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CHARACTERIZATION OF CANINE LEISHMANIASIS BY PCR-RFLP IN CUIABA, MATO GROSSO, BRAZIL

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ABSTRACT: Leishmaniases are neglected zoonoses that are increasing in Brazil. The dog is considered the main reservoir of the visceral form in urban areas of Brazil and also important in maintaining the cycle of transmission of the cutaneous form in endemic areas. We used PCR-RFLP to identify the species of *Leishmania* involved in canine infection in Cuiaba City, Mato Grosso. Samples of bone marrow and lymph node were collected from 181 dogs, of which 7.2% tested positive with indirect immunofluorescence and 24.9% using PCR-RFLP with a significant difference (P≤0.05). It was possible to characterize the species *Leishmania (L.) chagasi.* This will aid in developing prevention measures and in the control of disease in Cuiaba and the surrounding area.
Key Words: molecular diagnosis, dog, *Leishmania*, Mato Grosso

CARACTERIZAÇÃO DA LEISHMANIOSE CANINA POR PCR-RFLP EM CUIABÁ, MATO GROSSO, BRASIL

RESUMO: Leishmanioses são zoonoses negligenciadas que estão aumentando no Brasil. O cão é considerado o principal reservatório da forma visceral em áreas urbanas do Brasil e também importantes na manutenção do ciclo de transmissão da forma cutânea em áreas endêmicas. Usaram-se PCR-RFLP para identificar as espécies de *Leishmania* envolvidas na infecção canina na cidade de Cuiabá, Estado de Mato Grosso. Amostras de medula óssea e linfonodos foram coletadas de 181 cães, dos quais 7.2% foram positivos na imunofluorescência indireta e 24.9% pela PCR-RFLP, com diferença significativa (P≤0,05), sendo possível caracterizar as espécies como *Leishmania (L.) chagasi*. Isso ajuda no desenvolvimento de medidas de prevenção e no controle da doença em Cuiabá e arredores.
Palavras-chave: cão, diagnóstico molecular, *Leishmania*, Mato Grosso

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INTRODUCTION

The leishmaniases are a major public health concern, causing cutaneous infections and systemic involvement, and transmitted by sandflies of the genus *Lutzomyia* in the Americas. Visceral leishmaniasis caused by *Leishmania (Leishmania) chagasi* (= syn *L. infantum*) is the most serious form of the disease, while cutaneous leishmaniasis in Brazil is caused by infection with *Leishmania (Viannia) braziliensis*. In recent years, leishmaniasis in Brazil has undergone changes in its epidemiology. Initially, the disease was typically rural but is now an urban zoonosis (Ashford, 2000), with reports of human cases in four regions, due the deforestation and migration to the major population centers (Silva *et al.*, 2005).

Several species of mammals are subject to infection by *Leishmania* spp in Brazil, but the dog is considered the main reservoir for transmission of *L. (L.) chagasi* (Dantas-Torres, 2007). The serological techniques are recommended for epidemiological investigations by the Ministry of Health in Brazil (MS, 2006). In recent years, PCR has been used as a sensitive and specific technique for detecting DNA of *Leishmania* in biological samples (Ikonomopoulos *et al.*, 2003).

Mato Grosso State is considered endemic for both visceral and cutaneous leishmaniases, showing an increase in reporting of these diseases (SES-MT, 2008). This study aimed to characterize the species of *Leishmania* involved in canine leishmaniasis in Cuiaba city by PCR, through the use of restriction enzymes (PCR-RFLP). The identification of the species is important to the control of the disease in this area.

MATERIAL AND METHODS

Samples of blood, bone marrow, and lymph node were obtained from 181 dogs of various breeds, aged more than six months old and of both sexes, from four districts of Cuiaba, where cases of diseased dogs had been diagnosed at the Veterinary Hospital of the Federal University of Mato Grosso (HOVET-UFMT).

