

# A comparison of four DNA extraction protocols for the analysis of urine from patients with visceral leishmaniasis

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## ABSTRACT

**Introduction:** Polymerase chain reaction (PCR) may offer an alternative diagnostic option when clinical signs and symptoms suggest visceral leishmaniasis (VL) but microscopic scanning and serological tests provide negative results. PCR using urine is sensitive enough to diagnose human visceral leishmaniasis (VL). However, DNA quality is a crucial factor for successful amplification. **Methods:** A comparative performance evaluation of DNA extraction methods from the urine of patients with VL using two commercially available extraction kits and two phenol-chloroform protocols was conducted to determine which method produces the highest quality DNA suitable for PCR amplification, as well as the most sensitive, fast and inexpensive method. All commercially available kits were able to shorten the duration of DNA extraction. **Results:** With regard to detection limits, both phenol: chloroform extraction and the QIAamp DNA Mini Kit provided good results (0.1 pg of DNA) for the extraction of DNA from a parasite smaller than *Leishmania (Leishmania) infantum* (< 100fg of DNA). However, among 11 urine samples from subjects with VL, better performance was achieved with the phenol:chloroform method (8/11) relative to the QIAamp DNA Mini Kit (4/11), with a greater number of positive samples detected at a lower cost using PCR. **Conclusion:** Our results demonstrate that phenol:chloroform with an ethanol precipitation prior to extraction is the most efficient method in terms of yield and cost, using urine as a non-invasive source of DNA and providing an alternative diagnostic method at a low cost.

**Keywords:** Urine. Extraction. DNA. Visceral leishmaniasis.

## INTRODUCTION

Visceral leishmaniasis is endemic in 62 countries in tropical and subtropical regions. There are 500,000 new cases reported annually, 90% of which are in India, Sudan, Bangladesh, and Brazil<sup>1</sup>. The common symptoms include fever, weight loss, hepatomegaly, splenomegaly, hypergammaglobulinemia, and pancytopenia. If untreated, visceral leishmaniasis (VL) is fatal in 90% of cases<sup>2</sup>.

In resolution WHA60.13 on the control of leishmaniasis, the Health Assembly urged member states to strengthen the prevention, active detection, and treatment of visceral leishmaniasis to reduce the burden of the disease<sup>3</sup>. However, an effective diagnostic method to detect leishmaniasis is not yet available. The original technique of identifying amastigotes

in smears of spleen, bone marrow, and lymph node aspirates remains the standard method for diagnosis<sup>4</sup>. However, this technique requires strict precautions, training, and technical expertise, hindering its application in routine and field studies<sup>5-7</sup>. Sero-diagnoses are not ideal because they are unable to discriminate between the disease state and asymptomatic infection, and they are susceptible to cross-reactions<sup>8,9</sup>.

Polymerase chain reaction (PCR) may be an alternative option when clinical signs and symptoms suggest VL but microscopic scanning and serological tests provide negative results<sup>10</sup>. Assays that test various body fluids, such as blood, bone marrow aspirate, serum, and urine, have been standardized<sup>11-15</sup>. Urine collection offers the advantages of being non-invasive, inexpensive, and easy to perform. Deoxyribonucleic acid (DNA) extraction from urine is preferable to extraction from blood and serum due to the low protein content of urine<sup>16</sup>. Viable cultures of amastigotes have been obtained from the urine of patients with VL, as well as parts of the parasite, yielding proteins and DNA<sup>14,17,18</sup>. The use of urine samples for PCR is promising, with a sensitivity of approximately 88-97% and a specificity of 100%<sup>14,18,19</sup>.

To perform PCR effectively, it is essential that DNA extraction is efficient and produces DNA that is pure and free of inhibitors. The aim of this study was to compare 4 methods of extracting DNA from urine for PCR analysis.

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**Received** 1 November 2013

**Accepted** 7 April 2014

## METHODS

### Detection limit

*Leishmania (Leishmania) infantum* (MHOM/BR/1974/PP75) promastigotes were grown in Schneider's medium and used as a source of DNA. DNA was extracted from the cultures using the GenomicPrep Tissue DNA Isolation Kit (GE, Amersham Place, Little Chalfont, Bucks, UK) according to the manufacturer's instructions. The amount of DNA was quantified by measuring the absorbance at 260nm. The detection limit of the protocol was determined by preparing seven urine samples containing known concentrations of genomic *L. (L.) infantum* DNA. These urine samples were from healthy parasite-negative donors and were subsequently used as negative controls. The final concentrations were 1.0, 0.8, 0.6, 0.4, 0.2, and 0.1pg of *L. (L.) infantum* DNA per microliter of urine.

