Comparison of real-time PCR and conventional PCR with two DNA targets for detection of *Leishmania (Leishmania) infantum* infection in human and dog blood samples

A. Mohammadiha, M. Mohebali, A. Haghighi, R. Mahdian, A.R. Abadi, Z. Zarei, F. Yeganeh, B. Kazemi, N. Taghipour, B. Akhoundi

Department of Medical Parasitology & Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Biotechnology Research Center, Molecular Medicine Department, Pasteur Institute of Iran, Tehran, Iran

Center for Research of Endemic Parasites of Iran (CREPI), Tehran University of Medical Sciences, Tehran, Iran

Department of Community & Health, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Cellular & Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

**Highlights**

- *Leishmania infantum* infection in blood samples of 100 dogs and 100 humans by real time PCR were analyzed.
- A sensitivity of 100% and 93.9%, specificity of 96.4% and 100%, were found for dog and human samples, respectively.
- The highest concordance was obtained between DAT and real-time PCR results.

**Abstract**

Zoonotic visceral leishmaniasis (VL) is endemic in northwestern Iran. Real-time PCR, conventional PCR, and the direct agglutination test (DAT) were used to diagnose *Leishmania infantum* infection in blood samples from 100 domestic dogs and 100 humans. Based on clinical evaluation, 82 humans and 72 dogs from the endemic area were categorized as having asymptomatic infection, DAT positive with no clinical signs of VL, or symptomatic infection, DAT positive with at least one sign of VL. Eighteen human samples containing no *Leishmania* antibodies (DAT−) and 28 dog DAT− sera from non-endemic areas with no history of VL constituted negative controls. All 46 DAT− samples were also negative by Dipstick rK39. Bone marrow material was used for parasitological examinations in symptomatic VL, and peripheral blood samples were used for detection of *L. infantum* infection using conventional PCR and real-time PCR in non-symptomatic subjects. Two DNA targets (ITS1 kDNA) were used for conventional PCR. *L. infantum* antibodies in sera were detected by DAT. Parasitemia was measured by real-time PCR targeting kDNA using Taqman Assay. All 72 (100%) symptomatic (38/38) and asymptomatic (34/34) dog DAT+ samples, 45 of 48 (93.8%) symptomatic human DAT+ samples, and 32 of 34 (94.1%) human asymptomatic cases were identified by real-time PCR. The mean (59.19 vs 12.38 parasite equivalents/mL of blood) and median (16.15 vs 1 parasite equivalents/mL of blood) ranges of parasitemia were higher in dogs than in humans.
1. Introduction

Visceral leishmaniasis (VL) is a severe systemic zoonotic disease caused by the protozoan parasite *Leishmania infantum*, with dogs considered to be the main reservoir of the parasite in Iran (Mohebali et al., 2005, 2010). Leishmaniasis is endemic in at least 88 countries, and more than 350 million people are at risk (Desjeux, 2004). Half a million new cases of VL occur worldwide annually (WHO, 2010). In the Mediterranean basin, the Middle East, and Iran seroprevalence ranges from 10% to 37% (Sideris et al., 1999; Mohebali et al., 2005). Canine visceral leishmaniasis (CVL) is endemic in northwestern and southern Iran, where its prevalence ranges from 14.2% to 17.4% (Mohebali et al., 2001, 2002; Mohebali, 2012; Moshef et al., 2008). Some studies suggest that the rate of infection is higher than indicated from serological studies (Zaffaroni et al., 1999; Solano-Gallego et al., 2001). It has been demonstrated that infected, but asymptomatic, dogs (Mohebali et al., 2005; Moshef et al., 2009) and probably asymptomatic humans (Fakhar et al., 2008) are sources of the parasite for phlebotomine sandfly vectors. Deane and Deane (1962) showed that patients with active VL could infect sandflies, and Costa et al. (2000, 2002) confirmed the possibility that healthy human carriers may act as reservoirs, since sandflies feeding on such carriers can become infected. Fakhar et al. (2008) reported that many asymptomatic human carriers of *L. infantum* potentially act as reservoirs of infection. Such carriers must be considered in the development of VL control programs in the region.

