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Comparison of parasitological, immunological and molecular methods for the diagnosis of leishmaniasis in dogs with different clinical signs

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Abstract

Aiming to improve the diagnosis of canine leishmaniasis (CanL) in an endemic area of the Northwest region of São Paulo State, Brazil, the efficacy of parasitological, immunological and molecular diagnostic methods were studied. Dogs with and without clinical signs of the disease and positive for *Leishmania*, by direct parasite identification on lymph node smears and/or specific antibody detection by ELISA, were selected for the study. According to the clinical signs, 89 dogs attending the Veterinary Hospital of UNESP in Araçatuba (SP, Brazil) were divided into three groups: symptomatic (36%), oligosymptomatic (22%) and asymptomatic (22%). Twenty-six dogs from an area non-endemic for CanL were used as negative controls (20%). Fine-needle aspiration biopsies (FNA) of popliteal lymph nodes were collected and Diff-Quick®-stained for optical microscopy. Direct immunofluorescence, immunocytochemistry and parasite DNA amplification by PCR were also performed. After euthanasia, fragments of popliteal lymph nodes, spleen, bone marrow and liver were collected and processed for HE and immunohistochemistry. Parasite detection by both HE and immunohistochemistry was specifically more effective in lymph nodes, when compared with the other organs. Immunolabeling provided higher sensitivity for parasite detection in the tissues. In the symptomatic group, assay sensitivity was 75.61% for direct parasite search on Diff-Quick®-stained FNAs, 92.68% for direct immunofluorescence, 92.68% for immunocytochemistry and 100% for PCR; the corresponding values in the other clinical groups were: 32, 60, 76 and 96% (oligosymptomatic), and 39.13, 73.91, 100 and 95.65% (asymptomatic). Results of the control animals from the CanL non-endemic area were all negative, indicating that the methods used were 100% specific.

Keywords: Canine leishmaniasis; *Leishmania* (*Leishmania*) *infantum chagasi*; Diagnosis; Enzyme-linked immunosorbent assay (ELISA); Polymerase chain reaction (PCR); Immunohistochemistry

1. Introduction

American Visceral Leishmaniasis (AVL) is an infectious disease of chronic evolution caused by the protozoan *Leishmania* (*Leishmania*) *infantum chagasi*,

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which is transmitted mainly by the vector *Lutzomyia longipalpis*, with incrimination of *Lutzomyia evansi* in parts of Colombia and Venezuela and *Lutzomyia cruzi* in Mato Grosso State in Brazil (Santos et al., 1998).

Dogs are considered the main domestic reservoir of the parasite, constituting part of the epidemiological cycle of human transmission (Deane and Deane, 1955a,b; Laison and Shaw, 1987), with Brazil accounting for more than 90% of VL cases described in the New World (Grimaldi and Tesh, 1993). Canine leishmaniasis (CanL) and human visceral leishmaniasis (VL) occur endemically in many states of the North, Northeast and Mid-West regions of Brazil. They are also found in the Southeast states of Minas Gerais, Rio de Janeiro and, more recently, in the Northwest area of São Paulo State (Yamamoto et al., 1988; Evans et al., 1990; Costa et al., 1991; Paranhos-Silva et al., 1996; Tafuri et al., 1996; Feitosa et al., 2000).

According to the clinical signs, Mancianti et al., 1988 classified dogs infected with *L. infantum* as: symptomatic, those presenting more than three clinical signs; oligosymptomatic, from one to three clinical signs; and asymptomatic dogs, with no clinical signs. The most frequent clinical signs found were lymphadenomegaly, weight loss, dermatological changes and onychogryphosis. However, CanL is considered to have an extraordinary pleomorphism of clinical signs and mimesis with other diseases, showing nonspecific microscopic lesions similar to those observed in other infectious and immunomediated disorders (Ferrer, 1999).

