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Molecular and parasitological detection of *Leishmania* spp. in dogs caught in Palmas, TO, Brazil

Detecção molecular e parasitológica de *Leishmania* spp. em cães capturados em Palmas, TO, Brasil

Natália Melquie Monteiro Teles[1]; Maria Augusta Paes Agostini[1]; Júlio Gomes Bigeli[1]; Rosalba Valadares Noleto[1]; Jaqueline Dias Oliveira[1]; Waldesse Piragé de Oliveira Junior[1]*

[1]Laboratório de Biotecnologia – LABIOTEC, Universidade Federal do Tocantins – UFT, Palmas, TO, Brasil

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Abstract

This study evaluated occurrences of *Leishmania infantum* in dogs in the municipality of Palmas, Tocantins, comparing diagnostic data obtained using the polymerase chain reaction (PCR) and parasitological diagnosis. Blood samples and lymph node aspirates were collected from 63 dogs of males and females and various ages and races, with or without owners, between August 2009 and June 2010. Slides containing smears of lymph node aspirates were stained with Giemsa stained. In PCR, the 145 bp target sequence of the LT1 fragment, located in the *Leishmania donovani* kDNA minicircle was detected using the RV1 and RV2 oligonucleotide primers. The chi-square test revealed that there was a significant relationship between the symptoms and dogs that were positive for visceral leishmaniasis (VL). The parasitological investigation showed concordance of 66.7% with PCR on blood and 84.1% with PCR on lymph node aspirate. In addition to these tests, evaluations of the diagnoses in parallel and in series were conducted, which showed concordances with the parasitological test of 76.2% and 74.6%, respectively. The results make it possible to suggest that PCR on lymph nodes should be used in evaluating large populations (surveys) and that the parasitological test should be used for initial clinical evaluations in veterinary consultation offices.

Keywords: PCR, blood, lymph node aspirate, parasite, diagnosis.

Resumo

Avaliou-se a ocorrência de *Leishmania infantum* em cães do município de Palmas-TO, comparando dados diagnósticos obtidos pela Reação em Cadeia da Polimerase (PCR) e pelo diagnóstico parasitológico. Foram coletadas amostras de sangue e de aspirado de linfonodo de 63 cães machos e fêmeas, várias idades e raças, domiciliares ou não de agosto de 2009 a junho de 2010. As lâminas contendo esfregaço dos aspirados de linfonodos foram coradas pelo corante Giemsa. Na PCR, a sequência alvo de 145 pb do fragmento LT1, situado no minicírculo do kDNA do grupo *Leishmania donovani*, foi detectada através dos oligonucleotídeos iniciadores RV1 e RV2. O teste χ^2 (Qui-quadrado), demonstrou haver relação significativa entre a sintomatologia e a positividade dos cães para Leishmaniose Visceral (LV). O exame parasitológico mostrou uma concordância de 66,7% com a PCR em sangue e 84,1% com a PCR de aspirado de linfonodo. Além destas análises, houve a avaliação dos diagnósticos em paralelo e em série, onde as concordâncias com o exame parasitológico foram de 76,2% e 74,6%, respectivamente. Os resultados permitem sugerir a utilização da PCR de linfonodos na avaliação de grandes populações (inquéritos), e o exame parasitológico para a avaliação clínica inicial em consultórios veterinários.

Palavras-chaves: PCR, sangue, aspirado de linfonodos, parasita, diagnóstico.

*__Corresponding author:__ Waldesse Piragé de Oliveira Junior
Laboratório de Biotecnologia – LABIOTEC,
Universidade Federal do Tocantins – UFT, Av. Ns 15, s/n,
ALCNO 14 (109 Norte), CP 114, CEP 77001-090, Palmas, TO, Brasil
e-mail: waldessejunior@mail.uft.edu.br

Introduction

Visceral leishmaniasis (VL), also called *kala-azar*, is an infectious disease with chronic progression (SHAW, 2006). The causal agent of VL is the protozoon *Leishmania (Leishmania) infantum*. In Brazil, as in other places, VL transmission takes place during the heteroxenous life cycle of the parasite. The major VL infection route for humans or animals is through the bites of female phlebotomine sand flies, particularly of the species *Lutzomyia longipalpis* (LAISON; SHAW, 1987). Dogs are considered to be the main domestic reservoirs of the parasite, playing an important role in the epidemiological cycle of VL transmission to human hosts (DANTAS-TORRES, 2007).