Correct diagnosis and characterization of the parasite is important for evaluating prognosis and prescribing appropriate treatment (Blum et al., 2004). The routine diagnostic methods for leishmaniasis, such as direct smear examination and culture, have important limitations (Deniau et al., 2003). Direct examination is inexpensive and easy, but lacks sensitivity when the number of parasites present in the tissue is low and requires expertise (Nasereeddin et al., 2006). Culture is labor-intensive, particularly in field conditions, and requires an extended time to obtain results. Indirect immunofluorescence assay test (IFAT) is a routine diagnostic method for CVL, although asymptomatic dogs may yield false negative results (Almeida et al., 2005), and cross reaction with other parasites and other *Leishmania* species can occur (Schulz et al., 2003; Ferreira et al., 2007). The direct agglutination test (DAT) is used as a serodiagnostic tool, because it is a simple and valid test and does not require specialized equipment (Harith et al., 1989; Mohebali et al., 2005, 2006, 2011).

Polymerase chain reaction (PCR) is an alternative method of diagnosis. Several *Leishmania* DNA targets have been used for the identification of parasites, including minicircles from kDNA (Lachaud et al., 2002), the coding and intergenic noncoding regions of the gp63 gene locus (Cupolillo et al., 1995), small subunit rRNA (Lemarini et al., 2002), spliced leader mini-exon (SLME) (Harris et al., 1998), and internal transcribed spacer (ITS) (Nasereeddin et al., 2006). Their value in PCR-based techniques has been evaluated for various species of *Leishmania* and using different tissues, including blood, urine, skin, and bone marrow (Reale et al., 1999; Reithinger et al., 2000; Martin-Sanchez et al., 2001; Mohebali et al., 2002; Kazemi et al., 2008).

These targets have proven to be suitable for diagnosis, although real-time PCR can be more sensitive and reproducible in diagnostic routines. kDNA-PCR appears to also be a sensitive method (Strauss-Ayali et al., 2004). In the present study, we compared sensitivity and specificity of real-time PCR and two PCR primer pairs (kDNA-based and ITS1-based) for *L. infantum* identification. These techniques were evaluated for the molecular diagnosis of VL and the limits of detection for both PCR primer pairs were assessed by real-time PCR assay. “Finally, the performance of the three molecular assays were compared between human and dog samples”.

2. Materials and methods

2.1. Study design

This study was carried out in the Meshkin-Shahr district in an endemic area for CVL in northwestern Iran (Mohebali et al., 2006, 2011). Blood samples from 100 domestic dogs and 100 humans were analyzed. All human patients were examined by a physician, and the dogs were physically examined by a veterinarian. Based on clinical evaluation, 82 humans and 72 dogs from the endemic area were categorized as having asymptomatic infection presenting DAT positive with no clinical signs of VL or with symptomatic infection presenting DAT positive with at least one sign of VL. Eighteen human samples exhibiting no *Leishmania* antibodies (DAT−) and 28 dog DAT− sera from non-endemic areas with no history of VL constituted negative controls. All 46 samples were also negative by Dipstick rk39.

2.2. Sample preparation

At least 3 mL blood samples were collected by venipuncture into 10 mL polypropylene tubes and processed 4–10 h after collection. Approximately 2 mL of blood was used for serum separation. Blood was centrifuged at 800 g for 5–10 min, and the serum was stored at −20 °C.

2.3. Parasitological examination

Bone marrow aspirates were only used for symptomatic cases. Microscope slides were prepared from bone marrow materials, fixed with methanol, stained with 10% Giemsa, and examined under high magnification (1000×) for the presence of *Leishmania* amastigotes.

