Whole blood PCR test and laboratorial findings in natural canine visceral leishmaniasis in Brazil

1Giane Regina Paludo, 1Larissa Campos Aquino, 1Bruna de Carvalho Cabral Lopes, 1Patrícia Helena Caldeira Silva, 1Thaís Sermoud Borges, 2Mady M. Barbeitas, 3César Omar Carranza-Tamayo, 4Maria Isabel Rao Bofill, 5Márcio Botelho de Castro

1Laboratories of Veterinary Clinical Pathology; 
2Veterinary Pathology, Agronomy and Veterinary Medicine College, University of Brasília, Brazil; 
3Tropical Medicine Center, Medicine College, University of Brasília, Brazil; and 
4Zoonosis Control Center of Distrito Federal, Brasília, Brazil.

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ABSTRACT
Leishmaniasis is a worldwide spread zoonosis. The agent responsible for the disease is the protozoan Leishmania spp which is transmitted by the vector of genus Lutzomyia. In urban areas, the domestic dog is the main reservoir host for human infection. This study was carried out in order to evaluate a molecular essay (PCR) as a tool in diagnostic of canine visceral leishmaniasis, and the mainly hematological and biochemical alterations observed in infected dogs from Brasília, Brazil. Whole blood PCR, hemograms, ALT, AST, urea, creatinine, total protein, albumin and globulin were performed in thirty animals serologically positive to Leishmania spp. Lymph nodes, spleen and bone marrow were submitted to parasitological evaluation. Three negative animals were submitted to PCR to evaluate specificity. From those serological positive samples used in this study, twenty (67%) were also positive by PCR. Parasitological analysis resulted in 95% agreement with whole blood PCR results. None of negative controls tested had DNA fragments visualized, showing 100% specificity of PCR essay. The PCR-positive animals had a mild normocytic normochromic anemia, dysproteinemia, and azotemia. A mild normocytic normochromic anemia, dysproteinemia, and azotemia were observed in Leishmania infected dogs from Brazil, which are not canine visceral leishmaniasis exclusive, but they may be useful as a differential diagnostic from other diseases as well as they may stimulate the clinicians for considering visceral leishmaniasis in their diagnostic assumption. Whole blood PCR method could be used as a fast, sensible and specific tool in diagnostic of canine visceral leishmaniasis.

INTRODUCTION
Visceral leishmaniasis is a zoonotic, vector-borne protozoal disease that is associated with chronic, debilitating illness in humans and dogs. The main vector for leishmaniasis is the phlebotomine sandfly and both wild and domestic dogs are the primary reservoir hosts for human visceral leishmaniasis in endemic regions (Irwin 2002).

Leishmaniasis is also widespread disease and is endemic in 88 countries. The majority of visceral leishmaniasis cases occur in rural and suburban areas of 5 countries, including Brazil (Desjeux 2004). The first autochthonous human case in a suburban area of Brazil’s capital, Brasília, was diagnosed in 2005. As a control strategy recommended by the Brazilian Ministry of Health the whole dog population from that area was serologically tested and the positive ones euthanized.

Due to absence of pathognomonic signs of canine leishmaniasis and to the high proportion of asymptomatic animals diagnosis must be confirmed by serological and molecular methods (Ozbel et al. 2000).

The most frequent clinical signs found in infected animals are lymphadenomegaly, weight loss, dermatological changes and onychogryphosis. However, these clinical signs are variable, and are many times found in other diseases thus precluding doubtless clinical diagnosis. Among laboratorial findings of dogs with leishmaniasis, it is common to observe dysproteinemia, normocytic normochromic anemia, leukopenia, and lymphopenia (Corona et al. 2004; da Costa-Val et al. 2007; Reis et al. 2006).

