# PCR in the investigation of canine American tegumentary leishmaniasis in northwestern Paraná State, Brazil

## Aplicação do teste de reação em cadeia da polimerase (PCR) no estudo da leishmaniose tegumentar americana em cães, na região noroeste do Paraná, Brasil

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### Abstract

*American tegumentary leishmaniasis (ATL) was studied in 143 dogs in a rural area in the county of Mariluz, northwestern Paraná State, Brazil, using direct parasite search, indirect immunofluorescence (IIF), and polymerase chain reaction (PCR). Thirty-nine dogs (27.3%) presented lesions suggestive of the disease, 5 (12.8%) of which were positive in direct parasite search and PCR (lesion), and of these 5, 4 were also positive by IIF. Of the 34 dogs with negative direct parasite search, 12 (35.3%) had PCR- positive lesions, and of these, 5 were also IIF-positive. One hundred and four dogs had no lesions, but 17/101 (16.8%) were IIF-positive. PCR in blood was positive in 10/38 (26.3%) of the dogs with lesions and in 16/104 (15.4%) of dogs without lesions. The association between PCR (lesion or blood), direct parasite search, and IIF detected 24/39 (61.5%) positive results among symptomatic dogs and 31/104 (29.8%) among asymptomatic animals. PCR was useful for diagnosing ATL, but there was no correlation between lesions, serology, and plasma PCR. Furthermore, detection of parasite DNA in the blood may indicate hematogenous parasite dissemination.*

*Leishmaniasis;* Leishmania (Viannia) braziliensis*; Polymerase Chain Reaction; Indirect Fluorescent Antibody Technique; Dogs*

### Introduction

American tegumentary leishmaniasis (ATL) is a zoonosis caused by parasites of the *Leishmania* genus, transmitted by bites from infected Phlebotominae, genus *Lutzomyia* [1]. Within this genus, *Leishmania (Viannia) braziliensis* is one of the key causative species of tegumentary leishmaniasis, which can cause all the way from localized skin lesions to severe mucocutaneous mutilations [1].

Tegumentary leishmaniasis is currently concentrated in South America, the Middle East, and India [2]. In Brazil, ATL has been found in all of the States [3,4] due to the intense destruction of native forests and adaptation of vectors to the peri-domiciliary environment [5,6].

Besides humans, ATL also affects domestic animals like canines and equids. The presence of dogs infected with *Leishmania* or with positive serology has been reported in endemic areas [7,8,9]. In the northwestern region of Paraná State, dogs infected with *L. (V.) braziliensis* [10] and those with positive ATL serology have been detected [3]. Various authors have shown that dogs may play a role in the ATL transmission cycle, acting as a possible secondary reservoir for the parasite [8,9,11,12,13,14]. However, many questions need still to be answered to elucidate the role of canines in the parasite's transmission cycle [13,14,15].

ATL diagnosis is performed by direct parasite search in lesions and antibody testing, be-

sides delayed hypersensitivity reaction (Montenegro skin test) [1,16,17,18]. However, these tests have certain limitations such as: low sensitivity for direct parasite search, mainly in chronic infections [13,18,19,20], impossibility of differentiating past from present infection with the Montenegro skin test [19,21,22], serological cross-reactivity with other parasites from the Tripanosomatidae family such as *Trypanosoma cruzi* [13,23], detection of antibodies against *Leishmania* in individuals who have had contact with the parasite but without developing the disease [20], and non-detection of antibody titers suggestive of infection, since the humoral response in tegumentary leishmaniasis is less intense than the cellular response [17].

Parasite DNA detection methodologies have become a useful tool for leishmaniasis diagnosis, using specific extra-nuclear DNA sequences in the parasite's kinetoplast (k-DNA) [19,24,25]. The DNA is divided into two distinct sequences, the maxi-circle and the mini-circle. In the mini-circle, base sequences are conserved among species of the *Leishmania* genus and also regions which vary among species [24]. Primers that amplify parasite DNA of the *Leishmania* genus have been used by various researchers and show high sensitivity and specificity [22,25,26,27,28].

To investigate *Leishmania* sp. infection in dogs from a rural area in northwestern Paraná State, Brazil, where there was an ATL outbreak in 2002, we performed polymerase chain reaction (PCR) and some commonly applied laboratory techniques such direct parasite search in lesions and indirect immunofluorescence (IIF).

