Comparison of DNA isolation methods to detect *Leishmania* parasites in blood samples

Karin Hagardson

Athens 2006
CONTENTS

1. ABSTRACT 3
2. INTRODUCTION 3-8
3. METHODS AND MATERIALS 8-11
  3.1. Preparation of seeded samples 8
  3.2. Isolation of white blood cells 8-9
    3.2.1. Ficoll separation method 8
    3.2.2. Erythrocyte lysis method with TE 8-9
  3.3. DNA isolation 9-10
    3.3.1. Proteinase K 9
    3.3.2. Sample preparation commercial kit 9-10
  3.4. PCR amplification 11
  3.5. PCR product analysis 11
  3.6. Precautions to avoid contamination 11
4. RESULTS 12-14
5. DISCUSSION 15-16
6. ACKNOWLEDGMENTS 17
7. REFERENCES 17-19
1. ABSTRACT
Leishmaniasis is a disease affecting more than 12 million people worldwide. It is caused by the protozoan parasite Leishmania, which is transmitted to humans and dog hosts through bites of infected sand flies belonging to genus Phlebotomine. Several studies have shown Polymerase Chain Reaction (PCR) to be effective for the diagnosis of VL in clinical samples compared to the classical methods. The aims of this study were first to compare four different sample preparation methods for the PCR diagnosis of visceral leishmaniasis (VL) using peripheral blood samples and furthermore to find a method that is sensitive, rapid, cost benefit, simple and easy to perform. Two preparation methods were compared for the isolation of leukocytes (with Ficoll and Tris –EDTA buffer) and two DNA isolation methods (with Proteinase K and QIAgen kit). From the methods that were compared, lysis of erythrocytes with TE and the QIAgen kit seems to be the most suitable to use.

Keywords: Leishmania species, Blood, Diagnosis, PCR, Sand fly, Leishmaniasis

2. INTRODUCTION
Leishmaniasis is a parasitic disease spread through the bite of infected sand flies [1], named after W.B Leishman, who discovered one of the first strains of Leishmania in 1901.

Leishmaniasis affects more than 12 million people worldwide and is found in parts of about 88 countries (22 in the New World and 66 in the Old World), therefore is a major public health problem [2,3]. Most of the affected countries are in the tropics and subtropics, for example Mexico, Central and South America, Southern Europe, Asia, Middle East and Africa. However, leishmaniasis is not found in Australia, Oceania [1].

The disease is caused by the protozoan parasite Leishmania, that is transmitted to human and dog hosts through bites of infected sand flies (subfamily phlebotominae). The sand fly is the vector of leishmaniasis and is a small dipteran [2], that becomes infected by biting an infected animal (for example, a rodent, a dog or person) [1].

Since sand flies do not make noise when they fly, they can be hard to detect. They are very small, only about one-third the size of typical mosquitoes, most active in twilight, evening, and night-time hours (from dusk to dawn) and less active during the hottest day
intervals. However, they will bite if disturbed, for example when a person brushes up against the trunk of a tree where sand flies are resting [4].

Transmission depends on the uptake of amastigotes by a feeding sand fly. The *Leishmania* parasite multiplies in the female sand fly in a flagellate form (promastigote). Promastigotes are then inoculated intradermally when the sand fly bites a new host and takes a blood meal. They are injected along with the insect saliva [4]. In mammalian hosts like humans, macrophages and other phagocytes take up the promastigotes, which then become the rounded, non-flagellate forms called amastigotes. These structures remain intact intracellularly [5], while the parasitic organism is retained in the cell parasitophorous vacuoles. In this way, the parasite is protected from digestion and can escape the host defense system [2]. Infected macrophages eventually burst (lyse) and release the pathogenic organism, which is now ready to infect new phagocytes [5].

There are different species of the genus *Leishmania* [6], normally 20 known *Leishmania* species. In Greece there are three species that are more abundant than others, these are *L. tropica*, *L. major* and *L. infantum*.