The blood, bone marrow and lymph node samples were collected by jugular venipuncture, xiphoid process of the sternum following antisepsis and local anesthesia with 2% lidocaine and popliteal lymph node, respectively, upon free consent of the pet owner, the latter two were added to 0.5 mL sterile 0.9% NaCl, subsequently stored frozen at -20°C until use. Indirect immunofluorescence was performed using a commercial kit (BioManguinhos ®/FIOCRUZ) following the manufacturer's directions.

To extract DNA, samples were dissolved in lysis buffer (10 mM Tris-HCl pH 8.0, 25 mM EDTA, 100 mM NaCl, 0.5% SDS, and 100 µg/ml proteinase K), and the mixture incubated at 56ºC for 12-18 h. DNA was extracted by the phenol-chloroform method and precipitated with isopropanol. After washing in 70% ethanol for 10 min at 10 000 x g, the DNA precipitate was dissolved in ultra pure water (Gomes *et al.*, 2007). The polymerase chain reaction was conducted using the primers 150 (sense) 5'-GGG (G/T) AGGGGCGTTCT (C/G) CGAA-3 'and 152 (antisense) 5' (C/G) (C/G) (C/G) (A/T) CTAT (A/T) TTACACCAACCCC-3' (Degrave *et al.*, 1994), which amplified a fragment of DNA from a 120 bp region of conserved minicircle kDNA present in all species of *Leishmania*. The amplification used 200 µM of dNTP, 1 µM of each primer, buffer solution (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 2 mM $MgCl_2$, 1.5 U of Taq DNA polymerase, and 5 µl of the

DNA sample in a final volume of 20 µl. Conditions were one cycle at 94ºC for 4 min, followed by 30 cycles at 94ºC for 30 s, 56ºC for 30 s, 72°C for 30 s, and a final step extension of one cycle at 72ºC for 10 min. The amplification product was fractionated by electrophoresis in 2.0% agarose gel stained with ethidium bromide and analyzed in a transilluminator at 300 nm.

PCR-RFLP mkDNA was carried out according to Andrade *et al.* (2006) with some modifications. Five µl of PCR product were digested by 1U HaeIII enzyme and incubated for 3 h at 37ºC. Restriction fragments were separated in 10% polyacrylamide gel and stained with ethidium bromide. The fragments obtained were compared with standard strains of *L. (L.) chagasi* (MHOM/BR/74/PP75) and *L. (V.) braziliensis* (MHOM/BR/75/M2903) from the Oswaldo Cruz Institute/FIOCRUZ, Rio de Janeiro.

Statistical analysis was performed using Epi Info, version 3.3.2 (CDC, Atlanta), through the χ^2 test, adopted significance level was 5%, and Kappa index (k) was calculated for analysis of concordance between tests. Sensitivity (S) and specificity (SP) of PCR were calculated by indirect fluorescent antibody test – IFAT as the gold standard.

RESULTS

Of the 181 sera tested, 13 (7.2%) showed anti-*Leishmania* using IFAT. Forty-five dogs (24.9%) tested positive with PCR. PCR showed a sensitivity of 53.8% and specificity of 77% compared with IFAT, with a low correlation between the two tests (*k* 0,14) and significant difference P≤0.05 (Table 1). Sensitivity in detection of DNA of *Leishmania* spp. in an individual dog was significantly higher (P=0.0039) when using bone marrow (37) compared to lymph node for PCR (15).

Table 1 - Performance of polymerase chain reaction (PCR) taking indirect immunofluorescence (IFAT) as the gold standard to diagnose canine leishmaniasis.

PCR	IFAT		S (%)	SP (%)	*k*	P
	Positive	Negative				
Global**						
Positive	7	38	53.84	77	0.14	0.029*
Negative	6	130				
Bone marrow						
Positive	6	31	53.84	82	0.16	0.04*
Negative	7	137				
Lymph node						
Positive	4	11	30.76	93	0.21	
Negative	9	157				

S, sensitivity, SP, specificity, *k*, Kappa index, * Significant at 5% probability by χ^2, ** Both bone marrow and lymph node.

