Polymerase Chain Reaction Using Noninvasively Obtained Samples, for the Detection of *Leishmania infantum* DNA in Dogs

Dalit Strauss-Ayali,1,2 Charles L. Jaffe,2 Ofer Burshtain,1 Liat Gonen,1 and Gad Baneth1

1School of Veterinary Medicine, Hebrew University of Jerusalem, Rehovot, and 2Kuvin Center for the Study of Tropical and Infectious Diseases, Hebrew University–Hadassah Medical School, Jerusalem, Israel

A polymerase chain reaction (PCR) procedure using noninvasively obtained samples, for the identification of *Leishmania infantum* in canine tissues, was evaluated and compared with serologic testing and culture. A total of 92% of naturally infected, symptomatic, seropositive dogs were found to be positive by use of DNA from conjunctival swabs. Spleen or lymph node aspirates were found to be positive by PCR in 86% and by culture in 74% of these dogs. The sensitivity and specificity of conjunctival PCR were 92% and 100%, respectively. Experimentally infected dogs were found to be positive by conjunctival PCR already at 45 days of infection (83%) and before seroconversion. PCR using noninvasively obtained conjunctival samples will be useful for epidemiological studies and for direct diagnosis of canine visceral leishmaniasis.

Canine visceral leishmaniasis (CVL), caused by *Leishmania infantum* (synonymous with *L. chagasi*), is widespread in the Mediterranean Basin, the Middle East, and South America. Dogs are the main reservoir host for this parasite, which also causes the potentially fatal human visceral leishmaniasis (HVL). Since a combined risk exists for both canine and human infections in areas where VL is endemic, there is a need for sensitive and specific diagnostic techniques. Serologic testing can identify exposure to the parasite but cannot indicate an active infection. In previous years, the reference standard for diagnosis was the demonstration of the parasite by either microscopy or culture of aspirates obtained from the spleen, lymph nodes, or bone marrow. However, the overall sensitivity of these methods in humans and dogs is variable and relatively poor [1]. In the last decade, the use of polymerase chain reaction (PCR) for demonstration of *Leishmania* DNA was shown to be sensitive and specific. A variety of canine tissues, including bone marrow, spleen, lymph nodes, skin, and conjunctival biopsy specimens, have been used for diagnosis. However, these samples were obtained in an invasive manner. Drawing blood is considered to be less invasive. Although some studies have shown that PCR using blood has good sensitivity [2, 3], others have indicated that it is a relatively insensitive source of leishmanial DNA [4, 5]. The present study was designed to identify a sensitive PCR procedure using noninvasively obtained samples that is suitable for the identification of *Leishmania* DNA in canine tissues and to compare its efficiency with that of PCR using invasively obtained samples, serologic testing, and culture.

**MATERIALS AND METHODS**

**Dogs.** Ninety-eight dogs were examined in the present study and were divided into 4 groups. Group A included dogs (*n* = 24) that were admitted to the Hebrew University Veterinary Teaching Hospital for evaluation of possible leishmanial infections. The dogs pre-
viously underwent serologic testing by ELISA, because of clinicopathological abnormalities typical of leishmaniasis, and were identified as seropositive. Group B included seronegative dogs \((n = 65)\) from the village of Nataf, an endemic focus of CVL. Group C included 5-month-old male beagle dogs \((n = 6)\) that were experimentally infected with \(L.\ infantum\) amastigotes \((8.6 \times 10^6/day\) intravenously [iv]; MCAN/IL/2001/LRC-L1020) and evaluated during the first 3 months after infection. Group D \((n = 9)\) included 4-month-old male \(Leishmania\)-seronegative beagle dogs with no clinical signs of leishmaniasis. The present study was approved by the Animal Ethics Committee of the Hebrew University of Jerusalem.

