

Diagnostic Value of Conjunctival Swab Sampling Associated with Nested PCR for Different Categories of Dogs Naturally Exposed to *Leishmania infantum* Infection

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The objective of the present study was to evaluate the diagnostic performance of a noninvasive assay, conjunctival swab (CS) nested-PCR (n-PCR), for diagnosing canine leishmaniasis (CanL) in different stages of infection in comparison to the performance of the indirect immunofluorescence antibody test (IFAT), lymph node microscopy, and buffy coat n-PCR. To this end, we performed a cross-sectional survey among 253 nonselected dogs in areas of endemicity in central Italy. We also performed a longitudinal study of CS n-PCR among 20 sick dogs undergoing antileishmanial treatment. In the first study, among the 72 animals that were positive by at least one test (28.45%), CS n-PCR showed the best relative performance (76.38%), with a high concordance in comparison to standard IFAT serology ($\kappa = 0.75$). The highest positivity rates using CS n-PCR were found in asymptomatic infected dogs (84.2%) and sick dogs (77.8%); however, the sensitivity of the assay was not associated with the presence of clinical signs. In the follow-up study on treated sick dogs, CS n-PCR was the most sensitive assay, with promising prognostic value for relapses. The univariate analysis of risk factors for CanL based on CS n-PCR findings showed a significant correlation with age ($P = 0.012$), breed size ($P = 0.026$), habitat ($P = 4.9 \times 10^{-4}$), and previous therapy ($P = 0.014$). Overall, the results indicated that CS n-PCR was the most sensitive assay of the less invasive diagnostic methods and could represent a good option for the early and simple diagnosis of CanL infection in asymptomatic animals and for monitoring relapses in drug-treated dogs.

Zoonotic visceral leishmaniasis, which is caused by the protozoan parasite *Leishmania infantum* (= *L. chagasi*), is a sand fly-borne disease which is endemic in the Mediterranean area, Asia, and Latin America (16). In most of this range, the domestic dog is the main reservoir host, and it plays a central role in *L. infantum* transmission to humans by sand fly bites. Canine leishmaniasis (CanL) is a major veterinary and public-health problem not only in areas of endemicity but also in areas where it is not endemic, such as northern Europe, the United States, and Canada, where individual clinical cases or outbreaks of disease are occasionally reported (41, 42, 46). However, there is much evidence that the burden of CanL has been underestimated. After infection, while some dogs show progressive disease, others can control the parasite and do not develop the disease in the short term, in some cases for years or even for life. The presence of latent infections in dogs contributes to maintaining the long-term transmission of the parasite in regions of endemicity. Disease management represents a serious problem, since both symptomatic and asymptomatic dogs can be infectious to phlebotomine vectors (26, 28), and the available antileishmanial drugs have limited efficacy in dogs. Furthermore, although progress has been made in CanL vaccination in the past decade, an effective *Leishmania* vaccine against CanL infection is not available worldwide (20, 34).

As in human leishmaniasis, the accepted standards for diagnosing CanL include serological tests and the microscopic demonstration of parasites in tissue smears or cultures from spleen, lymph node, or bone marrow aspirates. However, the sensitivity of these methods varies depending on the infection stage and the individual immune response. Molecular assays have greatly improved the sensitivity of the standard assays (4). For CanL diag-

nosis, PCR can be performed on samples from a broad range of tissues, including bone marrow, lymph node, buffy coat (BC), whole blood, and skin, with various degrees of sensitivity (22). Despite the fact that invasive samples, such as bone marrow and spleen aspirates, have very high sensitivity, less invasive samples are assuming greater importance because they are easier to obtain and more acceptable to dog owners. In fact, CanL monitoring in asymptomatic dogs exposed to *Leishmania* transmission or sick animals treated for leishmaniasis may require frequent samplings. Furthermore, less invasive samplings may be useful in experimental investigations on canine vaccines, drugs, or topical insecticides, which entail periodic assessments to evaluate their effectiveness.

Conjunctival swab (CS) sampling associated with a sensitive and specific PCR assay was tested for CanL diagnosis in experimentally infected (48), symptomatic untreated (11), and naturally *Leishmania*-exposed dogs (17, 19, 21), and it was found to have a high sensitivity. These findings suggested that careful evaluation of the diagnostic performance of CS nested (n)-PCR analysis was warranted.

To this end, we conducted a cross-sectional study to compare

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CS n-PCR analysis to indirect immunofluorescence antibody test (IFAT) and other less invasive procedures, particularly lymph node cytological examination (LN-CE) and n-PCR on peripheral blood BC, among a sample of dogs with different stages of CanL (35). We also conducted a longitudinal study to evaluate the sensitivity of CS n-PCR in naturally infected dogs that were treated for CanL and followed for 6 months. Finally, we determined whether the effectiveness of CS n-PCR varied by risk factor for CanL in the study area.

MATERIALS AND METHODS

Canine population. A total of 273 dogs from four regions of central Italy were enrolled in two studies. The collection of biological samples from dogs was performed in accordance with the national guidelines for animal welfare.

Cross-sectional survey. The study sample for the cross-sectional study consisted of all 253 dogs which from November 2008 through September 2009 were seen at the Veterinary Hospital of the University of Perugia or private veterinary clinics or which were housed in kennels, regardless of the presence of clinical conditions. This sample represented approximately 1/1,000 of the estimated total canine population (222,500 dogs) in the investigated areas, which have recently been estimated to be at medium-high risk (estimated prevalence of 10 to 20%) or high risk (20 to 30% estimated prevalence) for CanL (12). Specifically, the dogs consisted of 63 strays (housed in kennels for less than 6 months) (24.9%), 66 kennel dogs (housed in kennels for more than 6 months) (26.09%), and 124 privately owned dogs (49.01%) of both genders and different ages. The dog owners and kennel managers were asked to complete an individual standardized questionnaire developed by the FP6 integrated project of the European Union, Emerging Diseases in a changing European environment (EDEN), which includes items on the main CanL risk factors of gender, age (1 to 15, <3, 3 to 8, and >8 years), breed size (small, medium, and large), *Leishmania* exposure in terms of occupation, habitat, and location during the day and at night, use of topical insecticides against sand flies, presence of CanL-seropositive dogs in the household, and past therapy for CanL.