The classic methodology for leishmaniasis diagnosis includes direct microscopic identification of the parasites on Diff-Quick®-stained preparations, parasite isolation in culture medium and parasite inoculation in experimental animals. In endemic areas, serological tests are used for diagnosis and also as tools in epidemiological studies, the most employed techniques being complement fixation (Costa et al., 1991), indirect immunofluorescence (Vexenat et al., 1993) and ELISA (Mukerji et al., 1991). Recently, immunohistochemistry for specific identification of the parasite (Ferrer et al., 1988), and polymerase chain reaction (PCR) for the detection of *Leishmania* DNA (Harris et al., 1998), have been used with high sensitivity.

Increasing numbers of VL cases have been detected in the Northwest area of São Paulo State. Considering the involvement of dogs as a reservoir of the disease, the present study regarding the sensitivity and specificity of parasitological, immunological and molecular diagnostic methods was undertaken with the aim of improving disease diagnosis in dogs. To this end, both direct parasite exposure by conventional staining or specific parasite immunolabeling, as well as parasite DNA detection by PCR, were applied to fine-needle aspirate (FNA) biopsies of popliteal lymph nodes and samples of lymph node, spleen, bone marrow and liver fragments from dogs naturally infected with *L.* (*L.*) *infantum chagasi*.

2. Materials and methods

2.1. Animals

Dogs with or without clinical signs of the disease and showing parasites in lymph nodes smears and/or positive ELISA tests were selected for the study. According to clinical signs, 89 dogs attending the Veterinary Hospital of UNESP in Araçatuba (SP), Brazil were divided into three groups: symptomatic dogs, >3 clinical signs ($n = 41$); oligosymptomatic dogs, one to three clinical signs ($n = 25$), and asymptomatic dogs, with no clinical signs ($n = 23$) (Mancianti et al., 1988). Lymphadenomegaly, weight lost, skin changes, onychogryphosis and apathy were the main clinical signs observed. Dogs from the kennel of the Medical School of São Paulo University recovered from areas with no records of autochthonous cases of leishmaniasis ($n = 26$), were used as negative controls. The dogs were anesthetized with sodium thiopental 25 mg/kg; blood samples were drawn by jugular puncture. Euthanasia was performed by intravenous injection of 19.1% potassium chloride. The sera were stored at −20 °C for enzyme-linked immunosorbent assay (ELISA).

2.2. Tissue fragments

After euthanasia, fragments of popliteal lymph nodes, spleen, bone marrow and liver were collected and fixed in 0.01 M phosphate buffered 10% formaldehyde solution, pH 7.4. Paraffin embedded sections were stained with hematoxylin and eosin (HE), or processed for immunohistochemistry (IHC), in order to evaluate tissue parasitism.

2.3. Fine-needle aspiration of lymph node

Samples of popliteal lymph nodes were aspirated with a thin needle, transferred to polypropylene tubes and stored at −20 °C for PCR. Slide smears were also prepared, stained with Diff-Quick® and examined by optical microscopy, for parasitism evaluation. Additional smears were fixed in acetone/alcohol solution and

stored at −20 °C for direct immunofluorescence (DIF) and immunocytochemistry (ICC).

2.4. Enzyme-linked immunosorbent assay (ELISA)

Microplates were sensitized with 10 μl/well of *L.* (*L.*) *infantum chagasi* total antigen (20 μg/ml) in 0.05 M carbonate buffer pH 9.6, at 4 °C overnight (Riera et al., 1999). After blocking for 2 h with 200 μl/well of 0.01 M phosphate buffered saline (PBS) with 10% fetal calf serum (FCS), 100 μl/well of samples diluted at 1:400 in 0.01 M PBS with 0.05% Tween and 10% FCS were incubated at room temperature for 3 h. The microplates were then washed three times with 0.01 M PBS containing 0.05% Tween. Next, 100 μl of peroxidase labeled secondary antibody was added to each well and incubated at room temperature for 1 h. After further washing, 100 μl/well of 0.4 mg/ml *o*-phenylenediamine (OPD) and 0.4 μl/ml hydrogen peroxide was added. Color development was carried out in a dark chamber for 5 min at room temperature; the reaction was interrupted with 50 μl/well of 1 M H_2SO_4, and absorbance was measured at 492 nm using an ELISA reader. The reaction cut-off was determined as previously reported by Voller et al. (1980).