**Canine samples.** After clinical evaluation, dogs (groups A, C, and D) were anesthetized for invasive sampling, by use of iv ketamine hydrochloride \((10 \text{ mg/kg, adjusted for body weight})\) and diazepam \((1 \text{ mg/kg, adjusted for body weight})\). Invasively obtained fine-needle aspirates from the spleen (groups A, C, and D) and lymph nodes (group A) were put into sterile tubes or spotted onto filter paper. Blood anticoagulated with EDTA was also spotted onto filter paper, and buffy coat was prepared from 1 mL of EDTA-anticoagulated blood (all groups), as described elsewhere [6]. The conjunctivas of both eyes were sampled using sterile cotton swabs manufactured for bacteriological isolation (all groups). The swabs were rubbed against the surface of the lower eyelid, to collect the exfoliating cells, and then the cotton tip was bent and its shaft broken into a sterile tube. Skin scrapings (group A) were obtained, by use of a scalpel blade, from 2 areas in the back and from skin lesions when present. Tissue samples were kept at \(-20^\circ\text{C}\), and filter paper samples were dried and kept at room temperature until DNA extraction. Spleen (groups A, C, and D) and lymph node (group A) aspirates obtained in a sterile manner were cultured in NNN medium at 26°C and examined for parasite growth. Heparinized plasma or serum samples were obtained for serologic testing.

**DNA extraction from canine samples.** Samples spotted onto filter paper, corresponding to \(-20 \mu\text{L of fluid, were cut out by use of individual sterile scalpel blades and put into sterile tubes. A mixture (300 \mu\text{L}) of Proteinase K (250 \text{ g/mL}) and Triton X-100 (1\%) in Lysis buffer (50 mmol/L Tris, 50 mmol/L NaCl, and 10 mmol/L EDTA [pH 8.0]) was added to tubes containing filter papers, conjunctival swabs, 100 \mu\text{L of spleen or lymph node aspirates, and blood or buffy coats and was incubated for 2 h at 56°C. DNA was further extracted, by use of a minor modification of the phenol-chloroform-isoamylalcohol method [6]. Precipitation was done using 1 vol of isopropanol-sodium acetate, followed by single wash with 75% ethanol. The DNA pellet was suspended in 30 \mu\text{L of Tris-EDTA buffer (10 mmol/L Tris and 1 mmol/L EDTA [pH 8.0]) and was kept at 4°C until use.**

**PCR amplification.** PCR was performed as described elsewhere by Schonian et al. [7], with minor modifications (MgCl₂ and DMSO were added, to final concentrations of 2.5 mmol/L and 2.5%, respectively). The target for amplification was a 314-bp sequence in the ribosomal operon of \(L.\ infantum\), the internal transcribed spacer 1 (ITS1). It was amplified by use of the primers LISTR and L5.8 [8]. PCR was performed using a PTC-100 thermocycler (MJ Research). Amplification products were kept at 4°C until analyzed on 2% agarose gel and were stained with ethidium bromide. Samples were scored as positive whenever a 314-bp product was observed.

Restriction fragment–length polymorphism (RFLP) analysis of the reaction products, after enzymatic restriction with BsuRI (Hae III; MBI Fermentas), was visualized on 3% agarose gel by use of ethidium bromide. Conjugtival PCR products \((n = 10)\), as well as \(L.\ infantum\) (MCAN/IL/2001/LRC-L1020), \(L.\ tropica\) (MHOM/IL/1990/LRC-L590), and \(L.\ major\) (MHOM/IL/1979/Perlstein) amplification products, were analyzed.

**ELISA.** The presence of anti-\(Leishmania\) IgG was measured in canine serum samples by use of an ELISA, as described elsewhere [9], with minor modifications. Crude antigen \((30 \text{ g/mL})\) prepared from \(L.\ infantum\) promastigotes (MCAN/IL/1994/LRC-L369) was used for coating. Serum samples were considered to be positive at an OD of \(\geq 0.6\), as calculated elsewhere [9].