Longitudinal study. To evaluate the sensitivity of CS n-PCR in naturally infected dogs that were undergoing treatment for CanL, we performed a longitudinal study on an additional 20 dogs enrolled in the same facilities as the cross-sectional study. These dogs had been diagnosed with CanL based on clinical signs, high *Leishmania* antibody titers (IFAT \geq 1:160), and at least one positive result for parasitologic tests. All dogs had private owners. They were of different ages and breeds (3 mongrels and 17 guard or hunting breeds). They were submitted to pharmacological treatment using 2 different protocols: (i) 8 animals received a combination of meglumine antimoniate subcutaneously (100 mg/kg of body weight/day) and allopurinol (10 mg/kg/day) orally, for 60 days, followed by allopurinol alone for 4 months; and (ii) 12 dogs were treated orally with a combination of miltefosine (2 mg/kg/day) and allopurinol (10 mg/kg/day) for 28 days, followed by allopurinol alone for 6 months. With the consent of the owners, all of the dogs were examined at the onset of treatment (T_0) and reexamined at 3 (T_3) and 6 months (T_6) of treatment. Of the 20 dogs, 18 were enrolled in October 2008, so that the 6-month follow-up was completed in April 2009; the other 2 dogs were enrolled in March 2009, with follow-up completed in August 2009. Considering that the season of *Leishmania* transmission in Italy lasts from May to October (17), only the latter 2 dogs could have been reinfected during follow-up.

Clinical examination. All dogs were subjected to external clinical inspection. Furthermore, privately owned dogs were subjected to basic tests, including complete blood count, serum biochemical analysis, serum protein electrophoresis, and urinalysis. The dogs were classified based on whether or not they had at least one of the most common CanL signs, which, however, are largely aspecific and may be common for other canine diseases: weight loss, lymph node enlargement, hepatosplenomegaly,

skin alterations (exfoliative dermatitis, ulcers, and alopecia), and renal failure (22).

Canine samples. Peripheral blood (5 ml) was obtained from the jugular vein and equally distributed into EDTA-coated tubes and tubes without EDTA for BC and serum testing, respectively. The latter was performed within 24 h of sample collection at room temperature; both the sera and the BC samples were stored at -20°C until analysis.

Exfoliative epithelial cells were collected from the right and left conjunctiva of each animal using sterile cotton swabs manufactured for bacteriological isolation. The swabs were rubbed robustly back and forth once in the lower conjunctival sac. They were then immersed in 2 ml of sterile saline in 20-ml plastic tubes and stored at 4°C for 24 h. After the manual stirring of swabs, the saline containing the eluted exfoliating cells was transferred into sterile vials pending DNA extraction (17). Samples from the right and left eyelid conjunctivas were processed separately.

Popliteal lymph node tissue was sampled using needle aspiration and submitted to cytological examination (LN-CE); tissue smears were stained with Diff Quick (Medical Team Srl, Pescara, Italy) and microscopically examined for the presence of *Leishmania* amastigotes.

IFAT. The presence of anti-*Leishmania* antibodies (IgG) was investigated by IFAT, following the standard procedures recommended by the Office International des Epizooties (14). Promastigotes of *L. infantum* zymodeme MON-1 (MHOM/TN/80/IPT-1) were used as the antigen, while rabbit anti-dog IgG (Sigma-Aldrich Chemical, Germany) diluted 1:40 was used as the conjugate. Serial dilutions were performed. Positive titers were recorded from 1:80 to 1:1,280 or above. For the diagnosis of CanL, a cutoff of 1:160 was used (25, 32). However, in classifying the disease into stages (35), a cutoff of 1:80 was used to define the low-titer "exposed" dogs. In the longitudinal study, we also considered antibody titers below 1:160.

DNA extraction and n-PCR. BC samples were submitted to DNA extraction using the Easy-DNA kit (Invitrogen, San Diego, CA), following the manufacturer's instructions. The DNA samples were stored at -20°C until PCR assay.

The samples containing eluted conjunctival cells were centrifuged at $6,000 \times g$ for 10 min at 4°C , and the pellets were resuspended in 90 μl of lysis buffer plus 10 μl of 2 mg/ml proteinase K. After 2 h of incubation at 56°C , the proteinase K was inactivated at 95°C for 10 min; the samples were then centrifuged at $13,000 \times g$ for 10 min. Supernatants were collected in 1.5-ml vials and stored at -20°C pending PCR assay (17).

Total genomic DNA was submitted to n-PCR assay according to the method of Oliva et al. (32). Briefly, the first amplification was performed with the kinetoplastid-specific primers R221 and R332 of the small-subunit rRNA gene. For the second amplification, the *Leishmania*-specific primers R223 and R333 of the same gene were used (49). Three negative controls (no DNA and BC and CS DNA from a healthy dog) and one positive control (DNA from *L. infantum*-cultured promastigotes) were used. Amplification products were analyzed on 1.2% agarose gel and visualized under UV light. Positive samples yielded a predicted n-PCR product of 358 bp. Contamination of amplicons was avoided by using physical separation (rooms and materials), as well as decontamination procedures (UV exposure and bleaching of materials and surfaces). To exclude false-negative results due to scarce sample material, low DNA extraction efficiency, or the presence of PCR inhibitors, a random subset (approximately 10% of the total) of canine DNA samples that were n-PCR negative were submitted to conventional PCR for amplification of the 181-bp fragment of the canine glyceraldehyde-3-phosphate dehydrogenase gene, using primers and conditions reported by Ramiro et al. (38).