## Materials and methods

### Study area

The county of Mariluz is located in the northwestern Paraná State, 594km from the State capital Curitiba between 24º00'S and 53º10'W, with an area of 391.53km² and 500m altitude. It has a humid, subtropical, mesothermal climate with a mean temperature above 22ºC in summer and below 18ºC in winter.

### Dogs and biological samples

In July 2003, 143 dogs from a rural area in Mariluz were studied. The animals were investigated for the presence of ATL suggestive lesions and enlarged lymph nodes.

Blood was drawn from all the animals. An aliquot of 2ml blood was added to an equal volume of ACD solution (25mM citric acid; 50mM sodium citrate; 81mM glucose). The material was frozen at -20ºC for later DNA extraction. The remaining blood was used for serum extraction and was stored at -20ºC until use.

Dogs with lesions were submitted to scraping and biopsies of the lesions' borders. Scrapings were taken with a DNA-free metallic scraper (previously treated with 1.5% hypochlorite solution for 15 min). Smears were performed on glass slides for microscopic analysis for each type of biological sample taken from lesions. The remaining material collected from lesions (scrapings and biopsies) was placed in freezing vials with 100µl STE buffer (10mM TRIS; 1mM EDTA; 0.1M NaCl; pH 8.0) and stored in liquid nitrogen for later DNA extraction. Five animals presented palpable lymph nodes from which material was obtained by needle aspiration and submitted to the same treatment as that of lesions.

### Direct parasite search

Slides containing material from lesions were Giemsa stained and analyzed for presence of amastigotes under 1,000x magnification.

### Indirect immunofluorescence (IIF)

IIF was performed using as antigens promastigote forms of *L. (V.) braziliensis*, canine sera, and canine anti-immunoglobulin G conjugated with fluorescein (Sigma). Serum samples with titers equal to or greater than 40 were considered positive. Samples with significant *Leishmania* antibody titers were investigated for *Trypanosoma cruzi* antibodies by IIF, using Imunocruzi antigen (Biolab – Rio de Janeiro, Brazil).

### DNA extraction from lesional material and lymph node aspirates

DNA extraction from lesional material and lymph node aspirates was performed using the Gentra Purogene® kit. After thawing the samples, 300µl of the lyse solution and 1.5µl of 20mg/ml Proteinase K were added. Samples were homogenized and incubated overnight, at 56ºC. After that, 1.5µl of 4mg/ml RNAse were added and the samples were incubated for 1 hour, at 37ºC. Next, 100µl of the protein precipitation solution were added and the samples were shaken vigorously and centrifuged for 3 min., at 13,000g. The supernatant was transferred to another tube and the DNA was precipitated by the addition of 300µl of isopropanol. Samples were centrifuged at 13,000g, for 3 min.

The supernatant was discarded and 300µl of 70% ethanol were added to the sediment. Samples were centrifuged again at 13,000g, for 3 min. The supernatant was discarded, and the DNA was resuspended in 50µl of hydration solution. The final DNA was stored at 4ºC until use.

## DNA extraction from blood samples

The DNA was extracted from blood samples using the Phenol-Chloroform methodology described by Sambrook et al. [29] and modified as follows. Frozen blood was washed three times with 2ml of PBS (0.01M phosphate buffer, pH 7.2; 0.15M NaCl) at 2,200g, for 10 min. The cell sediment was broken and added of 200µl of the lyse solution (50mM Tris, pH 8.5; 50mM NaCl; 25mM $Na_2EDTA.2H_2O$, pH 8.0; 0.5% sodium dodecyl sulfate; 300µg/ml proteinase K). The resulting lysate was incubated for 4 hours, at 56ºC. After that, the lysate was added of 1.0µl of 4mg/ml RNAse, and incubated at 37ºC, for 1 hour. Next, 200µl of phenol-Tris (phenol equilibrated with Tris 0.1M, pH 8.0) were added and the samples were incubated for 1 hour, at room temperature (approximately 25ºC), in a tube homogenizer. The sample was centrifuged at 3,000g, for 10 min, and the upper phase was transferred to another tube and added of 200µl of phenol-Tris:chloroform-isoamylic alcohol (49:1; vol:vol) at 1:1 (vol:vol). Samples were incubated for an hour, in a tube homogenizer, and centrifuged at 3,000g, for 10 min. The supernatant was then transferred to another tube to which 200µl of chloroform: isoamylic alcohol (49:1; vol:vol) were added. The material was incubated under agitation for 1 hour and then centrifuged at 3,000g, for 10 min. The supernatant was then transferred to another tube and the DNA was precipitated by the addition of 25µl of 3M sodium acetate and 500µl of 100% ethanol. The tube was carefully inverted and subsequently centrifuged at 13,000g for 3 min. The sediment was washed once with 500µl of 70% ethanol followed by centrifugation (13,000g, for 3 min). The DNA pellet was resuspended in 50µl of TE buffer (TRIS 10mM; EDTA 1mM; pH 8.0) and stored at 4ºC until use. As an extraction control, blood samples that were added of *L. (V.) braziliensis* promastigotes were extracted in the same way.