Canine visceral leishmaniasis diagnosis is generally performed by serological tests, such as indirect immunofluorescence assay test (IFAT) and enzyme linked immunosorbent assay (ELISA), but they present a
broad range of cross-reactions with other infectious agents (Reis et al. 2006). In contrast, parasitological diagnosis presents a high specificity and is considered as the gold standard for visceral leishmaniasis, but this method is invasive and has a wide sensitivity range (from 58% to 95%) (Desjeux 2004). Molecular assays, as polymerase chain reaction (PCR), have been increasingly used as diagnostic tools. PCR has been performed from liver, spleen, lymph node (Gomes et al. 2007; Soares et al. 2005) and kidneys samples (Soares et al. 2005) presenting effective results as a tool for the diagnosis of Leishmania infection. At the same time only a few studies using PCR of peripheral blood have been described for canine leishmaniasis (Maia et al. 2007). Whole blood PCR is a non-invasive and rapid procedure that could be useful for the diagnosis of leishmaniasis. Thus this study was carried out to evaluate a molecular essay (PCR) using whole blood samples in diagnosis of canine visceral leishmaniasis, as well as to observe the main hematological and serum biochemical alterations in naturally infected dogs from Brasilia, Brazil.

MATERIAL AND METHODS

Animals:
Thirty dogs positive by serological test (IFAT) to visceral leishmaniasis were used in this study. These animals were from the first autochthonous human case area in Brasilia (Sobradinho II), Center West Region of Brazil. Samples collection as well as clinical evaluation of animals was done from October, 2006 to June, 2007.

Blood Samples:
Blood samples were obtained from either cephalic or jugular veins and collected into tubes containing EDTA for DNA extraction and hematologic analysis or into tubes containing no anticoagulant for clinical biochemical analysis.

Parasitological Evaluation:
Parasitological evaluation was performed by the analysis of lymph node, spleen and bone marrow cell smears. Fine-needle biopsy (FNB) of popliteal lymph nodes was performed during clinical inspection. Bone marrow and spleen samples were collected during necropsy procedure (all dogs were euthanized in the Zoonosis Control Center following Brazilian Health Department recommendation). At least three slides smears were prepared from each tissue and stained with Giemsa. Direct Leishmania amastigotes search was then performed by light microscopy examination of cell smears of lymph nodes. When parasitological evaluation was negative in lymph node aspirations, spleen and bone marrow smears were analyzed.

PCR:
Whole blood samples were submitted to DNA extraction using a commercial kit (QIAamp DNA blood mini kit, Qiagen, USA).

For the standardization of PCR, DNA from a dog with parasitological diagnostic was used. One healthy dog DNA and two ehrlichiosis positive dog DNA were tested as negative controls to confirm PCR specificity.

The PCR was performed using 3 oligonucleotides (one sense: GGG TAG GGG CGT TCT GCG AA and a 1:1 mixture of antisense primers: CCG CCC CTA TTT TAC ACC AAC CCC/ GGC CCA CTA TAT TAC ACC AAC CCC) that anneal to the origin of replication of both strands of the minicircle molecule of kDNA of the parasite (Disch et al. 2003). Reaction mixtures consisted of approximately 10 ng of template DNA, one time PCR buffer, 2,0 mM MgCl2, 250 µM of each deoxynucleotide, 0,6µM of each primer, and 1,5 unit Taq DNA polymerase in a 25 µL reaction volume. PCR was performed by using a thermal cycler (FTGene5D – Techgene, UK) with 94°C for 30 s, 63°C for 30 s, and 72°C for 10 s for 40 cycles. The products were electrophoresed on a 2% agarose gel and stained with ethidium bromide, resulting in a 120 bp band in the positive ones.

Hematologic and Clinical Biochemical Analysis:
For hematology, erythrocyte and leukocyte counts and the concentration of hemoglobin were obtained using an semi-automatic cell counter (CC 550 – CELM™, Brazil) while the number of platelets was obtained by manual counting on a hemocytometer. The PCV was determined by microhematocrit centrifugation. Differential leukocyte counts were obtained by direct observation of 100 leukocytes in Giemsa-stained blood smears with a light microscope (CX40RF200, Olympus, Japan) and absolute counts were calculated. Mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were calculated.

Plasma protein concentration was determined by refractometry.

Serum samples were analyzed for concentrations of total protein, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, and creatinine, using commercial kits (Labtest™, Brazil) performed in a semi-automatic biochemistry analyzer (Bio2000 – Bioplus™, Brazil). Values for the concentration of globulins and the albumin-globulin ratio were calculated.
Statistical Analysis:
The data were compared using a Student’s t-test and considered statistically significant if p ≤ 0.05.