Dog classification. The seropositive dogs were classified according to the guidelines of Paltrinieri et al. (35) as "exposed" (E, low IFAT titer plus negative cytology and negative PCR assay on relevant tissues), "infected" (I, low IFAT titer plus positive cytology and/or positive PCR assay on relevant tissues and without clinical signs), or "sick" (S, high IFAT titer plus a positive cytological examination and with at least one clinical sign).

Rate calculation and statistical analysis. The cross-sectional prevalence of CanL was calculated as the rate of animals positive by at least one test. The relative performance of the BC n-PCR, CS n-PCR (combining results from both conjunctivas), IFAT, and LN-CE assays was calculated as the rate of animals detected by each test out of all positive animals. The performance rates were then stratified by CanL stage as defined by Paltrinieri et al. (35) and by the presence/absence of clinical signs. The concordance between IFAT, assumed as the golden standard (14), and the other tests was determined using Cohen's kappa coefficient (κ); a κ value of 0.21 to 0.60 represents fair to moderate agreement, a κ value of 0.60 to 0.80 represents substantial agreement, and a κ value of >0.80 represents almost perfect agreement (10).

The explanatory variables regarding the dogs' characteristics and exposure to risk factors (Table 1) were used to build a logistic regression model in order to explore their effects on CS n-PCR findings and to evaluate the existence of possible interactions. The logistic model was built by adopting a stepwise forward procedure, starting from the null model and based on the minimization of the Akaike information criterion (AIC) (3). In particular, variables/interactions were added to the model if they had a positive impact on the AIC. The models were built using the glm and stepAIC functions of the R statistical environment (50, 37). Back-transformed odd ratios (OR) were derived from the estimated model parameters and are reported in the tables, together with back-transformed 95% confidence limits. Statistical analyses were performed with the statistics package SPSS, version 13.0 for Windows, and Epi Info software, version 6.04d.

RESULTS

Cross-sectional survey. The main characteristics of the canine population examined in the cross-sectional survey are shown in Table 1. The rates of *Leishmania* infection found among the 253 dogs were very similar when comparing CS n-PCR (21.73%; 95% confidence interval [CI], 16.8 to 27.3) to IFAT (at titers of 1:160 or above) (21.34%; 95% CI, 16.6 to 26.9), and these rates were much higher than those for LN-CE (14.22%; 95% CI, 10.2 to 19.2) and BC n-PCR (8.69%; 95% CI, 5.5 to 12.9). Specifically, 55 dogs were positive by CS n-PCR, 54 by IFAT, 36 by LN-CE, and 22 by BC n-PCR. Seventy-two dogs (28.45%; 95% CI, 23.0 to 34.4) were positive by at least one test and were thus considered CanL positive, whereas the remaining 181 dogs (71.54%; 95% CI, 65.6 to 77) were negative to all tests and classified as negative. The relative performance of the tests in the 72 CanL-positive animals was 76.38% for CS n-PCR, 75.0% for IFAT, 50.0% for LN-CE, and 30.55% for BC n-PCR (Table 2). Only 12 dogs were found to have been positive by all tests (16.66%). Fifteen animals (20.83%) were positive by CS n-PCR only, 7 (9.72%) by IFAT only, and 2 (2.77%) by BC n-PCR only. The other 36 animals were positive to different combinations of tests.

These results were analyzed for their concordance with the IFAT results. As reported in Table 3, 38 discordant results were found with BC n-PCR ($\kappa = 0.70$), 31 with CS n-PCR ($\kappa = 0.75$), and 18 with LN-CE ($\kappa = 0.76$).

Test correlation with infection and clinical staging. The positivity rates for each test were analyzed after CanL staging in the 54 IFAT-seropositive dogs. Seven dogs were classified as E (IFAT range, 1:80 to 1:160), 38 as I (IFAT range, 1:320 to 1:640), and 9 as S (IFAT, $\geq 1:1,280$). As shown in Table 4, CS n-PCR showed the highest positivity rate in the I group (84.2%, 32/38 dogs) when compared to the E and S groups; this rate was significantly different from the rates in the I group for BC n-PCR (42.1%, 16/38 dogs) and LN-CE (71.1%, 27/38 dogs) ($P < 0.05$). CS n-PCR positivity was also high in the S group (77.8%, 7/9 dogs). More-

TABLE 1 Individual characteristics and behavioral/environmental data on dogs included in the cross-sectional survey

| Variable | No. (%) of dogs |
|---|-----------------|
| Region | |
| Umbria | 203 (80.24) |
| Marche | 27 (10.67) |
| Toscana | 17 (6.72) |
| Lazio | 6 (2.37) |
| Gender | |
| Male | 127 (50.20) |
| Female | 126 (49.80) |
| Age (yr) | |
| <3 | 97 (38.34) |
| 3–8 | 116 (45.85) |
| >8 | 40 (15.81) |
| Breed size | |
| Small (<15 kg) | 69 (27.27) |
| Medium (15–25 kg) | 151 (59.68) |
| Large (>25 kg) | 33 (13.05) |
| Origin | |
| Kennel | 66 (26.09) |
| Privately owned | 124 (49.01) |
| Stray | 63 (25.00) |
| Occupation of privately owned dogs | |
| Hunting dog | 52 (41.94) |
| Pet | 67 (54.03) |
| Guard dog | 5 (4.03) |
| Location during daytime | |
| Outdoors | 99 (39.13) |
| Indoors | 25 (9.88) |
| Kennel | 129 (50.99) |
| Use of topical insecticides against sand flies ^a | |
| Yes | 73 (38.42) |
| No | 117 (61.58) |
| Habitat | |
| Periurban | 101 (39.92) |
| Rural | 132 (52.17) |
| Urban | 20 (7.91) |
| Living with CanL-positive dogs in the household | |
| Yes | 102 (40.32) |
| No | 151 (59.68) |
| Location during night time | |
| Indoors | 82 (32.41) |
| Outdoors | 171 (67.59) |
| Previous therapy for CanL | |
| Yes | 13 (5.14) |
| No | 240 (94.86) |