2.5. Direct immunofluorescence

Smears of popliteal lymph nodes previously fixed and stored at −20 °C were incubated with fluorescein-conjugated hamster anti-*L.* (*L.*) *infantum chagasi* polyclonal antibody (Moreira et al., 2002) diluted 1:250 in Evans Blue solution; incubation was carried out in a humid chamber at 37 °C for 30 min. After this period, the slides were washed three times in 0.01 M PBS, mounted with cover slips using glycerine as a mounting medium and observed under a fluorescence microscope. The reactions were compared against both positive (lymph nodes smears with high numbers of amastigotes, as revealed by Diff-Quick®) and negative (lymph nodes smears of dogs from a non-endemic area) controls.

2.6. Immunohistochemistry

Paraffin-embedded sections were dewaxed and rehydrated. The antigen was retrieved by steaming in 10 mM citric acid solution pH 6.0 for 3 min in a pressure cooker. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (H_2O_2) in water (three changes, 10 min each) followed by 2.5% hydrogen peroxide in methanol (three changes, 10 min each). Nonspecific interactions of secondary antibody were blocked with 1.5% normal swine serum in 0.01 M PBS with incubation for 1 h in a humid chamber at 37 °C. Nonspecific ionic interactions were blocked with a solution of powdered skimmed milk, 60 g/L diluted in distilled water and incubated for 30 min at room temperature.

Immunolabeling was performed with mouse anti-*Leishmania* polyclonal antibody diluted 1/3000 in 0.01 M PBS containing 1% bovine serum albumin; incubation was carried out in a humid chamber, first at 37 °C for 1 h and then at 4 °C overnight. After washing, the sections were incubated with biotinylated secondary antibody and then with streptavidin–peroxidase complex from LSAB kit (Dako, USA), both incubations were performed at 37 °C for 30 min. Color reaction was developed for 5 min at room temperature using as substrate 3-3-diamenobenzidine (Sigma, USA) at 60 mg/100 ml 0.01 M PBS containing 1% hydrogen peroxide. The sections were counterstained with hematoxylin, dehydrated and mounted in resin.

For immunocytochemistry reaction, the smears of popliteal lymph nodes previously fixed and stored at −20 °C, were directly incubated with 3% hydrogen peroxide to block the endogenous peroxidase and the reaction followed the same steps as immunohistochemistry.

2.7. Polymerase chain reaction (PCR)

2.7.1. DNA purification of parasites in culture and clinical samples

Promastigotes of *L.* (*L.*) *infantum chagasi*, *L.* (*V.*) *braziliensis* and *L.* (*L.*) *amazonensis* obtained from culture were used as positive controls for the reaction. Samples of FNA popliteal lymph node and controls were washed by centrifugation in sodium citrate (SSC) buffer at 1400 rpm for 5 min. Lysis buffer (375 μl of 0.2 M sodium acetate, 25 μl of 10% sodium dodecylsulfate and 5 μl of 20 mg/ml K proteinase) was added to the pellets, followed by incubation at 37 °C overnight. The lysates were treated with phenol/chloroform/isoamyl alcohol (25:24:1); DNA was precipitated with ethanol, dried and resuspended in 200 μl of TE buffer.

2.7.2. Primers

The pair of primers was prepared according to Rodgers et al. (1990), with initiators (13A and 13B) that amplify the DNA fragments of kinetoplast minicircles; this region is preserved in the different species of *Leishmania*.

2.7.3. DNA amplification

PCR consisted of: Taq buffer (50 mM KCl, 10 mM Tris pH 8.4), $MgCl_2$ standardized concentration of 1.5 mM, dNTPs (dATP, dCTP, dGTP and dTTP), initiators 13A and 13B, Taq DNA polymerase and DNA, in a final volume of 50 μl.