## DNA amplification

The DNA amplification was carried out using the MP3H (GAA CGG GGT TTC TGT ATG C) and the MP1L (TAC TCC CCG ACA TGC CTC TG) primers described by Lopez et al. [25], which amplify a fragment of 70 base pairs from the minicircles present in the kDNA of members of the *L. braziliensis* complex The PCR reaction mixture (25µl) contained 1µM of each MP1L and MP3H (Invitrogen®), 0.2mM dNTP (Invitrogen®), 1U *Taq* DNA Polymerase (Invitrogen®), 2µl of the extracted DNA, 3mM $MgCl_2$, and 1X enzyme buffer. DNA amplification was carried out in a Biometra PC Thermocycler, using an initial heating of 95ºC, for 3 min. Following the initial heating, 30 cycles were performed, each divided into three stages: denaturation (95ºC, 1.5 min), annealing (56ºC, 1.5 min), and elongation (72ºC, 2 min). After the reaction, the material was kept at 72°C for 10 min, and the amplified product was stored at 4ºC until use. Amplified samples were submitted to electrophoresis in a 3% agarose gel stained with ethidium bromide. For each six samples analyzed in a gel, one positive and one negative control were added. After the run, the presence of amplified DNA bands was verified in a UV transilluminator (Macro Vue UV-20 Hoefer).

## Evaluation of PCR specificity and sensitivity

The MP3H/MP1L primers specificity was evaluated in reactions using DNA from *T. cruzi* and from promastigotes of different *Leishmania* species that can cause ATL. *L. (V.) braziliensis* (MHOM/BR/1987/M11272) and *L. (L.) amazonensis* (MHOM/BR/1989/166MJO) were isolated from ATL patients at the Teaching and Research Laboratory for Clinical Analyses, State University of Maringá, Paraná, and identified at the Evandro Chagas Institute in Belém, Pará; *L. (V.) panamensis* (MHOM/PA/1967/BOYTON, *L. (V.) guyanensis* (MHOM/BR/1975/M4147), *L. (V.) naiffi* (MDAS/BR/1979/M5533), and *L. (V.) lainsoni* (MHOM/BR/1981/M6426) were kindly made available by Dr. J. J. Shaw, Evandro Chagas Institute/University of São Paulo, São Paulo. Promastigotes were cultured in 199 medium with 10% fetal bovine serum, 1% glutamine, and 1% human urine, at 25ºC, up to the stationary growth phase. Epimastigotes of *T. cruzi* strain Y grown in LIT medium were kindly made available by Dr. M. L. Gomes from the Basic Parasitology Laboratory, Department of Clinical Analyses, State University of Maringá.

The parasites were washed three times in PBS (720g for 15 min.) and the sediment was resuspended in TE buffer, aliquoted, and stored at -18ºC. DNA extraction was performed according to Belli et al. [19]. Fifty-microliter aliquots of a parasite suspension in TE buffer were incubated with 100µl sterile water at 100ºC, for

10 min. The sample was centrifuged at 13,000g, for 1 min, and the supernatant was stored at 4ºC. Supernatant aliquots (5µl) were used for the PCR reaction. DNA concentration was estimated by absorbance at 260nm, according to Sambrook et al. [29]. DNA was also extracted from lesional material obtained through scrapings and biopsies from 10 dogs presenting other types of skin diseases, which can be mistaken for tegumentary leishmaniasis, such as myiasis (1), demodectic scabies (3), mycosis (4), sarcoptic scabies (1), and seborrhea (1).