Different techniques may be used to diagnose VL in humans and dogs. However, these tests face limitations of various kinds, and differ in terms of sensitivity and specificity, practical applicability, requirements concerning field conditions, and availability of chemicals used (GONTIJO; MELO, 2004). PCR-based gene amplification techniques have gained increasing popularity for *Leishmania* diagnosis (CAMARGO; BARCINSKI, 2003), since these protocols are fast and sensitive, and do not require parasite culture growth (LACHAUD et al., 2002; ASSIS et al., 2010).

Nonetheless, parasitological methods carried out using biopsy specimens or even using material collected by puncture aspiration of the spleen, liver, bone marrow or lymph nodes, are still conventionally used to detect *L. infantum* parasitism. Although one of the possible disadvantages of this method is variable sensitivity, it is highly specific, and is considered to be the gold standard in diagnosing canine VL (BRASIL, 2003; GONTIJO; MELO, 2004).

In this light, the aim of the present study was to evaluate occurrences of the parasite *L. infantum* in blood and lymph node aspirates from dogs sampled in the municipality of Palmas, state of Tocantins, Brazil. The samples were analyzed by means of PCR and the results were compared with diagnoses made in accordance with conventional parasitological procedures.

Methods

1. Study area, canine population and collection of biological specimens

Blood samples and lymph node aspirates were collected from 63 male and female dogs of various ages and breeds that were either kept as pets or had been caught by the Zoonosis Control Center of the municipality of Palmas, state of Tocantins, Brazil. Sample collection took place between August 2009 and June 2010. The dogs were also evaluated in relation to coat length, presence of ectoparasites (*Rhipicephalus sanguineus*) and occurrences of visible signs like low weight, alopecia, scaliness, onychogryphosis and minor crusty injuries. The dogs were also clinically categorized as symptomatic, when they presented one or more clinical signs, and as asymptomatic, when no such signs were present, in accordance with previously defined criteria (MOLINA et al., 1994; MANCIANTI et al., 1988).

Blood samples (3 to 5 mL) were collected by puncturing the brachial or jugular veins. They were stored in sterile tubes containing anticoagulant (EDTA 27 mM) and transported to the laboratory. Lymph node aspirates were collected from the popliteal region using 10-mL syringes. Part of this material was smeared onto slides (in triplicate) and was used in the parasitological investigation, and part was sent to the laboratory for DNA analysis.

2. Parasitological investigation

The smears of lymph node aspirates were fixed in methyl alcohol and dried. Next, the smears were stained using Giemsa. After 30 minutes, the slides were washed in buffered water and left to dry at room temperature. Following this, they were inspected under a microscope. The smears were considered to be positive for VL when at least one parasite in the amastigote stage was present.

3. Molecular diagnosis

Initially, DNA was extracted in accordance with the phenol/chloroform method (SAMBROOK, 2001). DNA was then electrophoresed and quantified by means of spectrophotometry. The DNA specimens were stored in a freezer at –20 °C until PCR analysis.

The 145-bp target sequence of the LT1 fragment, in the kDNA minicircle of the *L. donovani* group (LE FICHOUX et al., 1999; GOMES et al., 2007) was detected by means of PCR using the primers RV1 (5' – CTT TTC TGG TCC CGC GGG TAG G – 3') and RV2 (– 3' CCA CCT GGG CTA TTT TAC ACC A – 5') (LE FICHOUX et al., 1999).

The reactions were conducted using a 20 μL final volume containing 1.5 U/μL of *Taq* DNA polymerase (Invitrogen), 20 mM of tris-HCl buffer (pH 8.4), 50 mM of KCl (Invitrogen), 1.5 mM of $MgCl_2$ (Invitrogen), 0.2 mM of deoxyribonucleotide triphosphate (dNTPs) (Invitrogen), RV1 and RV2 primers (10 pmol each), DNA from the individuals (between 80 and 150 ng/μL) and ultrapure water (12.7 μL) to complete the desired volume. Ultrapure water was used instead of the DNA sample in the negative control. The *L. infantum* DNA strain MHOM/BR2000/MER2 (FIOCRUZ/BA; Merivaldo strain, IOC-LC2455), which had been isolated from a VL patient in an endemic area of the town of Jequié, state of Bahia, Brazil, was used as the positive control (PARANHOS-SILVA et al., 2001). The reactions were carried out in a thermal cycler (PxE 0.2, Thermo Electron Corporation) with the following cycling parameters: 94 °C for 5 minutes; 35 cycles at 94 °C for 45 seconds, 58 °C for 45 seconds and 72 °C for 45 seconds; and an annealing cycle at 72 °C for 10 minutes and 4 °C for 10 minutes. Amplicons were electrophoresed in agarose gels stained with ethidium bromide (0.02 μL/mL). The gels were inspected under UV light and photographed. A 100-bp ladder (Amresco) was used for comparison. Samples that were diagnosed negative at the first examination were retested following the same routine as described above.