The samples were amplified in a thermocycler (PTC-100 MJ-Research) using an initial denaturation step of 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 54 °C for 45 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min; the samples were then kept at 4 °C until the next step. Amplification with primers 13A and 13B resulted in products of 120 base pairs (bp). Negative (tube without DNA) and positive (DNA from *Leishmania* culture) controls were performed for each experiment. After amplification, the PCR products were subjected to 2% agarose gel electrophoresis, stained with ethidium bromide and photographed under ultraviolet light.

2.8. Statistical analysis

The results of the different diagnostic methods were submitted to statistical analysis using the SPSS software (version 8.0) for Windows to determine the sensitivity and specificity of each test. The gold standard test was considered the direct search for parasites in fine-needle aspiration of popliteal lymph nodes.

In order to evaluate the of concordance index between two diagnostic methods, Kappa measurement was employed, where 0 indicates no concordance and 1 total concordance. Clinical status was compared based on the results from the different diagnostic methods using the Pearson chi square test. The Fisher test was applied when the expected values were below 5 and the results were considered significant, showing at p value <0.05.

3. Results

3.1. Enzyme-linked immunosorbent assay (ELISA)

The detection of anti-*Leishmania* antibodies in serum by ELISA was positive in 37/41, 17/25 and 22/23 dogs of the symptomatic, oligosymptomatic, and asymptomatic group, respectively. All 26 control dogs were negative. Thus, ELISA test sensitivity for each clinical group was 87.80%, symptomatic; 68.00%, oligosymptomatic; and 95.65%, asymptomatic; assay specificity was 100% (Table 1).

Table 1
Sensitivity of enzyme-linked immunosorbent assay (ELISA), cytology, direct immunofluorescence (DIF), immunocytochemistry (ICC) and polymerase chain reaction (PCR) of lymph node FNA biopsies of dogs with different clinical signs

Diagnostic method	Clinical signs		
	Symptomatic (%)	Oligosymptomatic (%)	Asymptomatic (%)
ELISA	87.80	68.00	95.65
Cytology	75.61	32.00	39.13
DIF	92.68	60.00	73.91
ICC	92.68	76.00	100.00
PCR	100.00	96.00	95.65

Assay specificity was 100% for all the diagnostic methods in all the clinical groups.

3.2. Tissue fragments

3.2.1. Popliteal lymph nodes

Optical microscopy of lymph node sections stained with HE showed *Leishmania* amastigotes in macrophage cytoplasm in 18/41 (43.90% sensitivity), 10/25 (40.00% sensitivity) and 9/23 (39.13% sensitivity) dogs of the symptomatic, oligosymptomatic and asymptomatic groups, respectively. In contrast, immunohistochemistry presented higher sensitivity, revealing parasite presence in 27/41 (65.85% sensitivity), 17/25 (68.00% sensitivity), 17/23 (72.73% sensitivity) dogs of the symptomatic, oligosymptomatic and asymptomatic groups, respectively. For both assays specificity was 100% (Table 2).

3.2.2. Spleen

HE from spleen samples showed positivity in 11/41, 7/25 and 8/23 dogs of the symptomatic, oligosymptomatic and asymptomatic groups, respectively, with corresponding sensitivities of 43.90, 40.00 and 38.13%. Immunohistochemistry of samples indicated positive diagnosis in 25/41, 16/25 and 16/23 dogs, respectively, and assay sensitivities of 60.98, 64.00 and 69.57%. For both assays specificity was 100% (Table 2).

3.2.3. Bone marrow

HE stained tissue revealed the presence of amastigotes in symptomatic, oligosymptomatic and asymptomatic dogs at ratios of 11/41, 3/25 and 5/23, with assay sensitivities of 26.83, 12.00, and 21.74%. Immunohistochemistry permitted the identification of amastigote parasites in 24/41 symptomatic, 8/25 oligosymptomatic and 11/23 asymptomatic dogs, with assay sensitivities of 58.54, 32.00 and 47.83%. For both assays specificity was 100% (Table 2).