PCR technical sensitivity with the primers MP3H and MP1L was evaluated with DNA extracted from suspensions with different concentrations of *L. (V.) braziliensis* promastigotes.

### Statistical analysis

Data were analyzed with the McNemar test using Statistica 6.0 software.

## Results

Of the 143 investigated dogs, 87 (60.8%) were male and 56 (39.2%) female, with ages around two years. Thirty-nine (27.3%) dogs presented lesions suggestive of ATL. Lesions were located in the ear in 23 dogs (58.9%), muzzle in 6 (15.4%), scrotum in 3 (7.7%), ear and scrotum in 4 (10.3%), muzzle and scrotum in 1 (2.6%), ear, scrotum and muzzle in 1 (2.6%), and ear, scrotum, muzzle, and paw in 1 (2.6%).

PCR detected 0.9ƒg of DNA or 3.2 parasites/µl of sample to be amplified. The MP3H/MP1L primers amplified a fragment of DNA of 70bp of *L. (V.) braziliensis*, *L. (V.) panamensis*, *L. (V.) guyanensis*, *L. (V.) naiffi*, and *L. (V.) lainsoni*, belonging to the subgenus *Viannia*, but did not identify the *L. (L.) amazonensis* DNA. These primers did not amplify the DNA in lesional material from dogs with other skin diseases (myiasis, demodectic and sarcoptic scabies, mycosis, and seborrhea), thus showing 100% specificity. Figure 1 shows a PCR reaction performed with different *L. (V.) braziliensis* (A) concentrations and with lesional material (B).

Direct parasite search (scraping and/or biopsy) was positive in 5 (12.8%) out of 39 dogs with lesions. Since some animals presented multiple lesions, 13 lesions were analyzed in these 5 dogs. Direct parasite search was positive in 4 out of 13 (30.8%) scrapings and in 7 out of 9 (77.8%) biopsies (p = 0.1336). PCR was positive in 10 out of 13 (76.9%) scrapings and in 8 out of 13 (61.5%) biopsies (p = 0.4795). The five dogs with positive direct parasite search in at least one lesion also had positive PCR in at least one lesion. In three of these animals the PCR was performed in lymph nodes, one of which was positive.

Of the remaining 34 dogs with lesions and negative direct parasite search, 12 (35.3%) had positive PCR. Eleven had positive PCR in lesional material, of which one was positive in both scraping and biopsy, five positive in scraping only, and five positive in biopsy only (p = 0.7518). Two dogs were submitted to lymph node aspirate PCR, one of which was positive.

Table 1 shows the association between lesional PCR, direct parasite search, and IIF in 39 dogs with lesions. PCR detected 17 (43.6%) and direct search 5 (12.8%) positive dogs out of 39 dogs with lesions (p = 0.0015). Lesional PCR was positive in: four dogs (100%) with positive direct search and IIF; the one (100%) with positive direct search and negative IIF; four (57.1%) with negative direct search and positive IIF; and eight (29.6%) with both negative direct search and negative IIF. All of the dogs had lower IIF titers for Chagas disease than for leishmaniasis. Positive lesional PCR was not statistically associated with positive IIF (p = 0.1489).

Blood PCR performed in 38 of 39 dogs with lesions was positive in 10 (26.3%) (Table 2). Four (4/11; 36.4%) had negative direct parasite search and positive lesional PCR. Another 27.3% (6/22) had both negative direct search and negative PCR. Only one was IIF-positive. No difference was found between parasite DNA detection in blood and either lesional direct search, lesional PCR (p = 0.2386), or IIF (p = 1.000), showing that there was no correlation between DNA detection in blood and either DNA parasite detection in the lesion or antibody detection.

One hundred and four dogs did not have lesions suggestive of *Leishmania* sp. infection (Table 3). IIF was performed in 101 dogs and was positive in 17 (16.8%). Of these, two (11.8%) had positive blood PCR. Of the 84 dogs with negative IIF, 14 (16.7%) had positive blood PCR. All animals with positive IIF were tested for Chagas disease, but the titers were lower than for leishmaniasis. Again, no relationship was found between parasite DNA detection in the blood and antibody detection (p = 1.0000) in dogs without lesions.