In six dogs showing antibodies detected by IFAT, there was no amplification of DNA of *Leishmania* sp. The titers of antibodies produced by these animals were 1:40 in four dogs and 1:80 and 1:160 in the remaining two. In dogs testing positive with PCR, three (6.7%) were symptomatic, 11 (24.4%) oligosymptomatic and 31 (68.9%) asymptomatic. In dogs testing positive with IIF the numbers were three, three, and seven, respectively, and these data were not significantly different from PCR results (P≥0.05). PCR-RFLP using the restriction enzyme HaeIII for identified species in these samples as *Leishmania (Leishmania) chagasi*.

DISCUSSION

Serological tests, such as ELISA and IFAT (MS, 2006), are traditionally used in Brazil to estimate the prevalence of leishmaniasis in dogs. These methods have been challenged, since the removal of seropositive dogs has not led to a decrease the occurrence of the disease (Silva *et al.*, 2005). Studies have shown PCR to be effective in the diagnosis of leishmaniasis in dogs and humans (Osman *et al.*, 1997; Fisa *et al.*, 2001). In the present study, PCR detected 45 of 181 (24.9%) dogs positive for leishmaniasis, compared to 13 (7.2%) using IFAT. The superiority of

PCR for detection of canine infection has been reported by others (Solano-Gallego *et al.*, 2001). The sensitivity and specificity of molecular methods are dependent on the DNA sequences used as primers for PCR. In this study, a pair of primers that amplify a sequence of 120 bp DNA of *Leishmania* spp. was used which Lachaud *et al.* (2002) reported to be the most effective in detecting infection of five assessed.

Six seropositive dogs tested negative with PCR; however antibody titration was at the lower limit level. The presence of *Leishmania* antibodies without detection of DNA may be attributed to antibodies remaining after elimination of the parasite or to a cross-reaction with other parasites such as *Trypanosoma*, as described by Vexenat *et al.* (1996). However, according to Francino *et al.* (2006), conventional PCR can result in false negatives when the number of parasites in a sample is small. In this study, the sensitivity of PCR may have been decreased in asymptomatic animals, where samples were of small volume, this was observed by Ikonomopoulos *et al.* (2003), who mention the ease of blood and bone marrow sample collection and obtained a greater volume of blood offering higher detection of parasite DNA.

The low sensitivity of epidemiological tests contributes to failure in control programs of leishmaniasis because asymptomatic dogs are not eliminated in endemic areas. Clinically, 31 (68.9%) of dogs with the amplification of DNA of *Leishmania* spp were asymptomatic at the time of collecting biological samples. This was true of nine identified by IFAT (69.2%), which was not significantly different. Francino *et al.* (2006) reported an increased capacity of PCR to detect asymptomatic dogs compared with serologic tests.

Various biological samples may be used in PCR for diagnosis of leishmaniasis (Fisa *et al.*, 2001). Manna *et al.* (2004) lymph node aspirate showed high sensitivity in diagnosing infection, but in this study the sensitivity of lymph node was significantly lower than bone marrow ($P \leq 0.05$).

According to Ikonomopoulos *et al.* (2003) it is more effective to diagnose leishmaniasis by PCR in symptomatic animals, due to the number of organs affected and consequent greater quantity of sample. However, this may difficult in epidemiological investigations with a greater proportion of asymptomatic dogs (Alvar *et al.*, 2004).

In this study, the restriction enzyme HaeIII, which, according to Volpini *et al.* (2004), can identify *Leishmania* species, indicated the species of *Leishmania* infecting dogs in Cuiaba to be *L.* (*L.*) *chagasi*, the agent of visceral leishmaniasis in Brazil. Despite being a cutaneous *Leishmania* tropism, *L.* (*V.*) *braziliensis* is often isolated from skin lesions (Madeira *et al.*, 2006). Andrade *et al.* (2006) identified it in samples of skin, bone marrow, liver and spleen of dogs in Belo Horizonte, Minas Gerais using the technique employed in this study, and demonstrating the higher sensitivity of molecular methods for detecting *Leishmania* spp infections compared to parasitological methods.

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INFORMATIVE NOTES

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