4. Data analysis

The characteristics of the canine population sampled and the respective positive results for VL were statistically analyzed using the chi-square (χ^2) test. The degree of agreement between the results obtained from PCR and parasitological investigation was analyzed using the kappa coefficient. Both of these tests were available through the freeware BioEstat version 5.0, and were used with 5% statistical significance. Co-positivity was calculated as a/(a + c), co-negativity was expressed as d/(b + b) and raw concordance was described as (a + d)/(a + b + c + d) (TÁVORA et al., 2007).

Results and Discussion

1. Clinical signs and characteristics of the canine population

As a rule, the characteristics of the canine population investigated varied considerably (Table 1). The signs most commonly noticed were alopecia, low weight, onychogryphosis and minor crusty injuries.

2. Parasitological findings

The parasitological examinations revealed that 26 dogs (36%) were positive for VL, of which 13 (50%) were males and 13 (50%) were females. However, the χ^2 test did not show any statistically significant association between the majority of the characteristics evaluated and positive results from the parasitological examination. This finding is in agreement with the results from Saridomichelakis et al. (2005), who also used the technique to analyze lymph node and bone marrow smears, and Azevedo et al. (2008), who equally did not observe any statistically significant difference between the sexes regarding positive canine VL diagnoses using serological methods. Likewise, the authors of that study did not find any significant association between positive VL results and the dogs' sex. Concerning infestation by ectoparasites, in spite of the potential role that ticks (*R. sanguineus*) may play as vectors in leishmaniasis, which was previously investigated in an interesting paper by Coutinho et al. (2005), no statistically significant difference in VL detection was observed in the present study between dogs with and without tick infestation.

The associations shown by clinical signs with positive VL results in dogs are presented in Table 2. A statistically significant association was observed between the signs and infection by *L. infantum*, though this result disagrees with the serological findings reported for canine VL in a previous study (SANTOS et al., 2010). Nevertheless, it is important to stress that not every symptomatic dog was diagnosed as VL-positive, which agrees with the conclusions reached by Osman et al. (1997) and Dantas-Torres et al. (2006). Azevedo et al. (2008) obtained a somewhat disturbing and opposite result, reporting that 55.2% of VL-positive dogs in their study were asymptomatic. Their findings are a matter of concern, because they suggested that VL could not be easily associated with infected dogs, since those animals did not show signs of the disease, even though they played an important role in VL transmission (AZEVEDO et al., 2008). These results indicate that characterization of VL-positive or negative status based on clinical examination should be made with caution (OSMAN et al., 1997).

Table 1. Characteristics and clinical signs of the canine population living in the municipality of Palmas, Tocantins, Brazil.

Characteristics	Class	N	(%)
Sex	Female	31	49.2
N = 63	Male	32	50.8
Breed	DB	19	30.2
N = 63	NDB	44	69.8
Size	Large	24	38.1
N = 63	Medium	23	36.5
	Small	16	25.4
Origin	Non-stray*	14	22.2
N = 63	Stray	49	77.8
Coat	Long	12	19
N = 63	Small	51	81
Ectoparasites	Presence	22	34.9
N = 63	Absent	41	65.1
Symptoms	Symptomatic	9	14.3
N = 63	Asymptomatic	54	85.7

DB: Defined breed; NDB: No defined breed; *Dogs under the care of their owners; N: Number.

Table 2. Positive findings of *L. infantum* in parasitological investigations using smears from dogs' lymph node aspirates.

Characteristics	N	NPD	χ^2	p-value
Sex			0.023	0.8805
Male	32	13		
Female	31	13		
Coat			0.127	0.7212
Short	51	20		
Long	12	6		
Ectoparasites			2.556	0.1099
Present	23	13		
Absent	40	13		
Symptoms			5.526	0.0187
Asymptomatic	9	0		
Symptomatic	54	26		

N: Number of dogs; NPD: Number of VL-positive dogs.