The association between blood and lesional PCR, direct parasite search, and IIF for ATL diagnosis showed 61.5% (24/39) positivity in symptomatic dogs and 29.8% (31/104) in asymptomatic.

## Discussion

This study investigated ATL in dogs from an endemic area where the causative agent for human infection is *L. (V.) braziliensis* [30]. PCR was applied and compared to both direct parasite search and IIF, aimed at overcoming some common limitations found in routine tests, such as low sensitivity in direct parasite search, especially in chronic infection [13,18,19,20]. This limitation makes it impossible to differentiate past from present infection [19,21,22], serological cross-reactivity [13,23], *Leishmania* antibody detection in individuals who have not developed the disease [20], or lack of antibody titer detection suggestive of infection.

Of the 17 dogs with ATL lesions, four had multiple lesions and 13 had single lesions, predominantly in the ears, followed by the muzzle and scrotum. Prevalence of single lesions has also been mentioned by other authors [11,12], and predominance of lesions in the ears has also occurred in other ATL endemic areas [9,11,12].

PCR proved quite sensitive, capable of detecting 3.2 parasites/µl or 0.9*f*g of DNA, similar to data described by Lopez et al. [25], who detected 0.14*f*g of DNA. The MP3H/MP1L primers amplified only DNA from species of *Leishmania (Viannia)* subgenus, including *L. (V.) naiffi* and *L. (V.) lainsoni*, not analyzed by Lopez et al. [25]. PCR did not present false-negative results, not amplifying DNA extracted from material of lesions involving other etiologies.

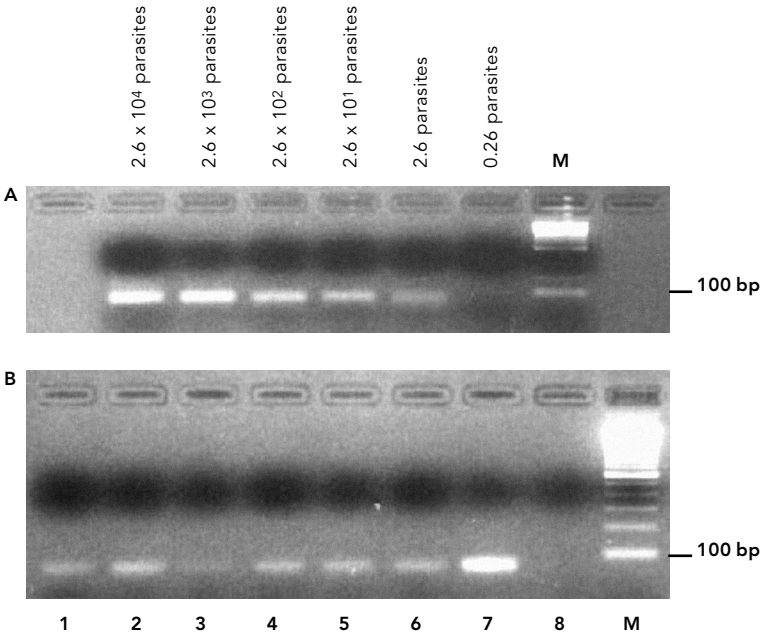
Lesional PCR did not show false-negative results, detecting all cases in which the direct parasite search was positive. PCR sensitivity in detecting the disease was higher than that of direct search. While direct search detected 12.8% of affected dogs with lesions, PCR detected 43.6% (p = 0.0015). Concordance between direct search and lesional PCR was 69.2%.

Comparison of the two methods for collecting material from lesions (biopsy or scraping) in the five dogs with positive direct search did not show any difference in positivity, either in direct search or PCR. However, of the 11 dogs with negative direct search and positive lesional PCR, ten had positive PCR in the material obtained by only one of the methods, showing that the association (p = 0.7518) between the two methods for collecting the material from lesions increases the likelihood of detecting infection, probably due to the scarce parasites in lesions [8].

PCR results from lymph node aspirate were not associated with either direct search or lesional PCR, and only contributed to the diagnosis in one of five dogs studied, which had

Figure 1

Representative gel showing 70 base pair (bp) fragment of the k-DNA mini-circle region of *Leishmania braziliensis* complex, amplified by polymerase chain reaction using MP1L/MP3H primers.