### DNA extraction protocols

Four extraction protocols using two commercial kits, the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and the Illustra Blood GenomicPrep Mini Spin Kit (GE, Amersham Place, Little Chalfont, Bucks, UK), as well as two methods based on proteinase K digestion, were evaluated for their capacity to extract DNA. All experiments were conducted in duplicate.

**Proteinase K digestion in a lysis buffer followed by phenol-chloroform extraction:** Protocols 1 and 2 were based on a solvent extraction using phenol: chloroform. The two methods differed in that Protocol 2 involved the precipitation of suspended solids, including DNA molecules, using ethanol and a reduction in volume by concentrating the samples in microtubes. In Protocol 1, 5mL of urine, 600µL of lysis solution (100mM NaCl, 10mM Tris-HCl, and 0.5% SDS, pH 8.0), and 40µL of proteinase K (20mg/mL) were added to a Falcon tube and incubated at 60°C for 1h. After incubation, an equal volume of buffer-saturated phenol was added to the DNA solution. The samples were centrifuged at 5,000rpm for 7min, and the aqueous layer was then carefully removed from each sample and placed into a new tube. An equal volume of a buffer-saturated phenol: chloroform (1:1) mixture was added to the aqueous layer. After centrifugation and the removal of the aqueous layer, an equal volume of chloroform was added to the aqueous layer to remove traces of phenol. This step was repeated twice. An equal volume of isopropyl alcohol was added to the aqueous layer. After centrifugation, the supernatant was discarded, and the pellet was washed with 70% ethanol, centrifuged, and dried at room temperature. The pellet was resuspended in 20µL of autoclaved Milli-Q water. In Protocol 2, each sample (5mL) was transferred to a 15-mL Falcon tube. Absolute ethanol (2 times the sample volume) was added, and samples were centrifuged at 5,000rpm for 10 min. For each sample, the supernatant was discarded, and 700µL of lysis buffer and 20µL of proteinase K (20mg/mL) were added to the pellet. After incubation (1h at 60°C), the DNA solution was transferred to a microtube, and DNA extraction was conducted using the phenol-chloroform method. However, in the case of Protocol 2, the samples

were centrifuged at 11,000rpm for 1 min for all steps with the exception of the isopropyl alcohol step, in which they were centrifuged at 11,000rpm for 3 min. The pellet was resuspended in 20µL of autoclaved Milli-Q water.

**Commercial kits:** In Protocol 3, the QIAamp DNA Mini Kit: Proteinase K (20µL) and Buffer AL (200µL) were added to urine samples (200µL) and incubated at 56°C for 10min. Absolute ethanol (200µL) was added, and the mixtures were transferred to the QIAamp spin columns. These were centrifuged at 6,000g for 1 min, and then the waste tube was discarded. The columns were washed with 500µL of buffer AW1 and then again with 500µL of AW2. The purified DNA was eluted from the spin columns with 200µL of buffer AE into clean sterile microfuge tubes and stored at -20°C until further analysis. In Protocol 4, the Illustra Blood GenomicPrep Mini Spin Kit: Proteinase K (20µL) and Lysis Buffer Type 10 (400µL) were added to urine samples (200µL) and incubated at 56°C for 30 min. The mixtures were loaded into assembled columns and collection tubes. These were centrifuged at 11,000g for 1 min, and the waste tubes were discarded. The columns were then washed again with 500µL of Lysis Buffer Type 10. Wash Buffer Type 6 (500µL) was added to the columns. These were centrifuged at 11,000g for 3 min. The purified DNA was eluted from the spin columns with 200µL of Elution Buffer Type 5, pre-heated to 70°C in clean sterile microfuge tubes, and stored at -20°C until further analysis.

### Urine collection

Urine samples from 11 individuals with active VL diagnosed using bone marrow aspiration were tested using Protocols 2, 3, and 4. Before anti-leishmanial treatment, urine was collected from each individual (50-60mL) in a Falcon tube with ethylenediaminetetraacetic acid (EDTA) to obtain a final concentration of 10mM EDTA. Urine specimens were stored at -50°C until use. All study participants gave written informed consent in accordance with local guidelines, and the study was approved by the Human Research Ethics Committees of the Centro de Pesquisas Aggeu Magalhães.

