Molecular diagnosis of leishmaniosis in dogs
Comparative application of traditional diagnostic methods and the proposed assay on clinical samples

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Received 15 August 2002; received in revised form 14 January 2003; accepted 25 January 2003

Abstract

Leishmaniosis is a zoonotic, parasitic disease caused by members of the genus Leishmania. The disadvantages of the traditional methods have currently rendered the polymerase chain reaction (PCR), the most reliable alternative for the laboratory diagnosis of this disease. Several relevant protocols have been described in the past but their application is in most cases limited to research use. The latter combined with the diagnostic problems that can be caused by the genetic variability of the different Leishmania strains or the presence of PCR inhibitors, indicate that an alternative approach should be followed for the development of a standard diagnostic tool for leishmaniosis.

In the present study, we have evaluated several PCR-based protocols, in order to identify a primer combination that would allow the reliable detection of Leishmania DNA from clinical material and the verification of its results, in a manner that could be applicable even for routine use.

The evaluation consisted of a BLAST verification of the specificity of the previously described primers, PCR testing, and optimisation of the reaction conditions. Our assessment was completed with the comparative evaluation of the results produced by the proposed PCR assay, light microscopy, and indirect fluorescent antibody technique (IFAT), on clinical samples collected from dogs suspected of leishmaniosis.

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The proposed assay which consists of a combination of two pairs of primers, targeted to different areas of the kinetoplast DNA of *Leishmania* spp., specific for *Leishmania infantum*, *Leishmania donovani* and *Leishmania chagasi*, showed optimum performance on our test samples, and detected 41.9% *Leishmania*-positive dogs from our 160 clinical cases. From the same number of cases, 46.25% were positive by IFAT (titre ≥200), and 19% by microscopic examination of lymph node aspirates.

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**Keywords:** *Leishmania* spp.; Diagnosis; Protozoa; PCR

1. Introduction

Leishmaniosis is a zoonotic, parasitic disease caused by members of the genus *Leishmania*. It is manifested in a number of forms in humans, i.e. cutaneous, mucocutaneous and visceral. The latter is considered a serious and often fatal health threat, especially in endemic areas (de Beer et al., 1990; Ferrer, 1999). The fact that there are almost 90 countries that fall within this category (Hu et al., 2000) necessitates constant alertness for leishmaniosis in order to control the disease in both animals and humans. To this end, a reliable and rapid diagnostic method would be a vital tool for the clinician.

The low sensitivity of the direct microscopic examination of bone marrow aspirates (Piarroux et al., 1994), the inefficiency of serology to differentiate active and cured cases (Hu et al., 2000) and the fact that culture of *Leishmania* cells is a laborious method with low sensitivity (Piarroux et al., 1994), compose a diagnostic problem that may be solved (Hu et al., 2000) by the application of the polymerase chain reaction (PCR). The high sensitivity and specificity, the ability to detect and identify the protozoa involved, and the fact that it can be applied directly on clinical samples and produce a reliable result within only a few hours, are undisputed advantages of PCR over traditional techniques. However, in most cases, these techniques are quite complex and laborious, hence applicable only for research use.

Our aim was to identify a reliable molecular tool to be used for the routine diagnosis of leishmaniosis, achieving the necessary, high level of repeatability and confirmation of results, in a simple and rapid manner. To this end, it was considered preferable to evaluate previously described PCR protocols (Smyth et al., 1992; Piarroux et al., 1994; Berrahal et al., 1996; Ramos et al., 1996; Aviles et al., 1999; Reale et al., 1999; Aransay et al., 2000; Hu et al., 2000; Lambson et al., 2000; Harris et al., 1998) and focus on *Leishmania infantum*, *Leishmania donovani* and *Leishmania major*, since our target area was the Mediterranean (Sideris et al., 1996; Tselentis et al., 1994). The most effective primer combination with regard to sensitivity, specificity, and simplicity, based on the results of the present study, and the information previously reported, would be proposed as suitable for incorporation in the standard assay. Clinical material (skin biopsies, blood, lymph node aspirates, and bone marrow samples) from dogs suspected of leishmaniosis, were examined by light microscopy, indirect fluorescent antibody technique (IFAT), and the standard PCR assay that was finally proposed, in order to complete its evaluation and assess its practical application.
2. Materials and methods