Table 2
Sensitivity of hematoxylin and eosin (HE) and immunohistochemistry (IHC) methods in lymph node, spleen, bone marrow and liver of dogs with different clinical signs

Organs	Diagnostic method	Clinical signs		
		Symptomatic (%)	Oligosymptomatic (%)	Asymptomatic (%)
Lymph node	HE	43.90	40.00	39.13
	IHC	65.85	68.00	72.73
Spleen	HE	36.59	28.00	34.78
	IHC	60.98	64.00	69.57
Bone marrow	HE	26.83	12.00	21.74
	IHC	58.54	32.00	47.83
Liver	HE	26.83	12.00	13.04
	IHC	53.66	40.00	56.52

Assay specificity was 100% for all the diagnostic methods in all the clinical groups.

3.2.4. Liver

HE staining of liver samples revealed the presence of *Leishmania* amastigotes in 11/41 symptomatic, 3/25 oligosymptomatic and 3/23 asymptomatic dogs, with corresponding sensitivities of 26.83, 12.00 and 13.04%, and 100% assay specificity. Immunohistochemistry of liver identified amastigotes in 22/41 symptomatic, 10/25 oligosymptomatic and 13/23 asymptomatic dogs, with corresponding sensitivities of 53.66, 40.00 and 56.52%; the assay specificity was also 100% (Table 2).

3.3. FNA of popliteal lymph node

3.3.1. Cytological examination

Diff-Quick® staining of popliteal lymph node FNA revealed the presence of typical *Leishmania* amastigotes in 34/41 symptomatic, 8/25 oligosymptomatic and 9/23 asymptomatic dogs, with assay sensitivities of 75.61, 32.00 and 39.13%, respectively; assay specificity was 100% (Table 1).

3.3.2. Direct immunofluorescence (DIF)

Parasite search by direct immunofluorescence in popliteal lymph node smears showed positive reaction in 38/41 symptomatic, 15/25 oligosymptomatic and 17/23 asymptomatic dogs. All the controls showed negative results. DIF sensitivities for the symptomatic, oligosymptomatic and asymptomatic groups were thus 92.68, 60.00 and 73.91%, respectively, and assay specificity was 100% (Table 1).

3.3.3. Immunocytochemistry (ICC)

Immunolabeling of *Leishmania* amastigotes by immunocytochemistry was positive in 38/41, 19/25 and 23/23 dogs of the symptomatic, oligosymptomatic and asymptomatic group, with assay sensitivities of 92.68, 76 and 100%, respectively. No reaction was observed in the control group, demonstrating 100% assay specificity (Table 1).

3.3.4. Polymerase chain reaction (PCR)

For the standardization of PCR, samples of *L.* (*L.*) *infantum chagasi*, *L.* (*L.*) *braziliensis e L.* (*L.*) *amazonensis* were utilized. Agarose gel electrophoresis revealed DNA amplification of a 120 bp from all species. DNA samples from popliteal lymph node FNA subjected to PCR demonstrated that 41/41 of the samples were positive in the symptomatic group (Table 1). In the oligosymptomatic and asymptomatic groups, PCR showed positive reactions in 24/25 and 22/23 of the samples, respectively. The presence of the 120 pb band was not found in any of the control samples. These results show that PCR sensitivity was 100.00, 96.00 and 95.65% for the symptomatic, oligosymptomatic and asymptomatic groups, respectively, and that assay specificity was 100%.

Comparative analysis of assay performance for the various diagnostic methods showed that ELISA and immunocytochemistry were most efficient in samples of asymptomatic dogs ($p < 0.05$). Direct identification of parasites by Diff-Quick® staining and direct immunofluorescence were more efficient in smears from symptomatic dogs, followed by asymptomatic and oligosymptomatic dogs ($p < 0.05$). PCR was found to be highly efficient for all the clinical manifestations of CanL.

