymptomatic Leishmania infections in dogs. J. Clin. Microbiol. **43**:5515-5519.

22. **Michalsky, E. M., M. F. Rocha, A. C. da Rocha Lima, J. C. França-Silva, M. Q. Pires, F. S. Oliveira, R. S. Pacheco, S. L. dos Santos, R. A. Barata, A. J. Romanha, C. L. Fortes-Dias, and E. S. Dias**. 2007. Infectivity of seropositive dogs, showing different clinical forms of leishmaniasis, to *Lutzomyia longipalpis* phlebotomine sand flies. Vet Parasitol. **147**:67-76.

23. Miró, G., L. Cardoso, M. G. Pennini, G. Oliva, and G. Baneth. 2008. Canine leishmaniosis – new concepts and insights on an expanding zoonosis: part two. Trends Parasitol. **24(8)**:371-7

442 **24. Molina, R., C. Amela, J. Nieto, M. San-Andrés, F. González, J. A. Castillo, J.**
443 **Lucientes, and J. Alvar.** 1994**.** Infectivity of dogs naturally infected with *Leishmania*
444 *infantum* to colonized *Phlebotomus perniciosus*. Trans. R .Soc. Trop. Med. Hyg.
445 **88:**491-493.
446 25. **Moreira, M. A., M. C. Luvizotto, J. F. Garcia, C. E. Corbett, and M. D. Laurenti**.
447 2007. Comparison of parasitological, immunological and molecular methods for the
448 diagnosis of leishmaniasis in dogs with different clinical signs. Vet. Parasitol.
449 **145**:245-252.
450 26. **Moreno, J., and J. Alvar** 2002. Canine leishmaniasis: epidemiological risk and the
451 experimental model. Trends Parasitol. **18**:399-405.
452 27. **Oliva, G., A. Scalone, V. Foglia Manzillo, M. Gramiccia, A. Pagano, T. Di**
453 **Muccio, and L. Gradoni**. 2006. Incidence and time course of *Leishmania infantum*
454 infections examined by parasitological, serologic, and nested-PCR techniques in a
455 cohort of naive dogs exposed to three consecutive transmission seasons. J. Clin.
456 Microbiol. **44:**1318-1322.
457 28. **Otranto, D., P. Paradies, M. Sasanelli, N. Leone, D. de Caprariis, J. Chirico, R.**
458 **Spinelli, G. Capelli, and O. Brandonisio.** 2005. Recombinant K39 dipstick
459 immunochromatographic test: a new tool for the serodiagnosis of canine
460 leishmaniasis. J. Vet. Diagn. Invest. **17:**32-37.
461 29. **Otranto, D., P. Paradies, R. P. Lia, M. S. Latrofa, G. Testini, C. Cantacessi, N.**
462 **Mencke, G. Galli, G. Capelli, and D. Stanneck.** 2007. Efficacy of a combination of
463 10% imidacloprid / 50% permethrin for the prevention of leishmaniasis in kennelled
464 dogs in an endemic area. Vet. Parasitol. **144:**270-278.

30. **Paradies, P., G. Capelli, C. Cafarchia, D. de Caprariis, M. T. Sasanelli, and D. Otranto**. 2006. Incidence of canine leishmaniasis in an endemic area of Southern Italy. J. Vet. Med. B Infect. Dis. Vet. Public Health **53:**295-298.

31. **Pinelli, E., V. P. Rutten, M. Bruysters, P. F. Moore, and E. J. Ruitenberg.** 1999. Compensation for decreased expression of B7 molecules on Leishmania infantum-infected canine macrophages results in restoration of parasite-specific T-cell proliferation and gamma interferon production. Infect. Immun. **67**:237-243.

32. **Pozio, E., M. Gramiccia, L. Gradoni, and P. Amerio.** 1985. Isolation of the agent causing cutaneous leishmaniasis in Italy and its visceralization in inbred hamsters. Trans. R. Soc. Trop. Med. Hyg. **79:**260-261.

33. **Reithinger, R., J. C. Espinoza, O. Courtenay, and C. R. Davies.** 2003. Evaluation of PCR as a diagnostic mass-screening tool to detect *Leishmania* (*Viannia*) spp. in domestic dogs (*Canis familiaris*). J. Clin. Microbiol. **41:**1486-1493.