^a Privately owned dogs plus kennel dogs ($n = 190$ animals).

over, 18 dogs were negative by IFAT and LN-CE and thus not categorized according to the guidelines of Paltrinieri et al. (35): of these dogs, 16 were positive by CS n-PCR and 3 by BC n-PCR (1 animal was positive to both); these dogs were categorized as “n-PCR positives in otherwise negative dogs.”

TABLE 2 Comparative performance of four tests in 72 dogs shown to be positive for CanL diagnosis by at least one test^a

| No. of dogs (%, 95% CI) | CS n-PCR | IFAT | LN-CE | BC n-PCR |
|----------------------------|--------------------------|-------------------------|-------------------------|--------------------------|
| 12 (16.66, 8.9–27.3) | + | + | + | + |
| 18 (25.0, 15.5–36.6) | + | + | + | – |
| 3 (4.16, 0.9–11.7) | + | + | – | + |
| 2 (2.77, 0.3–9.7) | – | + | + | + |
| 6 (8.33, 3.1–17.3) | + | + | – | – |
| 4 (5.55, 1.5–13.6) | – | + | + | – |
| 2 (2.77, 0.3–9.7) | – | + | – | + |
| 1 (1.38, 0.1–7.5) | + | – | – | + |
| 15 (20.83, 12.2–32.0) | + | – | – | – |
| 7 (9.72, 4.0–19.0) | – | + | – | – |
| 2 (2.77, 0.3–9.7) | – | – | – | + |
| Total (<i>n</i> = 72) | 55 (76.38, 64.9–85.6) | 54 (75.0, 63.4–84.5) | 36 (50.0, 38.0–62.0) | 22 (30.55, 20.2–42.5) |

^a CI, confidence interval; CS n-PCR, n-PCR on conjunctival swab sample; IFAT, indirect immunofluorescence antibody test; LN-CE, cytological examination of lymph node aspirate; BC n-PCR, n-PCR on buffy coat from peripheral blood; +, dogs were positive; –, dogs were negative.

These findings were also analyzed in relation to the presence or absence of clinical signs, which varied in terms of severity from slight lymph node enlargement to diffuse alopecia or severe weight loss, which could be referable to CanL. As shown in Table 5, 53 dogs (73.6%) showed clinical signs and 19 (26.4%) did not. In the dogs with clinical signs, IFAT represented the most sensitive test (92.45%), followed by CS n-PCR (75.5%), LN-CE (66.03%), and BC n-PCR (35.84%). For the dogs without clinical signs, CS n-PCR was the most sensitive test (78.9%).

Analysis of risk factors for CanL. The univariate analysis of risk factors based on CS n-PCR findings (Table 6) showed significant correlations between test positivity and age ($P = 0.012$), breed size ($P = 0.026$), habitat ($P = 4.9 \times 10^{-4}$), and previous therapy ($P = 0.017$). In particular, the positivity rate was higher for medium (OR = 1.86) and large breeds (OR = 3.81) than for small breeds, for dogs between 3 and 8 years of age (OR = 2.09) than for younger dogs (reference category) and older dogs (OR = 0.56), for dogs in periurban areas (OR = 3.43) than for those in urban areas (OR = 1.19) and rural areas (reference category), and for dogs that had received previous therapy for CanL (OR = 3.62) than for those not treated (reference category). None of the other variables showed significant associations.

In the final multivariate logistic regression model, age ($P = 0.016$) and habitat ($P = 5.4 \times 10^{-4}$) remained significantly associated with CS n-PCR positivity (Table 6), whereas breed size and previous therapy did not, given their strong association with other variables already in the model. Dogs between the ages of 3 and 8 years had a 2.41-fold greater risk of having a positive CS n-PCR than younger dogs (reference group) and older dogs. Dogs living in periurban areas had a 3.49-fold greater risk than dogs in rural areas (reference group) and urban areas.

Longitudinal study. The individual test results and the clinical assessment of the 20 dogs that were included in the posttreatment follow-up study are reported in Table 7, and the cumulative results at each time point are summarized in Table 8. At T_0 , all 20 dogs were IFAT positive, according to the study design (endpoint titer range, 1:160 to 1:2,560); all 20 dogs were also CS n-PCR positive, whereas 17 tested positive by LN-CE and 9 by BC n-PCR. At T_3 , both therapy protocols promoted a marked improvement, with total remission of clinical signs, a decrease in anti-*Leishmania* antibody titers (1:80 to 1:320), and a reduction in the positivity

TABLE 3 Comparison of IFAT and CS n-PCR, BC n-PCR, and LN-CE in the diagnosis of CanL^a

| Test | Result | IFAT (cutoff, 1:160) | | % LR (95% CI) | | κ value |
|----------|----------|-------------------------|----------|----------------|------------------|-------------------|
| | | Positive | Negative | Positive | Negative | |
| CS n-PCR | Positive | 39 | 16 | 71.6 (61.1–80) | 92.2 (89.2–94.6) | 0.75 |
| | Negative | 15 | 183 | | | |
| BC n-PCR | Positive | 19 | 3 | 50 (34.1–65.9) | 91.2 (87.4–95) | 0.70 |
| | Negative | 35 | 196 | | | |
| LN-CE | Positive | 36 | 0 | 80 (68.3–91.7) | 95.7 (92.9–98.4) | 0.76 |
| | Negative | 18 | 199 | | | |