### PCR assay

The extracted DNA was amplified using primers RV1 and RV2 (Le Fichoux et al.). The reaction mixture (25µL) contained 1X buffer (Invitrogen, Life Technologies, São Paulo, São Paulo, Brazil), 0.25mM dNTPs, 1.5mM MgCl<sub>2</sub>, 2.5 units of Taq polymerase (Invitrogen), 2µL of DNA, and 25pmol of each primer: RV1 (5'-CTTTTCTGGTCCC GCGGGTAGG-3') and RV2 (5'-CACCTGGCCTATTTTACACCA-3') to amplify a conserved fragment of the *Leishmania* minicircle kDNA. After an initial denaturation step (5 min at 94°C), 35 cycles (denaturation, 30s at 94°C; annealing, 1min at 67°C; polymerization, 30s at 72°C) were carried out, and PCR was concluded with a final extension (10 min at 70°C). DNA amplification was performed in a MasterCycler Gradient thermocycler (Eppendorf, Hamburger-Germany). All reactions included a positive and a negative control. The expected final product was 145bp. The amplification products were visualized after electrophoresis on a 2% agarose gel containing ethidium bromide.

### Calculation of cost and extraction time

The cost per test was calculated for each system. The time (number of minutes) taken to complete the steps in each protocol was estimated for each sample.

## RESULTS

### Detection limits, cost, and duration of extraction protocols

The detection limit for purified *L. (L.) infantum* DNA was 0.4pg using Protocol 1 and 0.2pg using Protocol 2. Despite this small difference in the detection limits, the changes introduced in Protocol 2 improved the performance (0.1pg) and yield of extraction (as demonstrated by the higher intensity of the bands in **Figure 1**) while reducing the cost (**Table 1**). Because the best results were achieved with Protocol 2, this protocol was chosen for DNA extraction from *L. (L.) infantum* in the urine of patients with visceral leishmaniasis.

The QIAamp DNA Mini Kit achieved a detection limit of 0.1pg for the extraction of DNA from urine, as the PCR products were visualized for all concentrations of DNA tested (**Figure 2**). This protocol required a reduced sample volume relative to those needed for Protocols 1 and 2 and was the fastest protocol tested. However, it was also the most expensive (**Table 1**). No amplification was observed for the Illustra Blood GenomicPrep Mini Spin Kit. Nevertheless, this kit was still tested using samples from patients with visceral leishmaniasis.

### Application of the 3 protocols for the detection of *L. (L.) infantum* in patient urine

Protocols 2, 3, and 4 were tested using urine samples from 11 patients who tested positive for the parasite. Protocol 2 detected DNA from *L. (L.) infantum* in 8 urine samples, whereas Protocols 3 and 4 detected DNA from *L. (L.) infantum* in 4 samples each (**Table 2**). Only one sample tested positive using all 3 protocols.

## DISCUSSION

Using urine as a source of DNA for diagnostic PCR is advantageous on several fronts. The collection of this biological fluid is practical, noninvasive, and inexpensive. Fisa et al.<sup>14</sup> described PCR from urine samples as a simple technique to diagnose VL during the acute phase, and the diagnostic performance of this method is similar to that of urinary antigen detection or PCR using blood or blood cultures. However, it should be noted that urine is dilute and contains factors that can potentially inhibit DNA amplification, such as urea and nitrites<sup>20</sup>. Furthermore, DNA may be degraded by endonucleases secreted by bacteria present in the urine<sup>21,22</sup>. DNA extraction is therefore the decisive step controlling the outcome of PCR, given that its aim is to recover DNA and eliminate potential PCR inhibitors that may be present in the sample. The degradation of DNA in urine can most likely be attributed to nucleases present in the fluid<sup>23</sup>.

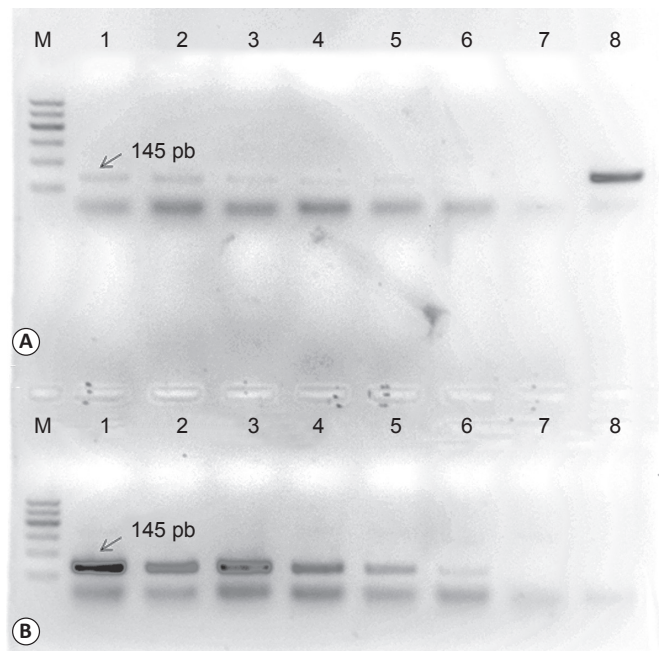


FIGURE 1 - RV1/RV2-PCR analyses of the kDNA region (145bp) amplified from the dilution curve of *Leishmania (Leishmania) chagasi* DNA after extraction using Protocol 1 (A) and Protocol 2 (B). Lanes 1-6: 1.0, 0.8, 0.6, 0.4, 0.2, and 0.1pg of *Leishmania (Leishmania) chagasi* DNA per microliter. Lane 7: negative control for the extraction. Lane 8a: positive control for PCR. Lane 8b: negative control for PCR. A 100-bp ladder was used as a molecular size marker (M). PCR: polymerase chain reaction; kDNA: kinetoplast DNA; DNA: deoxyribonucleic acid.