4. Discussion

Of the 89 dogs studied, 41 (46.1%) were diagnosed as symptomatic, 25 (28.1%) as oligosymptomatic and 23 (25.8%) as asymptomatic, according to the

classification of Mancianti et al. (1988). According to the present results, the most frequent clinical signs found for both symptomatic and oligosymptomatic dogs were lymphadenomegaly, weight loss, dermatological changes and onychogryphosis.

Serum testing for anti-*Leishmania* antibodies by indirect immunofluorescence (IF) and ELISA coupled with clinical evaluation, is a widely used strategy for CanL diagnosis. ELISA shows higher sensitivity and specificity in comparison with IF (Evans et al., 1990; Mancianti et al., 1995). In the present study, ELISA was employed in conjunction with routine histological parasite detection, as part of the procedure used to define symptomatic, oligosymptomatic and asymptomatic dogs. The reaction cut-off was based on the arithmetic mean of OD values from 26 healthy dog sera, plus three standard deviations (Voller et al., 1980). Since a CanL-positive serological test implied euthanasia of the canine in question and in view of the fact that contact with the parasite and the subsequent humoral response does not necessarily indicate active infection, a high cut-off value was selected in order to avoid false positives. In the present work, ELISA assay sensitivity indicated a more pronounced anti-*Leishmania* humoral response with specific antibody production among the asymptomatic dogs, followed by symptomatic and oligosymptomatic dogs. The between-group variation regarding the frequency of positive results in the ELISA assay presented here, agrees with observations reported by Pinelli et al. (1994), who detected the immunosuppression of antibody production by *Leishmania* antigen in symptomatic dogs. Serological results at variance with direct parasite evidence have been discussed by Lanotte et al. (1979) and Slappendel (1988), who observed that *Leishmania*-positive dogs, as defined by direct cytological evaluation, were negative for IF.

With respect to the etiological diagnosis of the disease, direct parasite detection in HE stained paraffin-embedded sections of lymphoid organs was more effective in popliteal lymph nodes, regardless of the clinical group analyzed, followed by the spleen and bone marrow. Such results agree with the findings of Keenan et al. (1984), who reported the identification of amastigote forms within lymphoid organ macrophages. However, immunohistochemistry proved to be a more sensitive assay for parasite detection when compared with the routine histological evaluation, especially in lymph nodes (Ferrer et al., 1988; Ramos et al., 1994; Azedeh et al., 1994; Ferrer, 1999).

Several authors pointed out that microscopic evaluation of liver sections stained with HE is not very sensitive for parasite detection (Keenan et al., 1984; Duarte et al., 1989). In the present study, immunolabeling of liver samples showed improved sensitivity in comparison with routine histological evaluation in all the clinical groups studied. Analyzing the present data regarding sensitivity, it is possible to conclude that immunohistochemistry provided improved diagnosis, especially for asymptomatic dogs. Sotto et al. (1989), while comparing the histopathology and immunohistochemistry of human skin biopsies for cutaneous leishmaniasis diagnosis, found 16% positivity by direct examination of skin scrapings from cutaneous lesions, 20% by histopathological evaluation and 64.5% by immunohistochemical evaluation, thus demonstrating that the last method presented far greater sensitivity than the others.

Present data on parasite identification by HE or immunohistochemical staining of paraffin-embedded tissue sections indicate that popliteal lymph nodes were the most suitable for a clearer etiological diagnosis of CanL, among the organs tested. Having established this, aspirates of popliteal lymph nodes from naturally infected dogs were used as a target to test additional methodologies, studied with the aim of facilitating and accelerating disease diagnosis.