2.4. Serological tests

The Iranian strain of *L. infantum* was used for preparation of DAT antigen in the Leishmaniasis Laboratory, at the School of Public Health and Institute of Public Health Research, Tehran University of Medical Sciences. The principal phases of the procedure for making DAT antigen were mass production of promastigotes of *L. infantum* [MCAN/IR/07/Moheb-gh. (GenBank accession no. FJ555210)] described previously by Mohebali et al. (2006). The cut-off value was determined in previous studies by experimental infection (Edrissian et al., 1996; Mohebali et al., 2005, 2010). All samples from human and dogs were examined by DAT.
2.5. DNA isolation

To completely remove interfering hemoglobin molecules from the samples prior to DNA extraction, 1 mL distilled water was added to 300 μL blood followed by vortexing and centrifuging at 4000g for 5 min. This step was repeated three times, and finally the pellets were washed with PBS. DNA was extracted with the DNG-plus Extraction Kit (Cinnagen, Iran) according to the manufacturer’s instructions. The DNA pellet was dissolved in 50 μL of sterile distilled water and incubated in a water bath at 65 °C for 5 min. DNA concentration and quality were determined using Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) at 260 and 280 nm. DNA samples with A260/A280 ratios between 1.8 and 2 were selected and stored at −20 °C for further analysis.

2.6. PCR for Leishmania kDNA

A conventional PCR for detection of L. infantum DNA was performed. Specific primers for kDNA of the L. infantum species were derived from RV1 (CTTTCTCGTCTCCTCCGGTGAGG) and RV2 (CCACCAGCCCTATTTACCAACA), described previously by Lachaud et al. (2002) and Mary et al. (2004). Kinetoplast DNA was chosen as the molecular target, and a 145 bp fragment was amplified. The amplification conditions were 94 °C for 4 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 40 s, with a final extension step at 72 °C for 5 min. 5 μL of the PCR products were visualized by EthBr staining following 2% agarose gel electrophoresis.

2.7. PCR for Leishmania ITS1-DNA

LITSR (5′-CTGGATCATTTTCCCATT-3′) and L5.8S (5′-TGATACC ACTTATCGCACTT-3′) primers were used to amplify the genes encoding the small subunit 5.8S rRNA, and PCR products were digested by HaeII restriction enzyme following protocol described previously (Schönian et al., 2003). The restriction fragments obtained were compared with the molecular profiles of the WHO reference strains of L. infantum (MCAN/IR/96/LON49), L. tropica (MHOM/IR/99/YAZ1), and L. major (MRHO/IR/75/ER). After using the restriction enzyme, banding patterns including the fragments of 220 and 140 bp for L. major, the fragments of 200, 80, and 60 bp for L. infantum, and 3 fragments of 200, 60, and <60 bp for L. tropica were observed with 2.5% agarose gel electrophoresis, stained with ethidium bromide.

2.8. Detection and quantification of kinetoplast DNA

Multiplex Real-time PCR was performed using hydrolysis TaqMan probes technology. The final 20 μL reaction mixture contained 10 pmol of forward and reverse primers and 50 pmol of TaqMan probe (FAM-TTCTCCGACACCGCCCATCACCAC-BHQ) with a 4 μL of sample DNA (Mary et al., 2004). To evaluate DNA extraction accuracy, β-actin was used as internal control in a multiplex format. Ten pmol primers (CTGGCAACCCACCTTACAA and GCCCTGGT CAGCACCA) and a 50 pmol of probe specific for the β-actin (VIC- CACCGCCAGCTCC-TAMRA) according to the method of Mamna et al. (2006) were used. The samples with β-actin cycle threshold (Ct) ≤ 22 (in VIC channel) were chosen for further analysis (on FAM channel), whereas those without Ct (no amplification) or with Cts higher than 22 were resubmitted to DNA extraction. All blood samples were positive for the β-actin. The Ct value was calculated for each sample by determining the point at which the fluorescence exceeded the threshold limit. Thermal cycling was performed on the Corbett 6000 system using the following cycling conditions: 30 s at 95 °C as a first denaturation step followed by 50 cycles at 95 °C for 5 s and 60 °C for 30 s.