2.1. Parasites

The promastigotes of *Leishmania* species incorporated in the present study (*L. major* LV39, *L. infantum* LA, *L. donovani* R2D2, *L. donovani* MON1, *L. infantum* MCAN/PT/98/IMT 244) were provided by the Scottish Parasite Diagnostic Laboratory Stobhill Hospital and the Department of Parasitology, Hellenic Pasteur Institute.

2.2. Clinical samples

A total of 417 clinical samples from 160 dogs suspected of leishmaniosis were collected by several veterinary clinics and hospitals operating in the broader area of Athens, Greece. Suspicion of leishmaniosis was established on clinical evidence and/or the results of the IFAT that was performed by the Hellenic Pasteur Institute, Athens, Greece, and the National Institute of Agricultural Research, Athens, Greece, in collaboration with the above-mentioned veterinary clinics. The latter also performed the microscopic examination (slides stained with Giemsa) of the lymph node aspirates (Table 1). A total of 215 samples were evaluated by the proposed standard PCR assay in our laboratory. These consisted of 160 whole blood samples (collected with EDTA or heparin), 25 bone marrow and 27 lymph node aspirates and 3 non-lesional skin biopsies (Table 1). Similar samples from healthy dogs and humans were used as negative controls (data not shown).

2.3. DNA extraction

DNA extraction of *Leishmania* promastigotes was performed by CTAB-proteinase K digestion (Maniatis et al., 1982). The extracted DNA was used directly in the PCR. The

<table>
<thead>
<tr>
<th>No. of cases</th>
<th>Serum samples tested by IFAT</th>
<th>Microscopy on lymph node aspirate</th>
<th>Samples tested by PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Blood</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>24</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>90</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>14</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>160</td>
<td>160</td>
<td>42</td>
<td>160</td>
</tr>
</tbody>
</table>

+ and −, availability of the certain type of clinical sample from the respective number of cases.

*Performed by the Hellenic Pasteur Institute, Athens, Greece, and the National Institute of Agricultural Research, Athens, Greece.*

*Performed in the veterinary clinics that provided the cases.*
same method, followed by isoamyl-propanol purification and ethanol precipitation, was used for bone marrow samples of 100–200 μl, and lymph node aspirates. The volume of the lymph node aspirates corresponded to the amount collected by two to three punctures of a thin biopsy needle. CTAB-proteinase K digestion, plus solvent purification and ethanol precipitation, proceeded by thin dissection of the samples was applied for DNA extraction from skin biopsies. Finally, for blood samples a volume of 200 μl was processed with the NucleoSpin Tissue Kit (Macherey-Nagel).

After ethanol precipitation, the purified DNA product was suspended in 50 μl TE buffer, and was evaluated by submerged gel electrophoresis and spectrophotometry, in order to assess DNA quantity, quality and integrity.

2.4. PCR evaluation

Based on the results of the BLAST search (Altschul et al., 1997) carried out for the primers under study, the PCR evaluation was restricted to the protocols described by Aransay et al. (2000) for the primers LINR4–LIN19, Hu et al. (2000) for the primers PrI–PrII, and CELBIO (as reported by G. Vesco, Instituto Zooprofilattico Sperimentalle della Sicilia) for the primers V1–V2 (Table 2). The evaluation of the PCR protocol was performed using approximately 0.25 pg of DNA extracted from Leishmania promastigotes. Additionally, we used total genomic DNA (aliquots of 1 μg), from lymph node aspirates of seven confirmed cases of leishmaniosis (typical clinical appearance, IFAT titres ≥500, lymph node aspirates producing positive results by microscopy). Finally, the PCR protocols were tested with DNA preparations from various bacterial species and fresh tissue samples from healthy dogs and humans, as negative controls (data not shown).