^a IFAT, indirect immunofluorescence antibody test; LR, likelihood ratio; CI, confidence interval; CS n-PCR, n-PCR on conjunctival swab; BC n-PCR, n-PCR on buffy coat; LN-CE, cytological examination of lymph node aspirate.

rates for CS n-PCR ($T_3 = 30\%$ versus $T_0 = 100\%$), BC n-PCR ($T_3 = 5\%$ versus $T_0 = 45\%$), and LN-CE ($T_3 = 10\%$ versus $T_0 = 85\%$). At T_6 , among the 18 dogs (2 were lost to follow-up), there was an increase in the positivity rate for BC n-PCR (44.44%) and CS n-PCR (88.89%), without the concomitant reappearance of clinical signs and/or an increase in serological titers, compared to the positivity rate at T_3 ; there was a less marked increase in LN-CE positivity (22.22%). No significant differences were observed between dogs treated with the two drug regimens.

DISCUSSION

A number of recent studies have established that CS can be useful for CanL diagnosis because it is both sensitive and noninvasive. We evaluated CS n-PCR as a diagnostic marker at different stages of infection in a heterogeneous canine population living in areas of endemicity and in dogs undergoing treatment for CanL. With regard to the study area, most of the dogs (93.67%) came from two regions of central Italy (Umbria and Marche). These areas have been traditionally assumed to have a moderately prevalent or unstable endemicity status. However, although several CanL serosurveys have been performed in these areas (7, 8, 9, 24, 30), the limited extent of the foci investigated and the low numbers of dogs examined have not allowed the current endemic status to be reliably determined. Very recently, a CanL seroprevalence prediction map carried out in three European regions (12) attributed to our study area a score of stable endemic focus (predicted range of seroprevalence, 10 to 30%), which is consistent with the 21.34% seroprevalence rate observed using IFAT in the present study. This rate is similar to the rates detected in neighboring regions of central and southern Italy, where CanL has traditionally been considered to be highly endemic (15, 23).

The CanL positivity rate using CS n-PCR (21.73%) fits nicely with the rate found in the same canine population using IFAT (21.34%), and it was found to have been more sensitive than cytology (14.22%) and n-PCR of peripheral blood (8.69%). These results demonstrate that CS sampling can play an important role in epidemiological surveys. When considering the rate of positivity to at least one test, the positivity rate increased to 28.45%, confirming that serology alone performed in a nonselected canine population underestimates the prevalence of infection (21).

The concordance between parasitological and molecular assays and standard IFAT serology differed depending on the specific method (Table 3). Of the 54 IFAT-positive dogs, many were found to have been negative by BC n-PCR ($n = 35$) or LN-CE ($n = 18$),

TABLE 4 Positivity rate using CS n-PCR, by stage of CanL infection^a

| Test ^b | No. of dogs (% , 95% CI) ^c | | | |
|-------------------|---------------------------------------|-----------------|----------------------|---------------------|
| | Negative | E | I | S |
| IFAT ^d | 0 (0, 0–18.5) | 7 (100, 59–100) | 38 (100, 90.7–100) | 9 (100, 66.4–100) |
| LN-CE | 0 (0, 0–18.5) | 0 (0, 0–41) | 27 (71.1, 54.1–84.6) | 9 (100, 66.4–100) |
| BC n-PCR | 3 (16.7, 3.6–41.4) ^e | 0 (0, 0–41) | 16 (42.1, 26.3–59.2) | 3 (33.3, 15.6–48.7) |
| CS n-PCR | 16 (88.9, 65.3–98.6) ^e | 0 (0, 0–41) | 32 (84.2, 68.7–94.0) | 7 (77.8, 40.0–97.2) |
| | 18 | 7 | 38 | 9 |

^a Stages of CanL infection were determined as proposed by Paltrinieri et al. (35). Note that according to these authors, attribution to various CanL stages does not include CS examination results.

^b IFAT, indirect immunofluorescence antibody test; LN-CE, cytological examination of lymph node aspirate; BC n-PCR, n-PCR on buffy coat from peripheral blood; CS n-PCR, n-PCR on conjunctival swab.

^c CI, confidence interval; E, exposed (low IFAT titer plus negative cytology and negative PCR on relevant tissue); I, infected (low IFAT titer plus positive cytology and/or positive PCR on relevant tissue and without clinical signs); S, sick (high IFAT titer plus positive cytology with at least one clinical sign).

^d IFAT titer ranges were 1:80 to 1:160 for exposed dogs; 1:320 to 1:640 for infected dogs; and \geq 1:1,280 for sick dogs.

^e n-PCR positives in otherwise negative dogs.

suggesting that these assays have a low sensitivity. However, their specificity was high, with few samples found to have been positive among the 199 IFAT-negative dogs. In contrast, CS n-PCR showed good concordance. Regarding discordance with IFAT, the number of seronegative dogs detected as positive by CS n-PCR ($n = 16$) was similar to the number of seropositive dogs detected as negative by CS n-PCR ($n = 15$). This finding probably reflects the inherent limits of both tests in detecting different stages of infection, suggesting that they do not have the same diagnostic value. Regarding concordant positive results from multiple tests, the highest proportion of animals (25.0%) was found to have been simultaneously positive by IFAT, CS n-PCR, and CE, whereas the lowest percentage (2.77%) was found for IFAT combined with LN-CE and/or BC n-PCR.