*Leishmania* is responsible for a range of different diseases. In some of them the amastigotes do not spread beyond the site of the vector’s bite. This results in a cutaneous form of leishmaniasis that often heals spontaneously. In other instances, the amastigotes may spread to the visceral organs (liver, spleen), resulting in visceral form of leishmaniasis (kala-azar or Dum-Dum fever), or to the mucous membranes of the mouth and nose, resulting in mucocutaneous leishmaniasis [7].

Their clinical presentations can be pleomorphic, and one species can be responsible for more than one clinical form. Most species cause cutaneous disease, while others are responsible for mucocutaneous and visceral disease [8].
The different forms of leishmaniasis are listed below:

- **Visceral leishmaniasis (VL).** *L. infantum* species is the pathogenic organism for human VL. The disease affects almost half million people annually [9]. It has caused a serious health problem in the greater Athens area in Greece [10]. VL is the most severe form of the disease, which if untreated, has a mortality rate of almost 100%. People with VL usually have fever, weight loss, anemia and swelling of the spleen and liver [1].

- **Cutaneous leishmaniasis (CL)** is the most common form of the disease and is caused by *L. tropica* and *L. major* species. It is a complex disease with a wide spectrum of clinical manifestations [11]. It causes 1-200 simple skin lesions which self-heal within a few months, leaving unsightly scars [12].

- **Mucosal leishmaniasis (MCL)** is a mutilating disease. It begins with skin ulcers (CL), which spread, causing dreadful and massive tissue destruction, especially of the nose and mouth [12].

- **Diffuse cutaneous leishmaniasis (DCL)** produces disseminated and chronic skin lesions resembling those of leprosy. The treatment of this form is difficult [12].

Cases of human leishmaniasis have been reported with an increasing incidence (Among others, the spread of the parasites to new geographical areas). Leishmaniasis is rather difficult to diagnose. This is due to the generally low number of parasites found in clinical samples [6,13].

People of all ages are at risk for leishmaniasis, especially if they live or travel in Leishmania - endemic regions. Leishmaniasis is predominantly a children’s disease and acts as an opportunistic pathogen in immunocompromised patients, such as AIDS patients. It can also be spread by blood transfusions, contaminated needles or even from a pregnant woman to the embryo [1].
Leishmaniasis is a treatable disease. Even today there is still no vaccine that is ready for general use to prevent leishmaniasis, but research is going on [1].

There are four categories of diagnostic methods:

- Parasitological methods (detection of amastigotes in stained smears of aspirates of bone marrow or lymph nodes)
- In vitro cultivation
- Serological methods (immunofluorescence (IF) and enzyme-linked immunosorbent assay (ELISA))
- Molecular methods (Polymerase chain reaction (PCR))

Over the last decade, several studies have shown PCR to be both highly specific and more sensitive than the classical methods for the diagnosis of leishmaniasis. PCR is more suitable for diagnosis, as it can be performed in any biological sample, including skin tissue, blood and bone marrow [3,8,13,14,15,16]. Additionally, it is always recommended to use more than one diagnostic test.

To diagnose leishmaniasis with PCR, several *Leishmania* DNA multi-copy targets have been identified, such as kinetoplast DNA (kDNA), ribosomal RNA genes (rRNA), mini-exon-derived RNA genes (medRNA) and genomic repeats. *Leishmania* are members of the order kinetoplastida, a group of organisms (which possess the kinetoplast, a unique DNA structure, a single mitochondrion which contains a characteristic DNA structure known as the kinetoplast DNA network). Kinetoplast DNA is comprised of two components, maxicircle and minicircle kDNA. Maxicircles, 20-40kb in length, are present in 30-50 copies and carry the genes encoding the mitochondrial enzymes. Minicircles are usually 1kb in length, they encode guide RNA (gRNA) molecules involved in the RNA editing of maxicircle crypogenes and are present in 10000-20000 copies. The kDNA is a good target because it contains multiple copies of the minicircles, each having regions with different rates of sequence evolution. Primers for the PCR reactions directed to amplify the conserved region can be used to detect all *Leishmania* species [5,10,15,17,18,19,20,21]