34. **Saridomichelakis, M. N., M. E. Mylonakis, L. S. Leontides, A. F. Koutinas, C. Billinis, and V. I. Kontos**. 2005. Evaluation of lymph node and bone marrow cytology in the diagnosis of canine leishmaniasis (*Leishmania infantum*) in symptomatic and asymptomatic dogs. Am. J. Trop. Med. Hyg. **73**:82-86.

35. **Singh, B.** 1997. Molecular methods for diagnosis and epidemiological studies of parasitic infections. Int. J. Parasitol. **27**:1135-114.

36. **Silva, E. S., C. M. Gontijo, C. Pirmez, O. Fernandes, and R. P. Brazil.** 2001. Short report: detection of Leishmania DNA by polymerase chain reaction on blood samples from dogs with visceral leishmaniasis. Am. J. Trop. Med. Hyg. **65**:896-898.

37. **Solano-Gallego, L., P. Morell, M. Arboix, J. Alberola, and L. Ferrer.** 2001. Prevalence of *Leishmania infantum* Infection in Dogs Living in an Area of Canine

Leishmaniasis Endemicity Using PCR on Several Tissues and Serology. J. Clin. Microbiol. **39**:560–563.

38. **Solano-Gallego, L., H., Fernández-Bellon, P., Morell, D., Fondevila, J., Alberola, A., Ramis, L., Ferrer.** 2004. Histological and immunohistochemical study of clinically normal skin of *Leishmania infantum*-infected dogs.. J. Comp. Pathol. **130**:7-12.

39. **Thrusfield, M**. 2000. Veterinary Epidemiology. Blackwell Science Ltd (ed.), UK.

40. **Trotz-Williams, L. A. and A. J. Trees**. 2003. Systematic review of the distribution of the major vector-borne parasitic infections in dogs and cats in Europe. Vet. Rec. **152**:97-105.

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 -*Leishmania infantum* antibodies	Anti -*Ehrlichia canis* 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 * $p<0.001$

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
	DS neg	**DS pos**	
IFAT neg	750	2	k=0.965 (“excellent”)
IFAT pos	3	76	p<0.001
	ELISA neg	**ELISA pos**	
IFAT neg	692	54	k=0.710 (“good”)
IFAT pos	0	79	p<0.001
	DS neg	**DS pos**	
ELISA neg	694	0	k=0.678 (“good”)
ELISA pos	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

	Leishmania				*Ehrlichia*
	ELISA	**IFAT**	**DS***	**PCR**	**IFAT**
	I (pos/N)	**I (pos/N)**	**I (pos/N)**	**I (pos/N)**	**I (pos/N)**
Prevalence at starting point					
Mar. 05	0% (0/204)	0% (0/204)	0% (0/204)	0% (0/204)	13.7% (28/204)
Intermediate incidence determinations					
Mar. 05 –Nov. 05	5.1% (9/178)	2.0% (4/196)	0% (0/196) 13 doubtful	7.2% (14/195)	20/168 (11.9%)
Nov. 05- Mar.06	2.0% (3/149)	1.1% (2/187)	2.2% (4/179) 4 doubtful	0.6% (1/178)	5/146 (3.4%)
Yearly incidence rates					
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)
Yearly recovery rates (neg'07/pos'06)					
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}
ELISA	33%^{aAB}	95%	50%^{bcAC}	95.5%	9%^{BC}	93%
IFI	14%^{aD}	99%	27%^{bDE}	99%	13%^{E}	92%
DS	-	-	18%^{CF}	99%	4.5%^{F}	100%

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

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
Group A (28), parasitologically positive animals														
15														+
4*				+					+					
3				+					+					+
3				+										
2													+	+
1								+	+					
Group B (18), serologically positive animals														
5												+		
2			+											
2			+				+							
3			+				+			+		+		
1										+				
1			+							+		+		
1							+							
1*			+							+				
1										+		+		
1	+				+	+				+		+		
Group C (9), parasitologically and serologically positive animals														
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**.**