Results:
At clinical inspection, most of animals showed poor body condition, emaciation, alopecia and lymphadenomegaly.
From the thirty IFAT positive for visceral leishmaniasis, only twenty (67%) were PCR positive. None of negative controls tested had Leishmania DNA fragments detected showing 100% specificity of PCR essay (Table 1).
Leishmania amastigotes forms were visualized in seventeen of lymph node preparations and additional two dogs were confirmed only after spleen and bone marrow smear evaluation, resulting in nineteen positive animals (63%). Parasitological analysis resulted in 95% agreement with whole blood PCR results.
Hematologic results are shown in Table 2. For comparison purposes dogs were grouped into either Leishmania positive (IFAT and PCR positive) or Leishmania negative (IFAT positive) group. Leishmania positive animals presented a normocytic normochromic anemia. Leukogram did not differ between negative and positive animals when compared with reference values. Despite of a slight thrombocytopenia observed in positive dogs, they did not differ significantly from the negative ones.
Serum biochemical results are shown in Table 3. The biochemical analysis indicated increased urea and creatinine serum levels, hyperproteinemia and hyperglobulinemia in Leishmania positive animals (p<0,05). Negative animals presented solely an increase in serum urea levels. Considering reference values, a discrete hypoalbuminemia was observed in positive animals, but there was no difference (p>0,05) between groups.

Discussion:
As most of dogs belonged to owners with lower socioeconomic conditions or were free-roaming dogs, the poor body condition, emaciation, alopecia and lymphadenomegaly observed at clinical inspection in most of animals could be either due leishmaniasis, unbalanced diet or other concurrent disease. However canine visceral
leishmaniasis diagnosis can be considered a major difficulty and a challenge for disease control, methodologies commonly used are based on serological (IFAT and ELISA) and parasitological assays (Desjeux 2004). IFAT is the main tool used in Brazil during screening programs. However, it was demonstrated that IFAT can either lead to false-positive and false-negative results (Desjeux 2004). Parasitological diagnosis based on lymph node and spleen cell smears require more invasive methods and their average sensitivity varies from 58% to 95%, respectively (Desjeux 2004). Molecular assays especially for infectious diseases diagnosis, including leishmaniasis, are being increasingly used in the field. In this study, whole blood PCR demonstrated to be a useful test in the diagnosis of canine visceral leishmaniasis. This statement was confirmed by 85% and 95% of agreement from lymph node and spleen/bone marrow preparations, respectively (Table 1). Another important fact is that PCR from a blood sample is a non-invasive technique and fast to perform. Its only drawback, its higher cost, is steadily decreasing over the years.

Parasitological examination is not always sensible enough, especially in asymptomatic dogs with no lymphadenopathy. In fact in two PCR positives animals, amastigotes forms were only found in bone marrow or spleen preparations. In the present study, lymph node cytology demonstrated a higher sensitivity (85%) than reported before (58%)(Desjeux 2004). This could be explained by the fact that the animals were from an endemic area during the first leishmaniasis outbreak in Brasília, which may have corroborated to the higher number of infected animals and, as a consequence, the amount of dogs cytology positive. It is also important to notice that the animals may have been infected for a long time without diagnosis, which could have increased the number of *Leishmania* amastigotes forms in tissues, improving parasitological evaluation results.

Several hematological and biochemical alterations have been described in dogs infected by *Leishmania spp.* A significant decrease in PCV and hemoglobin concentration, which characterize an anemia was observed in positive animals as reported previously (da Costa-Val et al. 2007; Reis et al. 2006). As showed in Table 2, the positive animals had a mild normocytic normochromic anemia, which could be associated with inflammatory processes and chronic infections. The pathogenesis of anemia of inflammatory disease is multifactorial, mediated by cytokines secreted during inflammation (Waner and Harrus 2000). Leukopenia, due to reduction in monocytes, eosinophils and mainly in lymphocytes, was also described (Reis et al. 2006). The animals of this study did not have any alterations in their leukograms, as was also reported previously (da Costa-Val et al. 2007). Thrombocytopenia observed could be due multiple factors involved, indicating that *Leishmania* infection may affect primary hemostasis (Gopegui 2000). In this study, positive and negative animals had hyperproteinemia and hyperglobulinemia characterizing a dysproteinemia, but positive animals showed higher medium protein and globulin levels than negative ones. Hyperproteinemia and decrease of albumin and increase of γ globulin are a remarkable characteristic of canine visceral leishmaniasis (Almeida et al. 2005; Gopegui 2000). On the other side, dysproteinemias due to hyperglobulinemias are observed in acute and chronic inflammatory diseases (Kaneko 1997). Considering that the population of dogs used in this study belonged to a poor suburban area of Brasília, and most of them were maintained in precarious sanitary condition, thus dysproteinemias may have occurred due other concurrent infections. Hypergammaglobulinemia associated to canine leishmaniasis is caused by polyclonal activation of B lymphocytes and dysregulation of T lymphocytes (Almeida et al. 2005; Gopegui 2000). Hypoalbuminemia observed in positive animals may be due to either albumin loss or failure of albumin synthesis due to any extensive inflammation (Kaneko 1997).