A = different concentrations of *Leishmania (Viannia) braziliensis* DNA were used. DNA amplified corresponding to 2.6 x $10^3$ to 0.26 parasites; B = lane 1 to 6, positive lesions from dogs; lane 7, positive control [DNA extracted from *Leishmania (Viannia) braziliensis* promastigotes]; lane 8, negative control (mixed reaction); M = molecular weight standards.

Table 1

Results of polymerase chain reaction (PCR) (lesion and/or lymph nodes) compared to direct parasite search (DP) and indirect immunofluorescence (IIF) in dogs with lesions suggestive of American tegumentary leishmaniasis.

| DP/IIF | Dogs with lesions (n) | PCR | |
|---|---|---|---|
| | | Positive (n) | Positivity (%) |
| Positive/Positive | 4 | 4 | 100.0 |
| Positive/Negative | 1 | 1 | 100.0 |
| Negative/Positive | 7 | 4 | 57.1 |
| Negative/Negative | 27 | 8 | 29.6 |
| **Total** | 39 | 17 | 43.7 |

p = 0.0015, McNemar test, PCR versus DP;
p = 0.1489, McNemar test, PCR versus IIF.

Table 2

Results of polymerase chain reaction (PCR) in blood compared to direct parasite search (DP), PCR in lesion, and indirect immunofluorescence (IIF) in dogs with lesions suggestive of American tegumentary leishmaniasis.

| DP/PCR (lesion)/IIF | Dogs with lesions (n) | PCR (blood) | |
|---|---|---|---|
| | | Positive (P/N) | Positivity (%) |
| Positive/Positive/Positive | 4 | 0/4 | 0.0 |
| Positive/Positive/Negative | 1 | 0/1 | 0.0 |
| Negative/Positive/Positive | 4 | 1/3 | 33.3 |
| Negative/Positive/Negative | 8 | 3/8 | 37.5 |
| Negative/Negative/Positive | 2 | 1/2 | 50.0 |
| Negative/Negative/Negative | 20 | 5/20 | 25.0 |
| **Total** | 39 | 10/38 | 26.3 |

P = number positive; N = number analyzed;
p = 0.2386, McNemar test, PCR in blood versus DP and/or PCR in lesion;
p = 1.0000, McNemar test, PCR in blood versus IIF.

Table 3

Results of polymerase chain reaction (PCR) in blood compared to indirect immunofluorescence (IIF) in dogs without lesions suggestive of American tegumentary leishmaniasis.

| IIF | Dogs without lesions (n) | PCR (blood) | |
|---|---|---|---|
| | | Positive (P/N) | Positivity (%) |
| Positive | 17 | 2/17 | 11.8 |
| Negative | 84 | 14/84 | 16.7 |
| Not done | 3 | 0/3 | 0.0 |
| **Total** | 104 | 16/104 | 15.4 |

P = number positive; N = number analyzed;
p = 1.0000, McNemar test.

both negative direct search and negative PCR from the lesion. Due to difficulty in obtaining this biological material, it is suggested that lymph node aspirate be used only in cases where lesional direct search and PCR are negative.

No association was found between IIF, direct search, and PCR in dogs with lesions. Positive lesional PCR in 57.1% of dogs with negative direct search and positive IIF showed that lesions were due to ATL, confirming the serological results. Positive PCR in 29.6% of dogs with both negative direct search and negative IIF confirms the previous observations that there is no association between serology and presence of lesions [9,31]. According to Uchôa et al. [31], this lack of association is due to the wide immunological window in ATL, whereby in some cases seroconversion takes several months to occur.

*Leishmania* DNA detection in the blood of dogs in endemic areas has been reported by other authors [15,32,33]. In the current study, detection of parasite DNA in the blood of 26.3% of dogs with lesions and 15.4% of those without lesions shows that presence of parasite DNA in blood does not correlate to presence of lesions (p = 0.0973). Reithinger et al. [15] found *Leishmania (Viannia)* DNA in the blood of only 7.11% of asymptomatic dogs in endemic areas of *L. (V). braziliensis* and *L. (V.) peruviana* in Peru, although 18.18% of symptomatic dogs had positive PCR in blood. Still, according to these authors, *Leishmania* DNA was detected in the blood of a large number of dogs that were investigated, suggesting that hematogenous dissemination is more common than previously thought. Blood samples from the majority of dogs with active lesions (positive biopsy smear) were negative in blood PCR [15]. This report agrees with our findings, since *Leishmania* DNA was not detected in the blood of any of the dogs with positive direct search, although 36.4% of the dogs with positive lesional PCR had positive blood PCR. A possible explanation is that *Leishmania (Viannia)* parasites locate first in the cutaneous infection site, and as the infection progresses they disappear from the lesion and spread via the bloodstream. If this explanation is true, dogs could be acting as a secondary ATL reservoir. However, the data accumulated thus far do not allow drawing this conclusion.