Each PCR assay was performed as described by the respective authors (Table 2), and reactions were run in a MJ Thermocycler (MJ Inc., Watertown, MA, USA) with two different brands of Taq polymerase, namely Promega Taq polymerase (Promega Corp., Madison, WI, USA) and Roche Taq polymerase (Roche Diagnostics, GmbH, Mannheim, Germany). The PCR products were analysed in a 2% agarose gel by submerged electrophoresis. Selected positive results were confirmed by sequencing (MWGAG BIOTECH Co.).

2.5. PCR assay

The method we selected for proposal as a standard assay, consists of a combination of two sets of primers. Each PCR reaction contained, 1× Taq polymerase buffer (Roche), 1.5 mM MgCl₂, 0.2 mM dNTPs, 1.5 U of Taq polymerase (Roche) and 1 μM of each of the primers LINR4–LIN19 (Aransay et al., 2000) or V1–V2 (CELBIO) in a total volume of 30 μl. The Taq polymerase was added after the completion of a 3 min denaturation step at 95 °C (hot start). PCR reactions with the primers LINR4–LIN19 were performed with 33 cycles of 30 s at 95 °C, 30 s at 58 °C, and 1 min at 72 °C followed by a 7 min completion step at 72 °C. The temperature profile for the primers V1–V2 consisted of 40 cycles of 30 s at 95 °C, 30 s at 56 °C, and 1 min at 72 °C followed again by a 7 min completion step at 72 °C. Reactions were overlaid with two drops of mineral oil. The DNA products were stained with ethidium bromide and photographed.
Table 2
The characteristics of the PCR assays that were currently evaluated

<table>
<thead>
<tr>
<th>Assay published by</th>
<th>Specificitya</th>
<th>Producta (bp)</th>
<th>Target areaa</th>
<th>Performed withb</th>
<th>Method assessed by</th>
<th>Sensitivity/methodc, b</th>
<th>Primersc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minicircle kinetoplast DNA</td>
<td>Taq polymerase</td>
<td>Boehringer</td>
<td>Biorocket, Eurogenetic, Liege Belgium</td>
<td>+−−</td>
<td>1 fg/PCR hybridisation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. infantum, L. chagasi, L. donovani</td>
<td>321</td>
<td>Minicircle kinetoplast DNA</td>
<td>Life Technologies, Gaithersburg, MD, USA</td>
<td>+−−</td>
<td>1 fg/PCR hybridisation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hu et al. (2000)</td>
<td>L. donovani, L. infantum</td>
<td>750</td>
<td>Promega Corp., Madison, WI</td>
<td>+−−</td>
<td>0.1 ng to 1 fg/PCR hybridisation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CELBIOd</td>
<td>L. infantum</td>
<td>720</td>
<td>GIBCO-BRL</td>
<td>+−−</td>
<td>5 parasites/PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Berrahal et al. (1996)</td>
<td>Leishmania spp.</td>
<td>125</td>
<td>Promega Corp., Madison, WI</td>
<td>+−−</td>
<td>0.1 ng to 1 fg/PCR hybridisation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aransay et al. (2000)</td>
<td>Leishmania spp.</td>
<td>720</td>
<td>GIBCO-BRL</td>
<td>+−−</td>
<td>5 parasites/PCR</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

+, performed; −, not performed/not reported.

a As reported by the authors.
b Relevant data are recorded only for those protocols that were assessed by PCR.
c 5‘−3‘ direction.
d As reported by G. Vesco, Instituto Zooprofilattico Sperimentale della Sicilia.
To identify false-positive results caused by a carryover effect, 20% of the samples tested in every assay were negative controls. An equal percentage of samples prepared with DNA from *Leishmania* promastigotes spiked into negative samples (of the same type with the ones examined), were utilised as positive controls. Control samples were processed identically to the cases under study with regard to pre-treatment, DNA extraction and preparation for PCR.