**RESULTS**

**Sensitivity of the ITS1-PCR.** The lower limit of detection of the PCR \((200 \text{ pg of DNA/reaction})\) was determined using serial dilutions of \(L.\ infantum\) promastigote DNA. In addition, the sensitivity of the assay in the presence of buffy coat DNA was evaluated, and it was possible to detect a minimum of 0.5 parasites/PCR. The efficiency of DNA extraction from parasite-spiked cotton swabs was similarly evaluated and was equivalent to a minimum of 6.6 parasites/PCR (data not shown).

**Identification of infecting Leishmania species, by RFLP.** All PCR products were visualized and were found to be 314 bp (data not shown), as reported elsewhere for the \(L.\ donovani\) complex [8]. Digestion of the ITS1-PCR product produces a unique RFLP pattern for each Eastern Hemisphere \(Leishmania\) species [7]. The RFLP pattern of PCR products obtained using conjunctival samples from the naturally infected dogs was identical to that of \(L.\ infantum\).

**Comparison of noninvasively and invasively obtained samples, for the detection of naturally occurring CVL.** All group A dogs were positive by ELISA (OD [mean \(\pm\) SE], 1.41 \(\pm\) 0.07). Eighty-three percent of samples obtained from either the right or the left conjunctiva of group A dogs were found to be positive by ITS1-PCR (figure 1). When positive results from both eyes were combined, 92% of the dogs were found to be positive. Skin scrapings obtained from 2 areas of the back of the dogs and from skin lesions were found to be positive for...
Diagnosis of CVL by Use of Noninvasively Obtained Samples

Figure 1. Sensitivity of noninvasively and invasively obtained samples, for the detection of *Leishmania* infection in naturally infected dogs (group A). Percentage of positive samples by polymerase chain reaction (PCR) or culture are plotted for individual sampling sites (white bars), or for combined sites (gray bars). Nos. above the bars indicate the percentage of positive samples, whereas nos. on the bottom indicate the total number of dogs tested for a specific sample. Back 1 and 2, two standardized healthy areas for the skin scrapings; LC, left conjunctiva; lesion, skin lesions area; LN, lymph node; RC, right conjunctiva; SPL, spleen.

29%, 41%, and 46% of the samples, respectively. When the results of all skin samples were combined, 65% of the dogs were found to be positive by at least 1 test. Other noninvasively obtained samples of buffy coat and blood were positive for 57% (5/8) and 17% (2/12) of the dogs, respectively (data not shown).

The presence of *L. infantum* DNA was demonstrated by a positive ITS1-PCR result, in invasively obtained spleen or lymph node aspirates from 86% of the dogs (figure 1). When considered individually, only 77% of spleen aspirates and 67% of lymph node aspirates were found to be positive. *L. infantum* parasites were cultured successfully from 61% and 37% of the spleen and lymph node samples, respectively. Seventy-four percent of the dogs were found to be positive by either spleen or lymph node culture (figure 1).

The mean ± SE optical density of the seronegative dogs from an endemic focus of CVL (group B), determined by use of ELISA, was 0.118 ± 0.015. Both conjunctival samples from all these dogs (100%) were found to be negative by ITS1-PCR. Their buffy coat samples were also found to be negative by ITS1-PCR. In the present study, the sensitivity and specificity of the conjunctival ITS1-PCR to detect *L. infantum* DNA in naturally occurring CVL were 92% and 100%, respectively.

Evaluation of conjunctival ITS1-PCR in experimental CVL. Experimentally infected dogs (group C) were evaluated by use of conjunctival ITS1-PCR every 2 weeks for the first 3 months after infection (figure 2). As early as 45 days after infection, 5 (83%) of the 6 experimentally infected beagle dogs had at least 1 conjunctival sample found to be positive by ITS1-PCR. Corresponding optical density values were still below the established cutoff value (OD [mean ± SE], 0.39 ± 0.055) at that sampling time (figure 2). At this time point, buffy coat DNA was found to be positive by use of ITS1-PCR for only 1 (17%) of 6 infected dogs. After 60 days of infection, the number of dogs with positive conjunctival samples remained identical (5 [83%] of 6). Buffy coat samples from 3 (50%) of 6 infected dogs became positive, and all dogs were considered to have seroconverted (OD [mean ± SE], 1.325 ± 0.122). At 75 and 90 days after infection, at least 1 conjunctival sample from 100% of the dogs was found to be positive. Of interest, none of theuffy coat samples was positive after 75 days of infection, and only 1 was positive after 90 days of infection. At 30 days after infection, cultures and ITS1-PCR of spleen aspirates were found to be positive for all experimentally infected dogs. Conjunctival, spleen, and buffy coat samples from seronegative beagle dogs (group D) (OD [mean ± SE], 0.0315 ± 0.01) were found to be negative for the presence of *L. infantum* DNA by use of ITS1-PCR. Spleen cultures from these dogs were found to be negative for *L. infantum*.