PCR efficiencies were calculated based on the slope of the standard curve and the following equation: Efficiency = 10(−1/Slope) − 1. Serial dilution analysis was performed on different DNA concentrations to drawing standard curve. Briefly, a series of extracted DNA (10−5–10−10 parasite/mL) from cultured Iranian strain of L. iainfantum promastigotes (MCAN/IR/07/Moheb-gh.), diluted by distilled water were used as standard DNA. Then, the logarithmic input amount of DNA samples was plotted against the corresponding CT values. The difference for each point of the curve was 1 log factor. The standard curve was linear over at least 7 log ranges of DNA concentration, so the standard curve allowed a detection limit of L. infantum DNA concentration corresponding to 0.0001 parasite/mL. Absolute slope values between −3.1 and −3.6, indicating PCR efficiency of more than 95%, were considered acceptable. Intra-assay analysis demonstrated good precision (R² = 0.9879).

Data evaluation was carried out using Rotor Gene Corbett 6000 software. A non-template control consisting of the reaction mixture and water instead of template DNA was added in each run. All reactions performed in triplicated manner.

2.9. Ethical approval

The trial was reviewed and approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences as well as the ethical committee of the Center of Diseases Control of Iran in accordance with the Helsinki Declaration and guidelines.

2.10. Statistical analysis

All data were compared using the χ² test and Fisher exact test with a conventional level of 95%, and a probability value of ≤0.05 was considered statistically significant. Results of real-time PCR with respect to the amounts of DNA present were analyzed using ANOVA. Analyses were performed with SPSS (version 16; SPSS Inc., Chicago, IL, USA). The degree of agreement between two tests was determined by calculating kappa (κ) values with 95% confidence intervals. Kappa values express the agreement between two tests. A value of 0.21–0.60 represents fair to moderate agreement, κ > 0.60–0.80 represents substantial agreement, κ > 0.80 represents almost total agreement (Altman, 2001). The sensitivity and specificity of the DAT and real-time PCR were calculated with the formula: Sensitivity = TP/(TP + FN) × 100%, specificity = TN/(TN + FP) × 100%. The positive predictive value (PPV) and negative predictive value (NPV) of the DAT and real-time PCR were calculated with the formula: PPV = TP/(TP + FP) × 100% and NPV = TN/(TN + FN) × 100% where TP represents true positive, TN true negative, FN false negative and FP false positive.

3. Results

Blood samples obtained from 100 dogs and 100 human patients were grouped by clinical status and DAT results (Tables 1 and 2). All 72 symptomatic dog and asymptomatic dog DAT positive samples (≥1:80) as well as 77 of 82 (93.9%) human symptomatic DAT positive (≥1:800) were identified by real-time (P < 0.05). One of 28 (3.6%) dogs in the negative control group was found positive by real-time PCR (Table 1). Positive results by kDNA-based-PCR and ITS1-based-PCR methods were 88.88% and 72.22% for dog and 75.61% and 53.66% for human samples, respectively (Table 1).

In dogs, parasitemia ranged from 0.01 to 690.02 parasites/mL, 0.34 to 256.11 parasites/mL, and 0.00 to 0.076 parasites/mL in symptomatic, asymptomatic, and negative control groups, respectively. In seropositive dogs, the median level of parasitemia was
16.2 parasites/mL. Ranges of 0.0–504.8 parasites/mL and 0.0–5.5 parasites/mL in human symptomatic and asymptomatic groups were obtained. The median value was 1 parasite/mL in seropositive human groups compared to 0.0 parasites/mL in seronegative subjects (Table 2). Sensitivity, specificity, positive predictive value, and negative predictive value for real-time PCR assay as well as other methods are shown in Table 3. The highest level of agreement was obtained between real-time PCR and conventional PCR with the kDNA target in both human (85%) and dog (92%) groups (data not shown). Sensitivity and specificity of 100% and 96.4% for dog samples and 93.5% and 100% for human samples, respectively, were observed by real-time PCR (Table 3). One of 28 (3.6%) control dogs was positive by real-time PCR and conventional PCR (kDNA), and 3/28 control dogs were positive by ITS conventional PCR but negative by DAT and Dipstick rK39 methods. All control humans were negative by real-time PCR assay and conventional PCR (kDNA), but two cases (11.1%) were positive by ITS-based-PCR.