Following *Leishmania* infection, the parasite is carried out to the lymph node, bone marrow, spleen, liver, kidney, lungs and gastrointestinal tract. An active chronic hepatitis may be observed in canine visceral leishmaniasis due to parasite multiplication within macrophages in the liver (Ikeda-Garcia et al. 2007). Herein, the canine population studied showed no significative (p>0.05) hepatic enzyme alteration despite an increasing activity of serum ALT and AST previously described in up to 82% of natural canine leishmaniasis cases (Slappendel 1988). No increase in ALT activity was also observed in 26 naturally infected dogs with leishmaniasis (Rallis et al. 2005). The absence of increased ALT activity probably indicated slowly progressive disease, rather than an extensive and acute inflammation with necrosis of hepatocytes (Rallis et al. 2005), indicating that the severity of hepatic lesions varies widely probably due to individual variations in the immunological response of the host (Gonzalez et al. 1988).

Immune complexes deposition in kidney capillaries occasionally results in proliferative glomerulonephritis and interstitialal nephritis, which may lead to azotemia (Ikeda-Garcia et al. 2007). Renal insufficiency may develop when moderate or severe renal lesions were present, or in patients with systemic complications such as secondary infections, sepsis, and hypotension insufficiency (Costa et al. 2003). Kidney involvement observed in this study was supported by the increase of urea and creatinine serum levels in positive animals (p<0.05) when compared with negative ones. Despite alterations in serum creatinine have been more related to tubulointerstitial alterations than to the severity of glomerular lesions, increased creatinemia was also reported in dogs with focal segmental glomerulosclerosis, membranoproliferative glomerulonephritis, and chronic glomerulonephritis(Costa et al. 2003). These observations suggest an important contribution of glomerular lesions to functional alterations, which may be related to the severity or chronicity of the lesions in leishmaniasis (Costa et al. 2003). The creatinine serum levels presented by infected dogs in this study suggest...
renal lesions caused by *Leishmania* infection. The discrete hypoalbuminemia previously described in positive animals may also be caused by this renal involvement.

Although serum urea increased in both groups, infected dogs had higher values (p<0.05) than non-infected ones. Urea levels may be influenced by nutritional and hydration status, renal function, fever, and other conditions (Kaneko *et al*. 1997). Dehydration may cause hypovolemia, which leads to impaired excretion of urea and creatinine secondary to reduced renal blood flow and glomerular filtration rate (Finco 1997). As nonrenal losses of urea are less than those for creatinine, urea increases due to renal failure may be earlier detected than creatinine ones. Another important fact that should be considered is that creatinine is easily diffusible through renal tissue (Finco 1995). Probably, the increase of urea serum levels in positive dogs may be due to renal lesion confirmed by concomitant increasing in creatinine levels. Increased urea levels observed in negative dogs should be attributed to metabolism, nutritional or hydration status since there was no alteration in serum creatinine.

**Conclusions:**

In conclusion, as serological techniques have variable specificity and sensitivity, and fine-needle aspiration of lymph nodes is an invasive technique, PCR performed from whole blood samples could be a useful routine tool for clinicians. The results indicate that PCR from blood samples can be used as a specific and sensible test for canine visceral leishmaniasis. According to higher concordance between parasitological analysis and PCR, the later method may be recommended as a diagnostic tool in canine visceral leishmaniasis. In endemic areas, leishmaniasis must be in the mind of clinician treating a patient with normocytic normochromic anemia, dysproteinemia and renal involvement.

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