The detection limit of the assay was assessed as previously described (Ikonomopoulos et al., 1998, 1999) using serial dilutions of DNA from *Leishmania* promastigotes.

2.6. Statistical analysis

Initially, descriptive statistics were calculated including cell frequencies of $2 \times 2$ tables and the percent agreement between different methods. Formal statistical evaluation was based on the Fisher’s exact test, performed by the software STATA 6.0 (STATA Corp.).

3. Results

3.1. PCR evaluation

PCR assays performed on DNA extracted from *Leishmania* promastigotes, and total genomic DNA extracted from our positive lymph node samples, amplified in most cases, the expected DNA products of around 720 bp for the primers LINR4–LIN19 (Figs. 1 and 2), and 750 bp for the primers V1–V2 (Figs. 3 and 4). Under the conditions originally described (Table 2), the primers PrI and PrII failed to produce any amplification from our positive control samples. In some cases, the expected 321 bp DNA product, was replaced by several non-specific products (Fig. 5).

Primers LINR4–LIN19 and V1–V2 produced consistent result with all our positive and negative controls (Figs. 1–4). PCR assays performed with the primers V1–V2, on serial dilutions of DNA from *Leishmania* promastigotes, produced a clearly visible product from a minimum of 31.5 ng of DNA, corresponding approximately to 300 parasitic cells (Piarroux et al., 1994) (Fig. 6). Similar results were also recorded for the primers LINR4–LIN19 (data not shown). However, for the primers V1–V2, these results were found after we increased the number of cycles from 30 to 40, and modified the temperature profile from 60 s at 94 °C, 60 s at 67.5 °C, and 1 min at 72 °C, to the one shown above. In each PCR assay, Promega Taq polymerase was substituted for Roche Taq polymerase. This increased the signal intensity and eliminated non-specific amplification products (Figs. 1–4).

3.2. Clinical samples

Of the 160 cases, 74 (46.25%) were positive by IFAT (titre $\geq$ 200), and 69 (43.12%) by PCR (Fig. 7). Of the 74 cases that were considered positive by serology (IFAT), 65 (87.8%) produced positive PCR results on at least one of the types of clinical samples that were tested (Table 3). Nine IFAT-positive cases, tested negative by PCR performed on blood samples. From the 86 cases that were IFAT-negative, 4 (4.6%) produced positive PCR
Figs. 1–4. Comparative performance evaluation of Promega (1 and 3) and Roche (2 and 4) Taq polymerase in a PCR assay performed with a number of reference *Leishmania* strains and the primers LINR4–LIN19 (1 and 2) and V1–V2 (3 and 4). The specific 720 bp DNA product is exhibiting size variability based on the *Leishmania* strain examined: (lane 1) 1 kb DNA ladder (BioLabs); (lane 2) *L. donovani* R2D2; (lane 3) *L. major* LV39; (lane 4) *L. infantum* MCAN/PT/98/IMT 244; (lane 5) *L. infantum* L4; (lane 6) negative control consisted solely of PCR reaction mixture.
results on at least 1 of the corresponding clinical samples: One IFAT-negative case tested positive by PCR performed on bone marrow, lymph node, and skin samples, one produced PCR-positive result on blood, and bone marrow samples, one just on bone marrow, and one produced a positive PCR result only on blood (Table 3).