### 4. Discussion

*Leishmania* species have been detected by PCR-based methods with high sensitivity compared to parasitological methods (Kumar et al., 2007). PCR can detect *Leishmania* parasite DNA in a variety of clinical sample types (e.g., skin biopsy, ulcer material, blood, bone marrow, and lymph node aspirates). Conventional PCR assays based on the *Leishmania* ITS1 and kDNA targets have been compared (Bensoussan et al., 2006). kDNA is considered to be the most sensitive target for diagnosing leishmaniasis, since kDNA contains in the range of 10,000 minicircles with an approximately 200 bp conserved region and 600 bp variable region. While a number of **Table 1**

Comparison of real-time PCR, conventional PCR, and parasitological methods on human and dog DAT positive and negative blood samples.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Groups</th>
<th>DAT* (n %)</th>
<th>Parasitology</th>
<th>Real-time PCR (Parasitemia)</th>
<th>kDNA-based PCR (Parasitemia)</th>
<th>ITS-based PCR (Parasitemia)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human</strong></td>
<td>Symptomatic</td>
<td>31/48 (64.6%)</td>
<td>20.61 (504.80)</td>
<td>93.8 (504.80)</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Asymptomatic</td>
<td>+</td>
<td>0.75 (5.50)</td>
<td>0.0 (0.0)</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>+</td>
<td>12.38 (504.80)</td>
<td>0 (0.0)</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>NC</td>
<td>–</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td><strong>Dog</strong></td>
<td>Symptomatic</td>
<td>19/38 (50%)</td>
<td>78.35 (690.02)</td>
<td>100</td>
<td>95</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Asymptomatic</td>
<td>+</td>
<td>37.78 (256.11)</td>
<td>0.0 (0.0)</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>+</td>
<td>59.19 (690.02)</td>
<td>0 (0.0)</td>
<td>100</td>
<td>95</td>
</tr>
</tbody>
</table>


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### Table 2

*Leishmania infantum* parasitemia in humans and dogs for symptomatic and asymptomatic groups.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Groups</th>
<th>Real-time PCR (Parasitemia)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DAT*</td>
</tr>
<tr>
<td><strong>Human</strong></td>
<td>Symptomatic</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Asymptomatic</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>NC</td>
<td>–</td>
</tr>
<tr>
<td><strong>Dog</strong></td>
<td>Symptomatic</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Asymptomatic</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>NC</td>
<td>–</td>
</tr>
</tbody>
</table>


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### Table 3

Validity of real-time PCR, conventional kDNA-PCR and ITS-PCR compared to the direct agglutination test for detection of canine and human visceral leishmaniasis.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Methods</th>
<th>Direct agglutination test (DAT)*</th>
<th>Sensitivity (%) (95% CI)</th>
<th>Specificity (%) (95% CI)</th>
<th>Agreement (%)</th>
<th>Positive predictive value (%) (95% CI)</th>
<th>Negative predictive value (%) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human</strong></td>
<td>Real-time PCR</td>
<td>93.9 (91.51–96.29)</td>
<td>100</td>
<td>95</td>
<td>100</td>
<td>78.3 (70.22–86.38)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Conventional kDNA-PCR</td>
<td>75.6 (71.31–79.9)</td>
<td>80</td>
<td>90</td>
<td>100</td>
<td>47.4 (37.61–57.19)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Conventional ITS-PCR</td>
<td>53.7 (49.07–58.33)</td>
<td>88.9 (85.76–92.04)</td>
<td>60</td>
<td>95.7 (91.72–99.68)</td>
<td>29.6 (20.65–38.53)</td>
<td></td>
</tr>
<tr>
<td><strong>Dog</strong></td>
<td>Real-time PCR</td>
<td>100</td>
<td>96.4 (94.54–98.26)</td>
<td>99</td>
<td>98.6 (96.3–100)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Conventional kDNA-PCR</td>
<td>89.5 (85.76–92.04)</td>
<td>94.6 (92.34–96.86)</td>
<td>91</td>
<td>98.5 (96.11–100)</td>
<td>77.1 (68.86–85.34)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Conventional ITS-PCR</td>
<td>72.2 (67.72–76.68)</td>
<td>89.3 (86.21–92.4)</td>
<td>77</td>
<td>94.5 (90.03–98.97)</td>
<td>55.6 (45.84–65.36)</td>
<td></td>
</tr>
</tbody>
</table>