The poor results obtained with BC n-PCR confirm that this test cannot be considered for mass screening for CanL (19, 40, 48), for several reasons. First, as reported in other studies, we had problems with DNA preparation and difficulties because of the presence of PCR inhibitors in blood samples (18, 31, 39, 45), and second, the parasite load in peripheral blood is inconsistent over the course of infection, with positive findings mainly associated with periods of vector transmission (17). With regard to LN-CE, this is a rapid and simple assay, yet it has a notoriously low sensitivity, particularly among asymptomatic dogs, and is thus not recommended for mass screening (22).

When considering the positivity rates among the 72 dogs that were positive by at least one test, the positivity rate for CS n-PCR (76.38%, the highest of all assays used) was similar to the rates observed in other studies using different PCR techniques or considering CS results for the right and left eyes separately. In infected asymptomatic dogs, Pilatti et al. (36) reported a positivity rate between 73.9% and 95.6% and Gramiccia et al. (17) found a rate of 80%. Other studies reported higher rates; in particular, Strauss-

Ayali et al. (48) reported a rate of 92%, and Ferreira et al. (11) reported 91.7%, but these studies were performed on, respectively, experimentally infected dogs and symptomatic infected dogs. A considerably lower rate for CS-PCR (43%) in a sample of seropositive dogs was reported recently by Lombardo et al. (21).

To evaluate the performance of CS as a diagnostic tool in different stages of CanL, we analyzed the results for three different categories of dogs (exposed, infected, and sick) (35). However, n-PCR alone was able to identify an additional group of dogs that were IFAT negative and, thus, were defined as n-PCR positives in otherwise negative dogs. This situation has been reported in previous studies, although no detailed analyses were conducted (17, 19, 21, 32). In our study, only 7 dogs were classified as exposed and 9 as sick. The largest CanL category was represented by infected dogs ($n = 38$), followed by n-PCR positives in otherwise negative dogs ($n = 18$), of which 16 (88.9%) were positive by CS n-PCR. With regard to the latter group, we can suppose that CS may reveal a parasite contact followed by one of three patterns of infection evolution: (i) establishment of a patent condition with seroconversion (infected dogs); (ii) a status of a prolonged subpatent condition (6, 32) with various antibody-mediated immune responses, depending on individual immune reactivity, or (iii) transient, self-resolving infection, leading to the parameters observed in exposed dogs (low antibody titers). Although it was not possible to verify this hypothesis because the dogs were not followed further, CS could provide an early marker in these patterns of evolution, before the conversion of other tissues, such as lymph node, peripheral blood, and serum. In fact, in a previous study on experimentally infected dogs, CS-PCR was found to have been positive at 45 days postinfection, before seroconversion (48). Apart from two dogs, all 16 CS n-PCR positives in otherwise negative dogs were sampled at the end of the transmission season; these dogs were younger than 5 years of age, and 7 of them had not yet lived

TABLE 5 Positivity rate using serologic and parasitological assays in CanL-positive dogs, by presence/absence of clinical signs

| Clinical signs (no. of dogs) | No. of positive dogs (% , 95% CI) ^a | | | |
|------------------------------|--|----------------------|-----------------------|-----------------------|
| | IFAT | CS n-PCR | BC n-PCR | LN-CE |
| Present (53) | 49 (92.45, 81.8–97.9) | 40 (75.5, 61.7–86.2) | 19 (35.84, 23.1–50.2) | 35 (66.03, 49.8–76.9) |
| Absent (19) | 5 (26.31, 9.1–51.2) | 15 (78.9, 54.4–93.9) | 3 (15.78, 3.4–39.6) | 1 (5.26, 1–26) |

^a CI, confidence interval; IFAT, indirect immunofluorescence antibody test; CS n-PCR, n-PCR on conjunctival swab; BC n-PCR, n-PCR on buffy coat; LN-CE, cytological examination of lymph node aspirate.

TABLE 6 Risk factors associated with CS n-PCR positivity in univariate analysis and final model^a

| Variable, category | Univariate analysis | | | | Final model | |
|---|-----------------------------------|-------------------------|---------|-------------|-------------------------|------------------|
| | % (no.) of CS n-PCR-positive dogs | P value | OR | 95% CI | P value | OR (95% CI) |
| Gender | | | | | | |
| Male | 25.19 (32) | 0.23 | 1 (Ref) | 0.35–1.32 | | |
| Female | 18.25 (23) | | 0.69 | | | |
| Age (in years) | | | | | | |
| <3 | 17.52 (17) | 0.012 ^d | 1 (Ref) | 0.71–4.89 | 0.016 | 1 (Ref) |
| 3–8 | 29.31 (34) | | 2.09 | | | 2.41 (1.74–4.71) |
| >8 | 10 (4) | | 0.56 | | | 0.66 (0.20–2.18) |
| Breed size | | | | | | |
| Small (<15 kg) | 14.49 (10) | 0.026 ^d | 1 (Ref) | 0.84–4.15 | | |
| Medium (15–25 kg) | 21.85 (33) | | 1.86 | | | |
| Large (>25 kg) | 36.36 (12) | | 3.81 | | | |
| Origin | | | | | | |
| Kennel | 12.12 (8) | 0.07 | 1 (Ref) | (1.25–4.83) | | |
| Privately owned | 26.61 (33) | | 2.72 | | | |
| Stray | 22.22 (14) | | 2.07 | | | |
| Occupation of privately owned dogs ^b | | | | | | |
| Hunting dog | 25 (13) | 0.22 | 1 (Ref) | (0.58–2.86) | | |
| Pet | 25.37 (17) | | 1.02 | | | |
| Guard dog | 60 (3) | | 4.5 | | | |
| Location during daytime | | | | | | |
| Outdoors | 28.28 (28) | 0.12 | 1 (Ref) | (0.15–1.29) | | |
| Indoors | 20 (5) | | 0.63 | | | |
| Kennel | 17.05 (22) | | 0.52 | | | |
| Use of topical insecticides against sand flies ^c | | | | | | |
| No | 18.8 (22) | 0.24 | 1 (Ref) | (1.07–3.12) | | |
| Yes | 26.02 (19) | | 1.52 | | | |
| Habitat | | | | | | |
| Rural | 12.87 (17) | 4.9 × 10 ^{-4d} | 1 (Ref) | 0.32–4.51 | 5.4 × 10 ^{-4d} | 1 (Ref) |
| Urban | 15 (3) | | 1.19 | | | 1.05 (0.27–4.05) |
| Periurban | 34.65 (35) | | 3.43 | | | 3.49 (1.78–6.83) |
| Living with CanL positive dogs in the household | | | | | | |
| Yes | 20.58 (21) | 0.83 | 1 (Ref) | 0.48–1.64 | | |
| No | 12.51 (34) | | 1.12 | | | |
| Location at night time | | | | | | |
| Indoors | 20.73 (17) | 0.87 | 1 (Ref) | 0.55–2.01 | | |
| Outdoors | 22.52 (38) | | 1.06 | | | |
| Previous therapy | | | | | | |
| No | 19.58 (47) | 0.017 ^d | 1 (Ref) | 1.23–10.30 | | |
| Yes | 61.53 (8) | | 3.62 | | | |