The purpose of a PCR (Polymerase Chain Reaction) is to amplify the initial number of template genomic region. There are three major steps in a PCR, which are repeated for 30 or 40 cycles. The steps are: *Denaturation* at 94°C, when the double strand melts open to single stranded DNA, *Annealing* at 54°C, when the primers form hydrogen bonds with complementary regions of the template DNA and finally *Extension* at 72°C which is the
ideal working temperature for the polymerase. The reaction is preformed on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time [22]. The bases (complementary to the template) are coupled to the primer on the 3' side as DNA polymerase adds dNTP's from 5' to 3', reading the template from 3’ to 5’ [22].

The PCR products are electrophoresed in agarose or polyacrylamide gels and the bands are visualised over a UV transilluminator. The ladder use in electrophoresis is a mixture of fragments with known size to compare with the PCR fragments.

In this study erythrocytes were removed from blood samples using ficoll separation or lysis with TE buffer and DNA was isolated, using Proteinase K and the QIAamp DNA Blood Mini Kit (Cat.No.51106 QIAGEN) according to the manufacturer’s instructions. Proteinase K is an enzyme commonly used for DNA isolation that degrades proteins so that the DNA is available for PCR. Tween 20 is included in the buffer for membrane disruption and protein denaturation.

The QIAamp kit is designed to provide a fast and easy method for purification of total DNA for reliable PCR and Southern blotting. Total DNA (e.g. genomic, viral, mitochondrial) can be purified from whole blood, plasma, serum, buffy coat, bone marrow, other body fluids, lymphocytes, cultured cells, tissue and forensic specimens. Samples may be either fresh or frozen. [23]. The purified DNA is free of protein, nucleases and other contaminants or inhibitors. DNA purified using QIAamp kit ranges in size up to 50kb, with fragments of approximately 20-30kb predominating. DNA is absorbed onto the QIAamp silica-gel membrane during a brief centrifugation. Salt and pH conditions in the lysate ensure that protein and other contaminants, which can inhibit PCR and other downstream enzymatic reactions, are not retained on the QIAamp membrane. The lysate buffering conditions are adjusted to allow optimal binding of the DNA to the QIAamp membrane before the sample is loaded onto the QIAamp spin column. DNA bound to the QIAamp membrane is washed in two centrifugation steps. The use of two different wash buffers, AW1 and AW2, significantly improves the purity of the eluted DNA. Wash conditions ensure complete removal of any residual contaminants without affecting DNA binding. Purified DNA is eluted from the QIAamp spin column in a concentrated form in Buffer AE [23].
3. MATERIALS AND METHODS

3.1. Preparation of seeded samples:
Seeded samples were made by adding live L. infantum promastigotes obtained for culture to peripheral blood from healthy donors. The concentrations of parasites tested were 1000, 100 and 10 parasites/ml of blood. The parasites were counted in Newbauer plates.

Throughout the duration of the project and because of the limited blood quantity from a single donor on a daily basis send from the hospital to our laboratory, more than one different blood samples were mixed to obtain the desired final volume. Furthermore, to avoid coagulations, all blood samples that were used had the same blood group and were fresh to ensure that the cells would be intact. In case the clinical specimen is not fresh hemolysis is observed, which is more profound when using the ficoll separation method, as the upper layer containing plasma is colored red.

The whole procedure of DNA isolation can be separated in two phases, isolation of white blood cells and disruption of leukocytes and complexes between DNA and proteins.

3.2. Isolation of white blood cells:

3.2.1. Ficoll separation method:
Ficoll separates leukocytes from erythrocytes. After centrifugation, the erythrocytes sediment to the bottom of the tube and the leukocytes can then be collected.

1) Add 1 ml Ficoll and on top, 1 – 2 ml of peripheral blood to an 8 ml plastic tube, gently, so that they don’t mix. Centrifuge at 600 x g for 30 mins.
2) The leukocytes are collected from the interphase and carefully applied to a new 8 ml tube with a Pasteur pipette. Fill it up with PBS and centrifuge at 1000 x g for 10 mins.
3) Remove the supernatant and transfer the pellet to a 1.65 ml Eppendorf tube. Fill it up with PBS. Centrifuge at 10 000 x g for 1 min.
4) Store the pellet at -20ºC for further use.