Direct parasite search by cytological analysis of lymph node smears was more sensitive in the symptomatic group, possibly because in this case the immune response triggered by infection may not have been sufficient to control parasite burden, as mentioned by Ferrer (1999) and Pinelli et al. (1999), thus facilitating detection due to the large number of intact parasites displayed in the tissue. In contrast, the lower sensitivity indexes found for this technique in the asymptomatic and oligosymptomatic groups, may reflect a large destruction of amastigote forms as a consequence of a more effective immune response (Pinelli et al., 1999).

Direct immunofluorescence evaluation of lymph node smears resulted in sensitivity values of 92.68, 73.91 and 60.00% for the symptomatic, oligosymptomatic and asymptomatic clinical groups, respectively, thus indicating improved diagnostic performance when compared with direct cytological evaluation. This was also observed by Moreira et al. (2002) in a comparative study of lymph node aspirates from dogs with negative, suspected or positive CanL diagnosis; and by Sotto et al. (1989) in tissue samples from patients with cutaneous leishmaniasis.

In the present study, immunohistochemistry proved to be highly efficient at revealing the presence of intact parasites in lymph node smears, as it gave positivity indexes of 100, 92.98 and 76.00% in the asymptomatic,

symptomatic and oligosymptomatic groups, respectively, with 100% specificity. Not only was its overall sensitivity high, but it specifically allowed for 100% correct diagnosis of asymptomatic dogs and should, therefore, be considered an important tool for inclusion in routine diagnostic practices. According to Ferrer (1999) and Gradoni (2002), the ideal diagnostic method for CanL should be low-cost, fast, and show high sensitivity and specificity ratios. Although immunohistochemistry is not considered a low-cost assay and requires considerable time to perform, its excellent diagnostic performance both in terms of specificity and sensitivity is a sound argument to grant its use as a routine diagnostic assay.

PCR applied to lymph node aspirates presented sensitivity indexes of 100, 96 and 95.65% in the symptomatic, oligosymptomatic and asymptomatic groups, respectively. This method did not show significant sensitivity differences between the clinical groups of dogs from the Araçatuba (SP) region, where CanL is endemic. Ashford et al. (1995) reported 100% sensitivity for PCR in dogs diagnosed by positive parasite culture, as did Reale et al. (1999), in a group of dogs that were serum-positive and -negative for CanL. These data might suggest that PCR should be considered a golden diagnostic test. However, according to the study performed by Berrahal et al. (1996) in Marseille, France, which is a CanL endemic region, the detection of *Leishmania* DNA in skin samples by PCR was efficient in 89% dogs with clinical and biological signs of the disease, in 63% with a history of leishmaniasis and diagnosed as clinically cured after treatment and in 80% of asymptomatic dogs, of which 56% were serum-positive for anti-*Leishmania* antibodies by immunoblotting. The primers described by Rodgers et al. (1990) and synthesized for the present analysis amplify regions of kinetoplast DNA that are considered efficient for the characterization of the *Leishmania* genus, as they contain multiple copies of minicircles common to all *Leishmania* species. High PCR sensitivity when amplifying *Leishmania* kinetoplast DNA was mentioned by Lachaud et al. (2002) in their work on symptomatic and asymptomatic dogs.

The comparative analysis of the diagnostic performance of cytological evaluation, direct immunofluorescence (DIF), immunocytochemistry (ICC) and PCR applied to lymph node aspirate showed that parasite detection by direct cytological examination, despite being a fast, low-cost method that does not require sophisticated equipment, presented low sensitivity. Greater sensitivity indexes were obtained with the immunolabeling methods DIF and ICC, especially with the latter (Sotto et al., 1989; Ferrer et al., 1988; Moreira et al., 2002). The best sensitivity results were obtained with PCR, showing that it is a very efficient method for CanL diagnosis. However, this test requires sophisticated equipment and well-trained laboratory personnel, particularly in order to avoid sample contamination, since this technique is able to detect small amounts of parasite DNA (Roura et al., 1999). Additionally, this method detects amplified parasite DNA and not the intact parasite and does not reflect the severity of the infection nor the disease stage.

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