The 8 positive results that were recorded from the total of 42 lymph node aspirates examined by microscopy, corresponded to cases producing positive results both by IFAT and PCR. Of the 34 negative cases of this group, 16 (47%) produced PCR- and IFAT-positive results, while 4 (11.8%) produced PCR-positive and IFAT-negative results (Table 4).
Fig. 5. PCR assay performed with the Promega Taq polymerase and the primers PrI–PrII on various types of samples: (lanes 1 and 13) 100 bp DNA ladder (BioLabs); (lanes 2 and 3) PCR performed on DNA preparations from *L. infantum* L4 and *L. infantum*, MCAN/PT/98/IMT 244, producing no visible DNA product; (lanes 4–6) PCR assay performed on DNA preparations derived from blood samples that had tested positive with the primers LINR4–LIN19 and V1–V2 producing several by-products but not the expected 320 bp DNA product; (lane 7) PCR assay performed on DNA preparations derived from lymph node samples that had tested positive with the primers LINR4–LIN19 and V1–V2, producing several by products but not the expected 320 bp DNA product; (lanes 8–11) PCR assay performed on DNA preparations derived from blood (lanes 8–10) and lymph node samples (lane 11) that had tested negative with the primers LINR4–LIN19 and V1–V2, currently producing no visible DNA product; (lane 12) negative control samples consisted solely of reaction mixtures with the primers PrI–PrII.

Fig. 6. PCR assay performed with the Roche Taq polymerase and the primers V1–V2, on serial two-fold dilution of *L. infantum* L4 purified DNA: (lane 1) negative control consisted solely of PCR reaction mixture; (lane 2) 1 kb DNA ladder (BioLabs); (lanes 3–7) 250, 125, 62.5, 31.25, and 16 ng of *L. infantum* L4 purified DNA, respectively.
Fig. 7. PCR assay performed with the Roche Taq polymerase and the primers LINR4–LIN19 and V1–V2, on canine blood samples: (lanes 1A and 1B) 1 kb DNA ladder (BioLabs); (lanes 2–4A) PCR results of blood samples tested with the primers LINR4–LIN19, showing the *Leishmania*-specific 720 bp DNA product; (lanes 2–4B) PCR results of blood samples tested with the primers V1–V2, showing the *Leishmania*-specific 750 bp DNA product; (lanes 5A and 5B) negative control blood samples tested with the primers LINR4–LIN19 and V1–V2; (lanes 6A and 6B) negative control samples consisted solely of PCR reaction mixtures with the primers LINR4–LIN19 and V1–V2.

Table 3
Comparison of results obtained by PCR and IFAT performed on clinical material collected from 160 dogs suspected of leishmaniasis

<table>
<thead>
<tr>
<th>Results produced by serology</th>
<th>Blood</th>
<th>Bone marrow</th>
<th>Lymph node</th>
<th>Skin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (74)</td>
<td>65</td>
<td>9</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Negative (86)</td>
<td>4</td>
<td>82</td>
<td>3</td>
<td>17</td>
</tr>
<tr>
<td>Percent agreement&lt;sup&gt;b&lt;/sup&gt;</td>
<td>91.8</td>
<td>84</td>
<td>88.9</td>
<td>33.3</td>
</tr>
<tr>
<td>Fisher’s exact <em>P</em>-value&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.012&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> The results of serology correspond to 160 blood samples, which equal the number of blood samples that were tested by PCR. Corresponding bone marrow (25), lymph node (27), and skin biopsy (3) samples from the same cases were also examined by PCR.

<sup>b</sup> Agreement between the results obtained by IFAT and the proposed PCR assay.

<sup>c</sup> Statistically significant difference.
Table 4
Comparison of results obtained by microscopic examination, IFAT, and PCR, performed on lymph node aspirates collected from 42 dogs suspected of leishmaniasis

<table>
<thead>
<tr>
<th>Results produced by microscopy</th>
<th>Results produced by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFAT</td>
</tr>
<tr>
<td>Positive (8)</td>
<td>+ 8</td>
</tr>
<tr>
<td>Negative (34)</td>
<td>− 16</td>
</tr>
</tbody>
</table>

Percent agreement<sup>b</sup> 61.9 52.3
Fisher’s exact P-value<sup>c</sup> 0.007<sup>c</sup> 0.037<sup>c</sup>

<sup>a</sup> Positive result from at least one type of the clinical samples tested by PCR.
<sup>b</sup> Agreement between the results obtained by microscopy, with those of IFAT and PCR, respectively.
<sup>c</sup> Statistically significant difference.

