Toward Diagnosing Leishmania infantum Infection in Asymptomatic Dogs in an Area Where Leishmaniasis Is Endemic

Otranto, D; Paradies, P; de Caprariis, D; Stanneck, D; Testini, G; Grimm, F; Deplazes, P; Capelli, G


Postprint available at:
http://www.zora.uzh.ch

Posted at the Zurich Open Repository and Archive, University of Zurich.
http://www.zora.uzh.ch

Originally published at:
Toward Diagnosing Leishmania infantum Infection in Asymptomatic Dogs in an Area Where Leishmaniasis Is Endemic

Abstract

The most frequently used diagnostic methods were compared in a longitudinal survey with Leishmania infantum-infected asymptomatic dogs from an area of Italy where leishmaniasis is endemic. In February and March 2005, 845 asymptomatic dogs were tested by an immunofluorescence antibody test (IFAT), a dipstick assay (DS), and an enzyme-linked immunosorbent assay (ELISA) for L. infantum and by IFAT for Ehrlichia canis. Dogs seronegative for L. infantum were further parasitologically evaluated by microscopic examination of lymph node tissues and PCR of skin samples. A total of 204 animals both serologically and parasitologically negative for L. infantum at the first sampling were enrolled in the trial and were further examined for canine leishmaniasis (CanL) and canine monocytic ehrlichiosis in November 2005 (i.e., the end of the first sandfly season) and March 2006 and 2007 (1- and 2-year follow-ups, respectively). At the initial screening, the overall rates of L. infantum seroprevalence were 9.5% by IFAT, 17.1% by ELISA, and 9.8% by DS and the overall rate of E. canis seroprevalence was 15%. The rates of concordance between the results of IFAT and DS were almost equal, whereas the rate of concordance between the results of IFAT and DS and those of the ELISA was lower. The results of the annual incidence of Leishmania infection were variable, depending on the test employed, with the highest values registered for PCR (i.e., 5.7% and 11.4% at the 1- and 2-year follow-ups, respectively), followed by ELISA, IFAT, and DS. Over the 2 years of observation, 55 animals (i.e., 26.9%) became positive for L. infantum by one or more diagnostic tests at different follow-up times, with 12.7% showing clinical signs related to CanL, while the remaining 87.3% were asymptomatic. A diagnostic scheme for assessment of the L. infantum infection status in asymptomatic dogs is suggested.
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. Capelli⁵

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

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

³Vet practitioner, Koln, Germany

⁴Institute of Parasitology, University of Zurich, Switzerland

⁵Istituto 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 parasitologically 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 assess 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, epistaxis, lymphadenolymphospleno-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)
under their usual housing conditions (29). Animals were handled and sampled with the owners’ consent and approval by the Ethical Committee of the Faculty of Veterinary Medicine of the University of Bari (Italy). Sex, age, weight and coat length were recorded for each dog.

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

In March 2005, 204 animals (i.e. 102 for each kennel) which were both serologically and parasitologically negative for \textit{L. infantum} were enrolled in a longitudinal follow up trial, irrespective of the presence of anti-\textit{E. canis} antibodies. All animals remained untreated with any ecto-parasiticide through the study. They were examined serologically and parasitologically for CanL and CME in November 2005 (i.e. the end of the first sandfly season), in March 2006 (one year follow-up) and in March 2007 (two years follow-up).

Specifically, serological tests for the presence of anti-\textit{Leishmania} antibodies (IFAT, ELISA, DS) and anti-\textit{E. canis} antibodies (IFAT) as well as PCR for the detection of \textit{Leishmania} DNA in dermal tissue samples were performed at all follow up times. Additionally, lymph node smears were examined microscopically in March 2006 and 2007 for the presence of amastigote \textit{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\textsubscript{405}) was above the arithmetic mean plus 3 SD of the A\textsubscript{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).
*E. canis* specific antibodies were detected by IFAT by using slides containing fixed *E. canis* in DH82 cells (Canine Ehrlichiosis FA Substrate Slide, VMRD, Pullmann, Washington, USA, Lot P060228-002032908). The parasitized cells were exposed to sera diluted (1:50) in phosphate buffered saline (pH 7.2) in a moist chamber and, after washing, to fluoresceinated rabbit anti-dog IgG (Rabbit anti-dog IgG; Sigma - Aldrich Chemie, Germany, Lot 125K4752) diluted 1:60; both incubations were done at 37°C for 30 min. Samples scored positive when they produced cytoplasmic inclusion bodies fluorescence at a dilution of 1:50 or higher. All positive sera were further titrated until the reaction became negative.

2.2.2. Parasitological diagnosis of CanL

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

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

After disruption in liquid nitrogen and pestling (i.e. two thaw-freezing cycles), genomic DNA was extracted from about 30 mg of skin samples using a commercial kit (Genomic DNA Purification Kit, Gentra Systems, Minnesota, USA). A *L. infantum* kinetoplastid minicircle DNA fragment was amplified using the MC1/MC2 primer pair (6). Genomic DNA solution (4 µl) was added to the PCR reaction mix (46 µl) containing 2.5 mM MgCl₂, 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 AmpliTaq Gold (Applied Biosystems, Milan, Italy). 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 gels (Ambion, Milan, Italy) upon staining with ethidium bromide.

