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Diagnosis of Canine Leishmaniasis in the Endemic Area of Belo Horizonte, Minas Gerais, Brazil by Parasite, Antibody and DNA Detection Assays

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ABSTRACT

Canine leishmaniasis caused by *Leishmania chagasi* (*L. infantum*) is found throughout the South American continent, including Brazil, and dogs are considered to be the main reservoir host for this parasite. To support the implementation of a diagnostic protocol for surveillance of the disease in the region of Belo Horizonte (Minas Gerais, Brazil) we have compared the sensitivity and specificity of two serological tests, indirect immunofluorescent antibody test (IFAT) and direct agglutination test (DAT), with the combination of direct microscopy–culture–PCR as the gold standard, using samples obtained from 103 dogs in the city of Belo Horizonte, Minas Gerais. The currently used standard serodiagnostic test, IFAT, had a sensitivity of 100% and its specificity was 74% compared to the gold standard of the study. The sensitivity and specificity of the DAT were 100% and 91%, respectively. On the basis of this study it is recommended to change from the IFAT to DAT for the serodiagnosis of canine leishmaniasis because of the superior specificity of the test combined with its user-friendliness.

Keywords: diagnosis, dog, leishmaniasis

Abbreviations: CanL, canine leishmaniasis; DAT, direct agglutination test; IFAT, indirect immunofluorescent antibody test; MRBH, Metropolitan Region of Belo Horizonte; NNN, Novy–McNeal–Nicolle; PCR, polymerase chain reaction

INTRODUCTION

Leishmaniasis is present in 88 countries and, worldwide, more than 350 million people are exposed to the infection (Desjeux, 2001). In Brazil, the visceral form of leishmaniasis, caused by *Leishmania chagasi* (*L. infantum*), is considered to be a zoonotic disease formerly restricted to rural and peri-urban areas. However, in the last few years the number of human cases of American visceral leishmaniasis (AVL) in the Metropolitan Region of Belo Horizonte (MRBH), Minas Gerais (MG) has increased, indicating an elevation in the transmission rate of the disease in the urban area (Silva *et al*., 2001). The domestic

dog is considered to be the main reservoir of *L. chagasi* in Brazil and thus for subsequent transmission of disease to humans (Cunha *et al*., 1995). In addition, canine leishmaniasis (CanL) is a severe disease of dogs with hair loss, skin lesions, epistaxis, anaemia, wasting, swollen limbs and joints, lameness, renal failure, lymphadenopathy, ocular lesions and diarrhoea. Severely diseased animals often die of renal failure and haemorrhagic disorders.

Accurate and rapid diagnosis of CanL is of great importance so that early treatment can be started and to prevent transmission. Several direct methods, i.e. culture of parasites or microscopy, and indirect tools, such as serology and PCR, are available for the diagnosis of leishmaniasis (Schallig and Oskam, 2002). However, currently there is no gold standard for the diagnosis of leishmaniasis and many laboratories use their own in-house protocol for the detection of *Leishmania* infection. Control and surveillance programmes would greatly benefit from the establishment of a sensitive, specific and simple diagnostic tool (or combination of tools) for leishmaniasis that can be used for the accurate diagnosis of canine infections (Reithinger *et al*., 2002). Such a test should ideally be easy to use and more cost-effective than currently used diagnostics, in particular when used in mass-screening surveys.

In the present study we compared the sensitivity and specificity of two serological tests with a combination of PCR and parasitology as the gold standard for the diagnosis of canine leishmaniasis in the region of Belo Horizonte, to support the implementation of a diagnostic protocol for surveillance of the disease.

MATERIALS AND METHODS

Study area

The city of Belo Horizonte is located in the Metropolitan Region of Belo Horizonte (MRBH); it is the state capital of Minas Gerais and has a population of 2 238 526 inhabitants (2000 census Instituto Brasileiro de Geografia e Estatística, http://www.ibge.gov.br). The total number of notified human cases in the MRBH since 1994, when the first case was identified, up to 1999 was 345, of which 223 (65%) were from the city itself, indicating an urbanization of the disease (Silva *et al*., 2001). The canine serum prevalence in this region was estimated to be 3.6% by the Secretary of Health of the State Minas Gerais (see also Silva *et al*., 2001).

Blood and serum sampling

The total number of domestic dogs enrolled in this study was 103. The selected dogs were animals suspected of canine leishmaniasis that were brought to a veterinary health clinic for examination. All the included dogs had at least one clinical sign compatible with CanL.

Blood samples from the dogs were collected on filter paper (Whatman 3) and, after drying, rounds of filter paper (5 mm diameter) were cut out and stored at -20°C until PCR analysis. Venous blood samples of the dogs were collected by venepuncture and serum, obtained after centrifugation, was kept at -20°C until further use.