^a OR, odds ratio; CI, confidence interval; Ref, reference category.

^b n = 124.

^c Privately owned dogs plus kennel dogs (n = 190).

^d P < 0.05—statistically significant difference.

through more than 2 transmission seasons. It can be argued that these animals were recently exposed to infected phlebotomine sand fly bites, and hence, the CS positivity may suggest an early infection stage. Similar results were obtained in a previous study using skin samples (47).

Apparently, the performance of CS n-PCR did not depend on

the animal's clinical condition, given that the presence of *Leishmania* DNA in CS was similar when comparing dogs with overt clinical signs (75.5%) and asymptomatic dogs (78.9%). The latter rate is similar to that reported by Leite et al. (21) in a sample of 30 asymptomatic dogs examined by CS n-PCR (83.3%). In contrast, LN-CE, BC n-PCR, and IFAT showed lower sensitivities in

TABLE 7 Results of serologic and parasitological tests performed on dogs treated for CanL^a

| IN | TP | T ₀ | | | | | T ₃ | | | | | T ₆ | | | | |
|----|------|----------------|--------|-------|----------|----------|----------------|-------|-------|----------|----------|----------------|-------|-------|----------|----------|
| | | Clin | IFAT | LN-CE | BC n-PCR | CS n-PCR | Clin | IFAT | LN-CE | BC n-PCR | CS n-PCR | Clin | IFAT | LN-CE | BC n-PCR | CS n-PCR |
| 1 | M+A | P | 1:640 | + | + | + | A | 1:320 | + | - | - | A | 1:640 | + | + | + |
| 2 | M+A | P | 1:640 | + | + | + | A | 1:160 | - | - | - | A | 1:160 | - | + | + |
| 3 | M+A | P | 1:640 | + | + | + | A | 1:320 | - | - | - | A | 1:160 | - | + | + |
| 4 | M+A | P | 1:1280 | + | - | + | A | 1:160 | - | - | - | A | 1:80 | - | - | - |
| 5 | M+A | P | 1:160 | + | - | + | A | 1:160 | - | - | - | A | 1:160 | - | - | + |
| 6 | M+A | P | 1:1280 | + | + | + | A | 1:320 | - | - | - | A | 1:160 | - | - | + |
| 7 | M+A | P | 1:2560 | + | + | + | A | 1:320 | - | + | - | A | 1:320 | + | + | + |
| 8 | M+A | P | 1:640 | + | - | + | A | 1:160 | - | - | - | A | 1:160 | + | + | - |
| 9 | M+A | P | 1:320 | + | - | + | A | 1:80 | - | - | - | A | 1:160 | - | - | + |
| 10 | M+A | P | 1:640 | - | - | + | A | 1:160 | - | - | - | A | 1:160 | - | - | + |
| 11 | M+A | P | 1:640 | + | + | + | A | 1:160 | - | - | + | ND | ND | ND | ND | ND |
| 12 | M+A | P | 1:320 | + | + | + | A | 1:160 | - | - | + | ND | ND | ND | ND | ND |
| 13 | MA+A | P | 1:1280 | + | - | + | A | 1:320 | - | - | + | A | 1:160 | - | - | + |
| 14 | MA+A | P | 1:320 | - | + | + | A | 1:160 | - | - | + | A | 1:160 | - | - | + |
| 15 | MA+A | P | 1:320 | + | - | + | A | 1:160 | - | - | + | A | 1:160 | - | - | + |
| 16 | MA+A | P | 1:160 | + | - | + | A | 1:80 | - | - | + | A | 1:80 | - | - | + |
| 17 | MA+A | P | 1:320 | + | - | + | A | 1:160 | - | - | - | A | 1:160 | - | + | + |
| 18 | MA+A | P | 1:640 | - | - | + | A | 1:320 | - | - | - | A | 1:160 | - | + | + |
| 19 | MA+A | P | 1:640 | + | - | + | A | 1:320 | + | - | - | A | 1:640 | + | + | + |
| 20 | MA+A | P | 1:1280 | + | + | + | A | 1:160 | - | - | - | A | 1:160 | - | - | + |

^a IN, identification number; TP, treatment protocol; T₀, before treatment; T₃, 3 months from treatment onset; T₆, 6 months from treatment onset; Clin, clinical signs; IFAT, indirect immunofluorescence antibody test; LN-CE, lymph node cytological examination; BC n-PCR, n-PCR on buffy coat; CS n-PCR, n-PCR on conjunctival swab; M+A, miltefosine plus allopurinol; MA+A, meglumine antimoniate plus allopurinol; A, absence; P, presence; ND, not done (dog lost to follow-up).