3.2.2. Erythrocyte lysis method with Tris – EDTA buffer:
Tris – EDTA (TE) is a hypotonic solution that lyses the cells, because the concentration of electrolyte is below that in the cells. In this situation, osmotic pressure leads to the migration of water into the cells, in an attempt to balance the electrolyte concentration inside and outside the cell wall. If the difference in concentration is significant, the cell wall may rupture, leading to cell death [25].
All samples consisted of 1 to 2 ml of peripheral blood. The parasites were then added at an adequate concentration to each sample as described above.

1) Take 2 ml peripheral blood, add 2-3 ml of TE and centrifuge at 1000 x g for 10 mins.
2) Remove the supernatant, keep the pellet and transfer it to a sterile 1.65 ml Eppendorf tube and fill it up with TE. Mix by pulse-vortexing and centrifuge at 10 000 x g for 1 min (This step is repeated 1 more time).
3) Store the pellet at -20ºC for further use.

3.3. DNA isolation:

3.3.1. Proteinase K
The pellet from either the Ficoll or the TE methods was resuspended in 100 µl of PCR buffer (50 mM KCl, 20 mM Tris-Cl pH 8.3, 2.5 mM MgCl₂, 0.5% Tween 20) containing Proteinase K (500µg/ml). After overnight incubation at 56ºC, the cell debris, insoluble particles were pelleted with centrifugation at 10 000 x g for 1 min. The supernatant was heated at 96ºC for 10 mins to inactivate proteinase K. 10 µl were used as the starting template for PCR.

3.3.2. Sample preparation commercial kit:
The QIAamp DNA Blood Mini Kit (QIAGEN) was used according to the supplier’s instructions, blood and body fluid spin protocol:

1) Pipette 200 µl sample (pellet from either the Ficoll or the TE methods) to a microcentrifuge tube. If the sample volume is less than 200 µl, add the appropriate volume of PBS.
2) Add 20 µl QIAgen Protease (or Proteinase K) and 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 secs. In order to ensure efficient lysis, it is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution.
3) Incubate at 56ºC for 15 mins. DNA yield reaches a maximum after lysis for 10 mins at 56ºC. Longer incubation times have no effect on yield or quality of the purified DNA.
4) Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
5) Add 200 µl ethanol (96-100%) to the sample and mix again by pulse-vortexing for 15 secs. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

6) Carefully apply the mixture from step 6 to the QIAamp spin column (in a 2 ml collection tube) without wetting the rim, close the cap and centrifuge at full speed: 20 000 x g (14000 rpm) for 1 min. Centrifugation at full speed will not affect the yield or purity of the DNA. Full speed centrifugation is recommended to avoid clogging. If the lysate has not completely passed through the column after centrifugation, centrifuge again until the QIAamp spin column is empty. Place the QIAamp spin column in a clean 2 ml collection tube and discard the tube containing the filtrate.

7) Carefully open the QIAamp spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 20 000 x g for 1 min. Place the QIAamp spin column in a clean 2 ml collection tube and discard the collection tube containing the filtrate.

8) Carefully open the QIAamp spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at 20 000 x g for 3 mins.

9) The final step is to place the QIAamp spin column in a clean 1.5 ml microcentrifuge tube and discard the collection tube containing the filtrate. Carefully open the QIAamp spin column and add 100 µl Buffer AE. Then centrifuge at 20 000 x g for 1 min. Take 10 µl to the PCR amplification.

3.4. PCR amplification

PCR with primers Lei70R and Lei70L, which produce a 345 bp fragment upon amplification, was performed in a final volume of 100 µl (10 µl of DNA sample) containing 1 x PCR Buffer, 0.2 mM of each deoxynucleotide (dNTPs), 3 units of Taq DNA Polymerase (both PCR buffer and Taq DNA Polymerase are from New England Biolabs Inc) and 1 mM of each primer (Syntezza).