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References:

- Kumar et al., 2007
- Bensoussan et al., 2006
studies have examined the sensitivity and specificity of PCR assays compared to conventional diagnostic techniques for VL, only a few studies have evaluated these parameters among differing PCR assays. In this study, we compared the sensitivity and specificity of quantitative and conventional PCR techniques. Furthermore, conventional PCR were evaluated for molecular diagnosis of VL, based on kDNA or ITS1 genes.

Based on the statistical data, both DAT and real-time PCR are recommended as effective methods for detection of *L. infantum* infection in humans and dogs (Table 3).

Although 64.58% of symptomatic humans and 50% of dogs infected with *L. infantum* were detected using parasitological methods (Table 1), these methods could not be used for the asymptomatic group in either humans or dogs. On the other hand, a sufficient degree of sensitivity and specificity with molecular and DAT methods was observed for diagnosis of disease. The superior sensitivity of kDNA-based-PCR compared to genomic-DNA-based (Lachaud et al., 2002) or ITS1-based-PCR (Fallah et al., 2011) was previously reported. kDNA-based-PCR has been reported to detect parasites in 100% of asymptomatic dogs vs 62% by genomic-DNA-based techniques for molecular diagnosis of *L. infantum*. Real-time PCR was calculated at 96.4%.

The detection limits for primer pairs were determined by Taqman-based real-time PCR. By conventional PCR, kDNA-based-PCR was more sensitive than ITS-based PCR (Table 3). ITS-PCR detected 1–6 parasites/mL of blood, while the method targeting kDNA could detect less than 1 × 10^−2 parasites/mL (Lachaud et al., 2002) designed a similar study with seeded blood sampling with known parasite concentrations and serial dilution assay (SDA). In their study, genomic DNA showed 2–5 parasites/mL, and the highly repetitive kDNA detected 10^−3 parasites/mL of blood. We did not have a sample with fewer than 0.01 parasites, although a cut-off of 0.01 for kDNA-based PCR was detected, and real-time PCR could detect 0.0001 parasites/mL (data not shown). Therefore the possibility of detection in samples with lower parasitemia using real-time and kDNA-based PCR was predicted. A low detection limit of PCR with 10 fg DNA or 1 parasite/180 μL blood amplified a 115-bp sequence within the 18S rRNA gene of *Leishmania*, has been reported by Deborggraeve et al. (2008), who found specificity of 95.6% for endemic control samples and sensitivity of 86% in blood of patients with VL.

A noteworthy result obtained in this study is concordance between DAT and kDNA-based-PCR (91–80%) in dog and between DAT and real-time PCR (95–99%) in human groups.

Based on the mean (59.19 parasite/mL) and median (16.15 parasite/mL) level of parasitemia in dogs and the mean (12.38 parasite/mL) and median (1.00 parasite/mL) level of parasitemia in humans (Table 2), dogs support a larger reservoir of the parasites than humans.

In conclusion, although the gold standard for VL diagnosis is detection of parasites in specimens of infected organs, these samples must be obtained by invasive procedures. Blood samples are less invasive compared to bone marrow biopsy and are easy to collect. A high level of agreement was obtained between DAT and real-time PCR. Since real-time PCR is a rapid, sensitive, reproducible, and quantitative assay for detection of *L. infantum* infection in dogs and humans, it constitutes a non-invasive alternative to bone marrow aspiration for initial diagnosis of disease.

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