2.3 Statistical analysis

2.3.1 Sample size

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

2.3.2 Incidence calculation and test performance

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

Concordance among serological tests performed in the initial screening (March 2005) on asymptomatic dogs was evaluated by k statistics (39). Kappa values were ranked as low (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 Everitt (8). At the follow up examinations (November 2005, March 2006 and March 2007),
relative sensitivity ($SE_{rel}$) and relative specificity ($SP_{rel}$) of the serological tests were assessed on the basis of the PCR results obtained at the same sampling time. $Se$ and $Sp$ were calculated according to Greiner and Gardner (11).

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

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

3. RESULTS

3.1 Initial screening

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

Parasites were detected microscopically in stained lymph node smears of 3 (i.e. 0.7%) out of 453 dogs. The above three dogs were positive at serological tests. Concordance between IFAT and DS was almost perfect (kappa =0.965, rating “excellent”), with only five discordant cases, whereas concordance with ELISA was lower (54 discordant cases, kappa =0.71, rating “good”). Test agreement between ELISA and DS was also rated “good” (63 discordant cases, 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 (\(\chi^2\) 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
parasitologically and serologically positive (group C). Out of the 18 serologically positive animals of group B, 9 were positive at the ELISA only.

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

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

4. DISCUSSION

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


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

<table>
<thead>
<tr>
<th>Test</th>
<th>Anti-<em>Leishmania infantum</em> antibodies</th>
<th>Anti-<em>Ehrlichia canis</em> antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L-pos dogs</td>
<td>L-neg dogs</td>
</tr>
<tr>
<td>IFAT</td>
<td>79/831 (9.5%)</td>
<td>14/79 (17.7%)</td>
</tr>
<tr>
<td>ELISA</td>
<td>143/837 (17.1%)</td>
<td>38/143 (26.6%)*</td>
</tr>
<tr>
<td>DS</td>
<td>83/845 (9.8%)</td>
<td>14/83 (16.9%)</td>
</tr>
</tbody>
</table>

* p<0.001
TABLE 2. Measure of agreement (kappa values) among results (expressed as negative -neg- and positive -pos) of IFAT, ELISA, dipstick (DS) *Leishmania* tests calculated on 845 dogs at the study starting point.

<table>
<thead>
<tr>
<th>Tests and results</th>
<th>Kappa value (rank) and significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DS neg</td>
</tr>
<tr>
<td>IFAT neg</td>
<td>750</td>
</tr>
<tr>
<td>IFAT pos</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>ELISA neg</td>
</tr>
<tr>
<td>IFAT neg</td>
<td>692</td>
</tr>
<tr>
<td>IFAT pos</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>DS neg</td>
</tr>
<tr>
<td>ELISA neg</td>
<td>694</td>
</tr>
<tr>
<td>ELISA pos</td>
<td>63</td>
</tr>
</tbody>
</table>
TABLE 3. Prevalence at starting point and incidence rates (I) of *Leishmania infantum* and *Ehrlichia canis* infection calculated for each diagnostic technique. Dogs previously positive at the same test were excluded from calculation.

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

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

<table>
<thead>
<tr>
<th></th>
<th>November 2005</th>
<th>March 2006</th>
<th>March 2007</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$SE_{rel}$</td>
<td>$SP_{rel}$</td>
<td>$SE_{rel}$</td>
</tr>
<tr>
<td>ELISA</td>
<td>33% $^{aAB}$</td>
<td>95%</td>
<td>50% $^{bAC}$</td>
</tr>
<tr>
<td>IFI</td>
<td>14% $^{aD}$</td>
<td>99%</td>
<td>27% $^{bDE}$</td>
</tr>
<tr>
<td>DS</td>
<td>-</td>
<td>-</td>
<td>18% $^{CP}$</td>
</tr>
</tbody>
</table>

Equal letters correspond to significant difference for $p<0.01$.
TABLE 5. Diagnostic follow up of the 55 dogs resulted positive for *Leishmania infantum* infection at one or more tests

<table>
<thead>
<tr>
<th>No. of dogs</th>
<th>November 05</th>
<th>March 06</th>
<th>March 07</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFAT  DS  ELISA  PCR</td>
<td>IFAT  DS  ELISA  Mic</td>
<td>IFAT  DS  ELISA  Mic</td>
</tr>
<tr>
<td>Group A (28), parasitologically positive animals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4*</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Group B (18), serologically positive animals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Group C (9), parasitologically and serologically positive animals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1*</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1*</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
| *Single animals which showed symptoms of leishmaniasis (i.e. dermatitis, lymphadenopathy, conjunctivitis, skin ulcers) and/or hypergammaglobulinemia in each line; d, dead animals.