DISCUSSION

Ocular abnormalities are found in up to 54% of dogs with CVL [10]. Similar lesions were found as the sole manifestation in a case of HVL [11]. Biopsy specimens obtained from the con-
kinetics of polymerase chain reaction (PCR) and serologic testing in beagle dogs experimentally infected with *Leishmania infantum*, during the first 3 months after infection. Mean optical density (OD) values (405 nm) for infected dogs, determined by ELISA, are plotted (triangles). Columns represent the percentage of infected dogs with positive PCR of at least 1 conjunctival (gray bars) or buffy coat (white bars) sample. Error bars indicate the SE calculated for the ODs.

The conjunctival PCR shows good sensitivity (92%) and specificity (100%) in detection of active CVL. This sensitivity is in the same range (89%–100%) as that reported elsewhere for PCR using invasively obtained samples from symptomatic dogs, and it substantiates the benefit of this noninvasive technique [4, 13]. The value of conjunctival DNA for diagnosis is further emphasized by the finding that 83% of the dogs experimentally infected with *L. infantum* were already positive by PCR at 6 weeks after infection. Since the parasites are assumed to reach the eye via the hematogenous route [12], it is surprising that only 17% of the buffy coat samples obtained at the same time were found to be positive. However, DNA extracted from buffy coat, unlike conjunctival DNA, may not posses PCR inhibitors, and, more likely, the lymphatic tissue of the conjunctiva, as observed in histological sections (authors’ unpublished data), is infiltrated by tissue histiocytes that harbor parasites. The finding that conjunctival PCR in these dogs is positive before seroconversion indicates that, for early diagnosis of experimental infection, this technique is superior to serologic testing. Moreover, in the naturally infected dogs, conjunctival PCR was more sensitive (92%) than was either PCR using other tissues (17%–77%) or detection of parasites in culture (74%). This finding is in agreement with those of a previous study showing that PCR using conjunctival biopsy specimens is more sensitive than is PCR using bone marrow samples [14]. Unlike the conjunctival samples, the skin scrapings used here were not as sensitive as were full-thickness skin punch biopsy specimens evaluated in other studies [3, 14]. This is probably due to the superficial nature of the skin scrapings, which yielded mostly keratinocytes and low amounts of parasite DNA.

Although CVL in the Eastern Hemisphere is usually caused by *L. infantum*, *L. tropica* has been identified in dogs with CVL in North Africa [15]. Hence, the usefulness of ITS1-PCR for direct diagnosis is augmented, after simple restriction analysis of the amplicon, by its ability to identify the *Leishmania* species causing the disease.

In the present study, we have shown that PCR using noninvasively obtained conjunctival samples with naturally occurring CVL is superior to parasite culture or PCR using invasively obtained tissues. We also showed that experimentally infected dogs become positive, by PCR using conjunctival samples, before seroconversion. If a similar pathogenesis can be assumed for natural *L. infantum* infection, then conjunctival PCR might be helpful in the early detection of this zoonosis, since infected dogs present an increased risk of exposure to the surrounding human population. Therefore, we recommend the combined
use of conjunctival ITS1- PCR and serologic testing for screening dogs in disease-control and epidemiological studies and for direct diagnosis. In addition, the potential application of this technique to human studies should be further examined.

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References