3. Comparison between parasitological findings and molecular results

Table 3 shows the comparison between the parasitological findings and PCR analysis relating to VL in 63 dogs. Identical parasitological findings and PCR analyses of blood samples was observed for 42 dogs, thus representing a concordance rate of 66.7%. However, 21 dogs presented discordant parasitological and PCR diagnoses for *L. infantum*. For the lymph node samples, concordance was observed for 53 dogs (84.1%), while for 10 animals, the two techniques produced discordant results.

Table 3. Comparison between parasitological results and blood and lymph node PCR results, in parallel and serially, for dogs living in the municipality of Palmas, Tocantins, Brazil.

PCR test	Parasitological examination				
	Concordant			Discordant	
	Co-positive	Co-negative	Concordance (%) (Kappa; p-value)	Positive parasite × negative PCR	Negative parasite × positive PCR
PCR blood	11	31	42 (66.7) (0.2751; 0.0108)	15	6
PCR Lymph nodes	26	27	53 (84.1) (0.6903; <0.0001)	0	10
PCR in parallel	26	22	48 (76.2) (0.5476; <0.0001)	0	15
PCR serial	11	36	47 (74.6) (0.4305; <0.0001)	24	5

As expected, the concordance between parasitological findings and blood PCR results was lower than for the lymph node PCR results. These differences may be explained in the light of the fact that infection by *Leishmania* starts in blood, a medium in which immune system cells are present, and quickly proceeds to infect the viscera, preferentially in the macrophagic system of the spleen and in the liver, bone marrow and lymphoid organs, thus demonstrating the influence of the VL stage on detection of the protozoan (REY, 2008). These differences were also observed when the PCR results from blood samples were compared to those from lymph node aspirates, with low percentage concordance (54%; kappa = 0.0833; p-value = 0.2183). Such divergences may be explained through considering that in lymph nodes, the parasite is subject to confinement during the dog's immune response, or to different stages of the infection, when the parasite may or may not be present in the blood flow.

Another reason for the divergence in the results may be the low sensitivity of PCR on blood samples, possibly due to the low numbers of parasites usually detected in these samples (FISA et al., 2001; MANNA et al., 2004; NUNES et al., 2007). On the other hand, other biological materials like skin, lymph node aspirates, bone marrow aspirates and leukocyte cream (also called buffy coat) present higher sensitivity in the PCR technique (REITHINGER et al., 2000; MANNA et al., 2004). Furthermore, there is evidence that inhibitors (heme groups) of *Taq* play an interfering role during PCR on blood samples (ABU AL-SOUD; RÂNDSTROM, 1998; REITHINGER et al., 2000). Nevertheless, use of blood as a biological analysis material is justified given the simplicity of collecting it, with less invasiveness, especially when a large number of animals are analyzed. However, independently of the biological analysis material used, it is important to stress that PCR does not produce false positive diagnoses (FALLAH et al., 2011).

In addition to these analyses, we also analyzed diagnoses in parallel. In this, the dogs that produced positive PCR results in at least one of the types of sample used (blood or lymph node aspirates) were considered to be VL-positive and any dog with negative PCR results in both samples at the same time was considered to be VL-negative. Using this method, 41 (65.1%) of the 63 dogs evaluated were VL-positive in PCR analyses on blood or lymph nodes and, among these, 15 (36.6%) had negative parasitological results. In turn, 26 animals (41.7%) had positive parasitological findings which were not confirmed by PCR using either blood or lymph node aspirates. In this regard, the concordance between the techniques was 76.2%.

We also analyzed these results serially, i.e. the animals were considered to be infected by *L. infantum* when both blood and lymph node aspirates produced positive PCR results. Here, the concordance with parasitological findings was 74.6% (Table 3). Analysis of the results in parallel did not show any difference of enough importance to justify its use, since the PCR results from lymph nodes were similar and therefore the technique can be used alone.

The results from the present study make it possible to suggest that diagnostic procedures like analysis of lymph nodes through PCR can be used to investigate large populations (surveys), while parasitological examinations for initial clinical evaluation can be used in veterinary consultation offices. PCR protocols are more automated, do not produce false positive results and allow analysis on large numbers of samples. In turn, parasitological investigation is technically easier, demands less training, is less costly and may be useful for examinations on individual animals, even though it is more labor-intensive.

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