Reaction mixtures were cycled in a PTC-200 (MJ Research), a MiniCycler (MJ Research) or a Mastercycler gradient (Eppendorf).

The ThermoCycler programme that was used had the following conditions: initial denaturation at 94ºC for 5 mins and 40 cycles at 94ºC for 30 secs, 65ºC for 30 secs and 72ºC for 30 secs, and a final elongation step at 72ºC for 5 mins.
For each experiment, a negative control was used, containing DNA from a healthy donor. Positive controls were not needed because the samples were seeded and therefore already positive.

3.5. PCR product analysis
PCR products were visualized under UV light after electrophoresis in a 2 % agarose gel, containing 0.6 g agarose, 30 ml of 1xTAE buffer (800 mM Tris, 40 mM disodium EDTA and 400 mM Sodium acetate, final pH 7.9) and 0.5 µg/ml ethidium bromide, for 15 mins at 100V.

3.6. Precautions to avoid contamination
It is important to avoid cross-contamination between sample preparations. To avoid contamination, DNA isolation, PCR assays and electrophoresis were carried out in separate areas using laminar flow cabinets and aerosol barrier tips for DNA isolation and PCR reaction preparation. It is imperative to wear gloves throughout the entire procedure.
4. RESULTS

The PCR results obtained with seeded blood samples are shown in tables 1 and 2. The sensitivity of each PCR test was assessed from the intensity signal of the respective PCR product from ethidium bromide – stained agarose gels (graded from + to +++).

Figure 9: Gel photograph of experiment 4 (for further details see table 1)

(Lane 1: Ficoll and PrK with 1000 parasites/ml of blood, lane 2: TE and PrK with 1000 parasites/ml of blood, lane 3: Ficoll and PrK with 100 parasites/ml of blood, lane 4: TE and Prk with 100 parasites/ml of blood, lane 5: Ficoll and PrK with 10 parasites/ml of blood, lane 6: TE and Prk with 10 parasites/ml of blood, lane 7: Ficoll and PrK negative sample (blank), lane 8: TE and PrK negative sample (blank), lane 9: 100 bp DNA ladder)

Figure 10: Gel photograph of experiment 2 (for further details see table 2)

(Lane 1: TE and PrK with 1000 parasites/ml of blood, lane 2: TE and QIAGEN with 1000 parasites/ml of blood, lane 3: TE and PrK with 100 parasites/ml of blood, lane 4: TE and QIAGEN with 100 parasites/ml of blood, lane 5: TE and PrK with 10 parasites/ml of blood, lane 6: TE and QIAGEN with 10 parasites/ml of blood, lane 7: TE and PrK negative sample (blank), lane 8: TE and QIAGEN negative sample (blank)
<table>
<thead>
<tr>
<th>SEED SAMPLE (PARASITES/ML)</th>
<th>FICOLL + PRK</th>
<th>TE + PRK</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td><strong>Experiment 3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>100</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td><strong>Experiment 4</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>100</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td><strong>Experiment 5</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>100</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>10</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Table1. Comparison of leukocyte isolation methods (Ficoll and TE)
(+ to +++: less intense to most intense, -: no pattern on the gel, PrK - Proteinase K)

Ficoll experiments 1 and 2 produce the same results, while experiments 3 and 4 produce an additional intensity signal for 100 *Leishmania* parasites per ml of blood donor. Experiment 5, on the other hand gives successful signal even for 10 *Leishmania* parasites per ml of blood donor. The inconsistency of the results obtained with ficoll could be attributed to blood donor differences.
<table>
<thead>
<tr>
<th>SEEDED SAMPLE (PARASITES/ML)</th>
<th>TE + QIAGEN</th>
<th>TE + PRK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>100</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>100</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>100</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Experiment 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>10</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>1</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Experiment 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>100</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2. Comparison of DNA isolation methods (QIAGEN kit and Proteinase K)
(+ to +++ : less intense to most intense, - : no pattern on the gel, PrK - Proteinase K)
5. DISCUSSION

Throughout the duration of this study we were interested in comparing four different laboratory methods and find a method that is rapid, reproducible, accurate, cost benefit and easy to perform for PCR diagnosis of visceral leishmaniasis (VL). Only peripheral blood samples were used, as this type of sampling is easy to repeat, less invasive and therefore easier for the patient. Nevertheless, the parasite load in the circulation blood is lower than in the bone marrow.