asymptomatic dogs than in symptomatic animals, which probably reflects the paucity of amastigote forms in tissues and the scanty antibody production in these animals. Our results confirm that the lymph node represents a suitable tissue for the direct detection of *L. infantum* in infected symptomatic animals, with 66% positivity in infected dogs with clinical signs (see Table 5) and 100% relative performance in dogs with elevated IFAT titers (sick dogs) (Table 4). This finding is consistent with the results of a previous study (29). In contrast, in infected asymptomatic dogs, the lymph node showed a much lower positivity rate of 5% in dogs without clinical signs and 71% relative performance in dogs categorized as infected (35). These results suggest that LN-CE should not be considered among the first-line tests for CanL diagnosis in asymptomatic dogs, because of poor aspiration performance in non-enlarged lymph nodes and the intrinsic low parasite burden resulting from an effective immune response in most of the asymptomatic animals (33).

With regard to the analysis of CanL risk factors, to the best of our knowledge, this is the first study in which they were evaluated among dogs diagnosed using CS; in fact, this type of analysis has only been performed among serologically tested animals (12).

With respect to the dogs' characteristics (i.e., gender, age, and breed size), CS n-PCR positivity was found to have been influenced by age; the increasing age-specific risk of infection until 8 years coincides with previous observations of a bimodal seroprevalence distribution, with a small peak appearing in young dogs (1 to 2 years) and a more pronounced peak among the older dogs (5 to 6 years) (1, 5, 27). Regarding behavioral/environmental factors, different from other studies, in which an outdoor sleeping habit was reported to increase the risk of CanL, in our study, this factor showed no association (2, 13, 52). This could reflect the statistically high interaction ($P = 1.76 \times 10^{-9}$) between the variables "location during night time" and "habitat." Living in peri-urban areas showed the strongest association of any variable (OR = 3.49), and the fact that a large proportion of CS n-PCR-positive dogs that slept outdoors lived in periurban areas (34/54, 62.96%) might have masked the influence of "location during nighttime." The risk analysis also revealed that the "use of antivectional measures" did not represent a protective factor against CS n-PCR positivity. A possible explanation for this may lie in the circumstantial evidence that dog owners tend to adopt preventive control measures only after a positive CanL diagnosis, with the

TABLE 8 Results of serologic and parasitological assays at baseline (T₀) and at two follow-up determinations (T₃ and T₆) in dogs treated for CanL^a

| Time (no. of animals) | No. of positive dogs (%; 95% CI) | | | | |
|-----------------------|----------------------------------|--------------------|----------------------|----------------------|------------------------|
| | Clinical signs | IFAT | LN-CE | BC n-PCR | CS n-PCR |
| T ₀ (20) | 20 (100, 83.2–100) | 20 (100, 83.2–100) | 17 (85, 62.1–96.8) | 9 (45, 23.1–68.5) | 20 (100, 83.2–100) |
| T ₃ (20) | 0 (0, 0–16.84) | 20 (100, 83.2–100) | 2 (10, 27.2–72.8) | 1 (5, 0.1–24.9) | 6 (30, 11.9–54.3) |
| T ₆ (18) | 0 (0, 0–19.5) | 18 (100, 83.2–100) | 4 (22.22, 14.3–31.4) | 8 (44.44, 34.1–54.3) | 16 (88.89, 77.18–99.5) |

^a IFAT, indirect immunofluorescence antibody test; LN-CE, cytological examination of lymph node aspirate; BC n-PCR, n-PCR on buffy coat from peripheral blood; CS n-PCR, n-PCR on conjunctival swab; T₀, before treatment; T₃, 3 months from treatment onset; T₆, 6 months from treatment onset.

aim of preventing transmission to other dogs and because they fear the potential risk for the household.

The lack of an association with rural areas in our study differs from the results of other studies, in which an association was found for rural areas (43, 44, 52). Worldwide, it appears that zoonotic visceral leishmaniasis tends to migrate from typically rural areas (e.g., isolated farms) to more populated areas, including residential zones surrounding urban centers (51). This is intrinsically linked to modified environmental conditions able to influence the sand fly species composition and, therefore, the species that act as the main vectors.

In treated dogs, IFAT is highly sensitive in detecting specific anti-*Leishmania* antibodies, yet it does not provide information on the possible persistence of parasites, nor does it have a good predictive value for relapses during the follow-up of treated dogs. PCR-based methods are a good alternative, in that they can be used to monitor the parasitological status of animals during and after treatment; given that this requires performing frequent assessments, samples obtained using noninvasive techniques are preferable. Our follow-up results showed that at T_3 , both of the drug regimens promoted full clinical remission, resulting in a marked decrease in serologic titers and BC n-PCR positivity rates (5%), whereas the positivity rates for both CS n-PCR analysis and LN-CE were still moderate (30% and 10%, respectively). At T_6 , when the dogs were still apparently healthy, a consistent increase in the *Leishmania* DNA detection rate was observed by n-PCR analysis of both BC and CS samples, although the analysis performed on CS showed a much higher sensitivity (88.89%) than that performed on BC (44.44%). Since LN aspiration is a more invasive procedure and it is sometimes difficult to perform because of a treatment-induced reduction in the size of the lymph node, this tissue cannot be considered optimal for CanL monitoring in follow-up evaluations. We can thus conclude that in longitudinal surveys of drug-treated dogs, CS n-PCR can be considered the most sensitive and simple assay for the early detection of relapses and thus be used to monitor the evolution of infection after therapy. A similar approach could be useful in the periodic CanL evaluations required by experimental studies on canine vaccines, drugs, or topical insecticides.

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