Parasitology

Bone-marrow samples (from the shinbone) of 102 dogs were used to make Giemsa-stained slides for microscopic examination and for culturing on NNN medium supplemented with 15% defibrinated rabbit blood and enriched with liver infusion tryptose. Only bone marrow samples of microscopically negative dogs were subjected to extensive culture. Parasite cultures were incubated at 26°C and parasite growth was observed weekly. Subcultures were made in NNN, incubated at 26°C and observed for 4 weeks before being considered negative.

PCR

DNA was extracted from filter paper as described (Osman *et al*., 1997) and subjected to PCR analysis targeting a part of the ssu rRNA gene (Van Eys *et al*., 1992) of *Leishmania*. *Leishmania*-specific oligonucleotide primers R174 (5′-GGTTCCTTTCCTGATTTACG-3′) and R798 (5′-GGCCGGTAAAGGCCGAATAG-3′) were used to amplify an approximately 600 bp fragment. Amplification reactions were performed in volumes of 25 μl final reaction mixture comprising 5 μl of isolated DNA, PCR buffer, 4 mmol/L $MgCl_2$, 250 μmol/L dNTP, 250 μmol/L dUTP, 1 μmol/L of each primer, 0.1 U Uracil-*N*-glycosylase and 1 U Gold star polymerase. Samples were overlaid with 50 μl mineral oil and amplified in an automatic thermocycler with a thermal cycling profile of 50°C for 5 min, 94°C for 10 min, 40 cycles at 94°C for 75 s, 60°C for 1 min, 72°C for 2 min, and finally 72°C for 50 min. A positive control containing 100 pg of genomic *L. donovani* DNA and a negative control without template DNA were included. Amplification products were analysed on a 1.0% agarose gel containing 0.5 μg/ml ethidium bromide.

Serological tests

Two commercially available serodiagnostic tests were included in the present study. The indirect immunofluoresence assay (IFAT) for the diagnosis of canine leishmaniasis (FIOCRUZ/Bio-Manguinhos, Rio de Janeiro, Brazil) was performed according to the instructions of the manufacturer for detection of antibodies in serum samples. The cut-off value of the test is 1:20.

The direct agglutination test (DAT) based on freeze-dried *L. donovani* promastigote antigen (K.I.T. Biomedical Research, Amsterdam, The Netherlands) was performed according to the manufacturer's instructions (Oskam *et al*., 1996; Schallig *et al*., 2002). The cut-off value of the test was set at >1:400 (Schallig *et al*., 2002).

Data analysis

The sensitivity (i.e. the probability that the assay will be positive when the infection is present) and the specificity (i.e. the probability that the assay will be negative when the infection is absent) of each serodiagnostic test (IFAT and DAT) in the present study were

calculated according to Altman (1991) and related to the parasitological status of the dogs as defined by a combination of microscopy, culture and PCR ('gold standard'). It is to be noted that in the present study it was not possible to determine in the strictest sense the sensitivity and specificity of the tests empolyed because the study data are derived from clinical suspects and not truly from groups identified as CanL cases and non-CanL dogs. The sensitivity/specificity of the employed DAT test has been assessed extensively in laboratory evaluations (Oskam *et al*., 1996; Schallig *et al*., 2002, 2004).

The degree of agreement between the evaluated tests and combined parasitology–PCR data was determined. The agreement between the tests was determined by calculating Kappa (κ) values with 95% confidence intervals using Epi-info version 6 (Altman, 1991).

RESULTS

Parasitological examination of 103 dogs by microscopy or culture found 35 animals infected with *Leishmania*. Five dog samples could not be analysed by PCR because DNA extraction failed. PCR analysis of the remaining 98 blood samples found 28 dogs parasite-positive. There were 5 microscopy/culture-positive PCR-negative dogs and there was one microscopy/culture-negative PCR-positive dog (Table I). In total, 36 dogs were considered *Leishmania*-positive on the basis of combined microscopy, culture and PCR results. The remaining 67 dogs were considered negative.

All *Leishmania*-positive dogs ($n = 36$) were found to be seropositive with IFAT (see Table IIA) and 44 *Leishmania*-negative dogs were also found seronegative with IFAT. However, a relatively high number ($n = 23$) of seropositive/parasite-negative dogs was observed with IFAT. The sensitivity of the IFAT was 100% and its specificity was 74% compared to the

TABLE I
Results of parasitology (direct microscopic examination or culture) and PCR of 103 dogs from the Metropolitan Region of Belo Horizonte that were enrolled in the study

Number of dogs	Result of parasitology	Result of PCR
27	+	+
64	−	−
5	+	−
1	−	+
3	+	NT
2	−	NT
1	NT	−

Total number of dogs considered *Leishmania*-positive = 36
Total number of dogs considered *Leishmania*-negative = 67

NT = not tested owing either to absence of bone marrow sample or to failure to extract DNA