Leukocytes should be isolated as erythrocytes don’t contain any DNA and furthermore the containing hemosphere is known to inhibit DNA polymerase. It is the leukocytes that may be infected by the parasites and therefore contain Leishmania DNA that can be used for PCR amplification.

We decided to work with a specific range in the concentration of Leishmania parasites (1000, 100 and 10 parasites/ml) in an attempt to specify the level of sensitivity of the working PCR protocol.

Differences between the five experiments using the ficoll method for separation of leukocytes can be explained by differences in the immune state of each blood donor, because in Ficoll the leukocytes are alive and functioning for about 30 minutes at least from the addition of the parasites. Maybe, in some of the samples, the leukocytes have destroyed a number of parasites, if the donor has been infected before and developed immunity.

TE is a better method than Ficoll since it has less cell losses, less smear, is faster and less expensive (table 1). Maybe one of the reasons that Ficoll presents less sensitivity than TE is because Ficoll is cytotoxic and therefore some of the leukocytes are lysed.

The advantage of the Proteinase K method is that we don’t lose any DNA, as the whole procedure is accomplished in the same tube. Furthermore, it takes less time than the QIAGEN kit, and it is more cost effective. One disadvantage in using the Proteinase K is that you may have DNA inhibitors. Inhibition of the PCR reaction in blood samples however is a rare phenomenon (1%).

The advantage in using the QIAGEN kit is that we don’t have any inhibitors in the PCR reaction but instead we lose DNA. Only about 80% of the total DNA (according to the manufacturer) is regained and Leishmania DNA may be lost and hence reduce the sensitivity of the method.
Comparing Proteinase K with the QIAGEN kit shows that the kit has clearer pattern and less smear, which is probably due to the high degree of purity of the DNA template.

To conclude, the QIAGEN kit method is simple, rapid, specific and sensitive enough to allow detection of the parasites in blood samples, even at very low numbers. Overall, this method seems to be the most suitable for diagnosis of visceral leishmaniasis and follow-up of the disease.
6. ACKNOWLEDGMENTS
I would like to thank the personnel of the National School of Hygiene, Department of Parasitology, Entomology and Tropical Diseases, Athens, Greece. I would especially like to thank Prof. Nicholas C. Vakalis and Gregory Spanakos.

7. REFERENCES
A highly sensitive and rapid procedure for direct PCR detection of Leishmania infantum within human peripheral blood mononuclear cells. Laboratories Retrovirus-parasites, France. 1995.
Detection and identification of *Leishmania* DNA within naturally infected sand flies by seminested PCR on minicircle kinetoplaste DNA. Laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographical Medicine, University of Crete, Heraklion, Greece. Applied and Environmental Microbiology Vol 66, No 5, p 1933-1938. 2000.


Use of PCR to Detect *Leishmania (Viannia)* spp, in Dog Blood and Bone Marrow. Department of Infectious and Tropical Diseases, London school of Hygiene and Tropical Medicine, Molteno Institute for Parasitology, Department of Pathology, Cambridge University, U.K. J. Clin. Microbiol. 38:748-751. 2000.


[18] Barker D, Lambson B and Smyth A.  
Sequence homology within a minicircle class of the *Leishmania donovani* complex. Department of Pathology, Molteno Laboratory of Parasitology, University of Cambridge, U.K. Molecular and Biochemical Parasitology 101, p 229-232. 1999.


[25] [http://physchem.ox.ac.uk/MSDS/glossary/hypotonic.html](http://physchem.ox.ac.uk/MSDS/glossary/hypotonic.html)