Parasite DNA detection in the blood of dogs with negative serology has also been reported previously. Reithinger et al. [32] also found dogs with positive PCR in the blood and negative ELISA and explained the finding by the fact that the dogs had still not developed their immune response to the infection (pre-patent period). Follow-up of these animals showed that 21% eventually became ELISA-positive.

Altogether, use of lesional or blood PCR, direct parasite search, and IIF detected 61.5% of positive dogs among the symptomatic animals and 29.8% among asymptomatic, thus increasing the efficiency of ATL diagnosis in dogs. These tests also showed a high ATL endemicity in the study area. The data show that the use of PCR for detecting *Leishmania* DNA in canine blood, associated with other diagnostic tests, may help determine the extent of ATL sub-clinical infections, in agreement with Reithinger & Davies [34].

The results also show that although lesional PCR can be more expensive when compared to direct parasite search, it may be useful for ATL diagnosis in dogs due to its higher positivity

(3.4 times). Material obtained from lesions by more than one method increases the likelihood of detecting infection in canine lesions, probably because of the scarcity of parasites in lesions. No relationship was observed between serology, presence of active lesions, and detection of parasite DNA in blood. ATL prevalence was high in both symptomatic and asymptomatic animals assessed by direct parasite search, IIF, and PCR. Moreover, these results corroborate previous reports that the parasite's hematogenous dissemination in dogs is not a rare occurrence, reinforcing the hypothesis that dogs may act as a possible secondary ATL reservoir in some areas of *L. (V.) braziliensis* transmission in Brazil. Follow-up studies in dogs in endemic areas are being conducted and may shed new light on the role of canines in the ATL transmission cycle.


## Resumo

*A leishmaniose tegumentar americana (LTA) foi estudada em 143 cães da área rural no Município de Mariluz, noroeste do Estado do Paraná, Brasil, utilizando a pesquisa direta do parasito (PD), a imunofluorescência indireta (IFI) e a reação em cadeia da polimerase (PCR). Trinta e nove cães (27,3%) apresentavam lesões sugestivas da doença, 5 (12,8%) deles foram positivos na PD e PCR em tecido de lesão, e quatro foram também positivos na IFI. Dos 34 cães com PD negativa, 12 (35,3%) tiveram a PCR (lesão) positiva, e cinco desses tiveram também IFI positiva. Cento e quatro cães não apresentavam lesão, mas 17/101 (16,8%) tiveram IFI positiva. A PCR no sangue foi positiva em 10/38 (26,3%) cães com lesão e em 16/104 (15,4%) sem lesão. A associação entre PCR (lesão ou sangue), PD e IFI detectou 24/39 (61,5%) positivos entre os cães sintomáticos e 31/104 (29,8%) positivos entre os assintomáticos. A PCR foi útil para o diagnóstico de LTA, não houve relação entre presença de lesão, sorologia e PCR no sangue, e a detecção de DNA do parasito no sangue pode indicar a ocorrência de disseminação hematogênica do parasito.*



*Leishmaniose;* Leishmania (Viannia) braziliensis; *Reação em Cadeia da Polimerase; Técnica Indireta de Fluorescência para Anticorpo; Cães*


## Contributors

L. G. Velasquez participated in the field collection of biological material, laboratory tests, and elaboration of the manuscript. N. Membrive, U. Membrive, G. Rodrigues, and N. Reis contributed to the field collection of biological material. M. V. C. Lonardoni and U. Teodoro contributed to the data analysis, elaboration of the manuscript, and final revision of the text. I. P. B. Tessmann contributed to the standardization of assays and elaboration of the manuscript. T. G. V. Silveira oriented the research work, contributed to the data analysis, elaboration of the manuscript, and final revision of the text.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