Under the specific reaction conditions described above, we recorded discrepant results between the two PCR assays currently incorporated to the diagnostic investigation of our clinical cases, in 10 out of 215 (5%) of our clinical samples.

4. Discussion

4.1. PCR evaluation

The PCR assays that were used on our clinical samples were selected on criteria of sensitivity, specificity and simplicity. A crucial parameter was the ability to identify the DNA product by simple visual observation without the need of DNA hybridisation, which adds to the complexity of the overall process. DNA hybridisation could increase the detection limit of the test, although this is naturally enhanced by the presence of multiple copies of the target area in each parasite. To facilitate visual identification of the amplicons, we tried to avoid primers that amplify short DNA sequences, and PCR assays that may produce DNA by-products of similar size with the expected amplicons. The latter was considered of special significance since the kinetoplast DNA that was chosen as the DNA target of the final combination of primers, includes a variable region that should be expected to cause considerable size variability of the amplified products, depending on the species or sometimes even the strains of Leishmania spp., under study (Rodgers et al., 1990; Spithill and Grumont, 1984).

Based on the validation of the previous reports on the assays under study, and the results obtained by us under the conditions mentioned above, the proposed combination of primers allows easy, rapid, and direct detection of DNA from L. infantum, L. donovani and L. major from clinical material, without the need of laborious and time consuming techniques.

4.2. Clinical samples

In order to complete the evaluation of the PCR assays currently assessed for the detection of Leishmania DNA from clinical material, we incorporated a considerable number of
clinical samples from dogs suspected of leishmaniosis from the broader area of Athens, Greece. Our sample size does not support the purpose of a clinical trial that was not within the scope of this study, but may substantiate the accuracy of the results acquired by the proposed standard method. The latter is supported by the concordance of the results obtained by ourselves and others, regarding the percentage of canine leishmaniosis in the same geographic area that was estimated to around 48% by IFAT (Sideris et al., 1996).

The comparison of the results obtained by IFAT and the proposed standard PCR assay performed on the same or corresponding blood samples (Table 3), show 91.8% agreement, with 147 out of 160 cases producing identical results by both PCR and serology. The agreement within the positive and the negative results produced by each method was 87.8% (65 positive cases by both methods out of 74 serology positive cases) and 95.3% (82 negative cases by both methods out of 86 serology negative cases), respectively. These results indicate higher discrepancy between PCR and IFAT for the IFAT-positive cases. This can be attributed to antibodies persisting even after the elimination of PCR detectable Leishmania DNA.

On the other hand, the immunodeficiency of some patients (Slappendel and Ferrer, 1998) and the resulting inability to produce sufficient amounts of antibodies, may account for the four PCR-positive results produced from cases reacting negative by IFAT, a discrepancy that has also been recorded by others (Reale et al., 1999; Berrahal et al., 1996). This result has to be recognised as a weakness of serology, since the PCR results were validated by the application of the method on more than one different types of clinical samples. The agreement of IFAT and PCR results performed on lymph node, bone marrow, and skin biopsy samples, was 84, 88.9 and 33.3%, respectively (Table 3), although the latter corresponds to only three skin biopsies. Discrepancy between the results of IFAT and PCR performed on corresponding clinical material can be attributed to immunodeficiency concurrent to leishmaniosis, possible sampling errors, quite common in lymph node and bone marrow collection, or finally, the fact that the antigens of the parasite are not uniformly distributed throughout the body.