TABLE II
Comparison between combined microscopy–culture–PCR and IFAT (A) or DAT (B), respectively, for the detection of *Leishmania* infections in dogs

Combined microscopy–culture–PCR	IFAT		
	+ (*n*)	− (*n*)	Total
A. Comparison between combined microscopy–culture–PCR and IFAT*			
+ (*n*)	36	0	36
− (*n*)	23	44	67
Total	59	44	103
Combined microscopy–culture–PCR	DAT		
	+ (*n*)	− (*n*)	Total
B. Comparison between combined microscopy–culture–PCR and DAT**			
+ (*n*)	36	0	36
− (*n*)	7	60	67
Total	43	60	103

*Agreement = 78%; κ value = 0.57
**Agreement = 93%; κ value = 0.86

gold standard of the study (the combination of direct microscopy, culture and PCR). A poor to moderate degree of agreement (78%) was observed between the IFAT and the gold standard of the study (Table II). The agreement beyond change (κ value) was < 0.60. The DAT found all *Leishmania*-positive dogs ($n = 36$) seropositive (see Table IIB). In addition, 7 *Leishmania*-negative dogs were seropositive with DAT (DAT titre >1:400). Four out of those 7 false-positive dogs had a DAT titre of 1:400 and can be considered borderlines. The other 3 false positives had substantial DAT titres. The remaining 60 *Leishmania*-negative dogs were also seronegative with DAT. There were no DAT-negative dogs that were found positive with either PCR or parasitology. The sensitivity of the DAT was calculated to be 100% (no false negatives) and the specificity 91% (7 false positives). A high degree of agreement (93%; κ value 0.86) was observed between DAT and the combination of direct microscopy, culture and PCR.

DISCUSSION

Accurate and rapid diagnosis of *Leishmania* infection in dogs is of great importance—to allow early treatment to be started (in order to prevent severe disease) and to prevent transmission from the canine reservoir to humans—but remains problematic. Clinical diagnosis

is difficult owing to variable disease symptoms. Furthermore, about half of all infected dogs lack clinical signs of leishmaniasis (Abranches *et al*., 1991), but these asymptomatic dogs are just as infective to the vector as symptomatic dogs (Alvar *et al*., 1994). Parasitological diagnosis relies on microscopic demonstration or culturing of *Leishmania* parasites from aspirates, but sample retrieval is painful to the dog, and the microscopic identification in smears and biopsy sections requires experienced personnel and the isolation of parasites by culturing is time-consuming, difficult and expensive. Serology is used for indirect diagnosis of CanL and several techniques have been developed to detect anti-*Leishmania* antibodies in clinical samples. However, currently available tests have inadequate sensitivity and/or specificity (Reithinger and Davies, 1999; Palatnik de Sousa *et al*., 2001; Reithinger *et al*., 2002), resulting in misdiagnosis and thus subsequent wrong treatment or, even worse, in unnecessary sacrifice of dogs since in some countries it is practice to kill seropositive dogs as a control measure. Elimination of dogs in endemic areas in China and Brazil has been correlated with decreased incidence of human disease (Leng, 1982; Palatnik de Sousa *et al*., 2001). This practice is resisted by dog-owners and its overall effectiveness is questionable (Dietze *et al*., 1997; Reithinger and Davies, 1999).

In order to circumvent unnecessary treatment or culling of dogs, diagnostic tools that are considered for implementation in surveillance or control programmes must have high sensitivity and specificity. In the present study we have evaluated two available serological tests and compared their sensitivity and specificity with a combination PCR and parasitology as gold standard. Both tests, IFAT and DAT, have a very good sensitivity and no false-negative results were obtained. However, the IFAT had a relatively high number of false-positive results (23 animals), resulting in a much lower specificity of 74% compared to the DAT. The observed specificity of the DAT in the present study, 91%, is in good agreement with previous findings (Oskam *et al*., 1996; Schallig *et al*., 2002). The cut-off value of the DAT was set beforehand at $\geq$1:400 (cf. Schallig *et al*., 2002). Four dogs had a DAT titre of 1:400 and were included in the false-positive results.

On the basis of our results we recommend the replacement of the currently used IFAT test for the serodiagnosis of canine leishmaniasis by the DAT. Besides its higher specificity, the DAT test has additional advantages (Schallig *et al*., 2001): (i) the test is very simple to perform and requires only little training of the operators; (ii) performance of the DAT does not require sophisticated equipment (such as a microscope) and electricity; (iii) cold storage of test reagents is not necessary; (iv) blood samples can be used in the test; (v) a large number of samples can be processed in a short time. These properties make the DAT a very suitable test for the serodiagnosis of canine leishmaniasis even under harsh field conditions.

Finally, we emphasize that serological tools are an *aid* in the diagnosis of canine leishmaniasis. The outcome of any serological test should always be judged judiciously in connection with clinical, epidemiological and other diagnostic data.

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