The comparison of the results obtained by the microscopic examination of lymph node smears to those obtained by PCR (Table 4), indicate that, as expected, the latter is far more sensitive. Supportive of this statement is the fact that 14 out of 34 samples (41%) that were considered negative by microscopy, produced PCR-positive results, 12 of which (35.3%) were also positive by IFAT. The latter has to be attributed to the low detection limit of microscopic examination or perhaps to the false interpretation of the results. Considering that all the samples that produced a positive result by microscopic examination have also produced positive PCR results, supports high agreement of these methods for positive samples.

In addition to blood samples, PCR was performed on corresponding clinical material consisting of lymph node, bone marrow, and skin biopsy samples (Table 1), in 46 out of 160 cases. Discrepancy of the PCR results between the different types of clinical material incorporated in the assay were recorded in 3 out of 46 cases for which corresponding samples of various types were available. The results obtained by PCR on blood samples were confirmed to a percentage that reached 93.5% (43 out of 46 cases) by an identical PCR result on at least one of the other types of clinical material (data not shown). The latter, and the fact that PCR amplified products were, in some cases, detected only in blood samples and not in the corresponding bone marrow samples, an observation that has also been reported by others (Hu et al., 2000), suggest that blood samples are a reliable clinical material type for the detection of Leishmania DNA by PCR. This observation is in agreement with previous
reports (Hu et al., 2000; Reale et al., 1999) and can be attributed to the simplicity of blood collection, which makes it a procedure less prone to errors. Furthermore, blood sampling allows the examination of a much larger sample volume than bone marrow sampling, which is often suggested as the ideal type of clinical material for detection of *Leishmania* DNA by PCR (Bettini et al., 1990). The above is of significant practical use, since blood collection is much less invasive and by far more acceptable to dog owners than bone marrow or lymph node collection. However, regardless of the specific type of clinical material that will be finally selected, the above results indicate that it is significant to apply repeated sampling, i.e. acquiring several samples over time, in order to increase the predictive value of PCR incorporated to the diagnosis of leishmaniosis.

Finally, disagreement between the PCR results recorded with each of the two sets of primers, was recorded in about 5% (10 out of 215) of our clinical samples. Given that positive controls of each assay produced the expected result, the above discrepancy can be attributed to false negative results possibly caused by even the small sequence divergence recognised among the minicircle classes of the constant region (Reale et al., 1999; Piarroux et al., 1993; Degrave et al., 1988; Spithill and Grumont, 1984), or perhaps by PCR inhibitors. This problem, which could be of considerable significance in routine diagnosis, was eliminated by the combined use of two sets of primers targeted to different areas of the *Leishmania* genome. This practice that offered a simple alternative for the necessary cross verification of the results allowed very high levels of sensitivity and specificity. Thus, if we acknowledge as confirmed cases of leishmaniosis those that produced positive results by serology and microscopic examination of lymph node smears, and as negative cases, those that consisted our negative controls, the sensitivity and specificity of the proposed PCR assay, have both reached 100%.

### 5. Conclusion

It can be suggested that the proposed standard PCR assay is a reliable tool for the detection of *Leishmania* DNA from clinical material. The method relies on a combination of two different sets of primers in order to increase its diagnostic spectrum and eliminate false negative results that could be caused by PCR inhibitors or the genetic variability of *Leishmania* spp., a problem that cannot be resolved even with the incorporation of hybridisation. The assay produces a result within only 12–24 h, depending on the nature of the sample, and it does not involve biological hazards, or laborious and complex procedures. Furthermore, the application of this method has currently proved that it may resolve the diagnostic problems that result by the low sensitivity of microscopic examination and the low positive and negative predictive values of serology, usually attributed to persistent antibodies or immuno-suppression, respectively.

### Acknowledgements

We would like to thank Dr. E. Dotsika, of the Athens Pasteur Institute, Greece, and Professor Huw V. Smith, of the Scottish Parasite Diagnostic Laboratory, Stobhill Hospital,
Glasgow, for their kind help and support. Finally we would also like to thank Dr. Nigel Cook of the Central Science Laboratory, Yorkshire, UK, for his valuable help in editing this text.

References