TABLE 1 - Comparison of the four protocols for DNA extraction in urine.

Protocol	Cost/sample (US\$)	Total processing time (min)	Sample size	Detection limit (pg of DNA)
Protocol 1	19.00	110	5mL	0.2
Protocol 2	1.75	75	5mL	0.1
Protocol 3	5.20	25	200μL	0.1
Protocol 4	2.25	40	200μL	No product

DNA: deoxyribonucleic acid.

In studies investigating cell-free transrenal DNA (i.e., DNA from the bloodstream that has passed through the kidney barrier), urine was collected and immediately mixed with EDTA (a chelating agent that sequesters metal ions) to a final concentration of 10mM. This step is important because high concentrations of metal ions are found in urine and can interfere with PCR<sup>24</sup>.

With regard to detection limits, both extraction using phenol: chloroform and the QIAamp DNA Mini Kit performed well in the DNA extraction from a parasite smaller than *Leishmania (Leishmania) infantum* because each *Leishmania* spp.

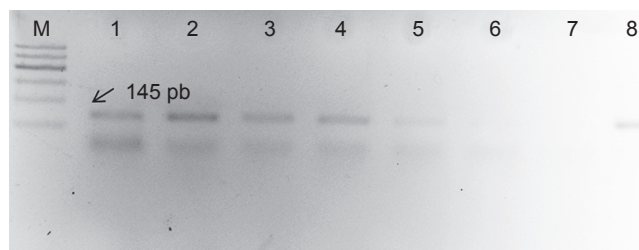


FIGURE 2 - RV1/RV2-PCR analyses of the kDNA region (145bp) amplified from the dilution curve of *Leishmania (Leishmania) chagasi* DNA after extraction using Protocol 3. Lanes 1-6: 1.0, 0.8, 0.6, 0.4, 0.2, and 0.1pg of DNA *Leishmania (Leishmania) chagasi* per microliter of urine. Lane 7: negative control for the extraction. Lane 8: positive control for PCR. A 100-bp ladder was used as a molecular size marker (M). PCR: polymerase chain reaction; kDNA: kinetoplast DNA; DNA: deoxyribonucleic acid.

cell contains 100fg of DNA<sup>25</sup>. Other studies have described the efficiency of these methods in removing PCR inhibitors<sup>18,26,27</sup>. However, in our study, the performance of Protocol 2 was better than that of Protocol 3 for urine samples from subjects with VL, with a greater number of positive samples detected using PCR. The extraction method involving phenol and chloroform is still widely used in research and diagnostics. We propose 2 modifications in the present study: the introduction of an ethanol precipitation prior to extraction (in contrast to the conventional method) and a reduction of the initial volume of the biological fluid. These changes improved the extraction, as demonstrated by the increased intensities of the products during electrophoresis. Compared to Protocol 3, Protocol 2 works with a larger volume of urine (5mL or more) without having to use the high-cost solvents that are necessary in the classic phenol-chloroform method. This greater volume increases the likelihood of detecting DNA in the sample and may have therefore been responsible for the difference between these 2 protocols in terms of the number of urine samples testing positive during the PCR analysis. Commercial kits are limited in terms of the volume of sample that can be used. The introduction of an ethanol precipitation prior to extraction may have also contributed to the superior performance of Protocol 2. This study was devoted to developing sample pretreatments to obtain adequate specimens or to enhance the efficiency of PCR. In this context, although it is less expensive than commercial kits, Protocol 2 is inconvenient because it requires the most time to run and uses toxic solvents. This protocol failed to diagnose 3 confirmed cases of VL in patients. This failure may be attributed to improper handling and storage conditions. It is necessary to perform more assays using larger sample volumes to better evaluate the reliability of the method.

In the present study, the commercial kits did not perform well when applied to urine samples from patients. Despite the poor performance of the GE kit during the detection limit test, it was able to purify DNA from four urine sample from subjects with VL. The GE kit was also able to purify DNA from a urine sample when the other protocols failed. These results were not reproducible, which may be attributed to the small sample

TABLE 2 - kDNA PCR for the detection of *Leishmania (Leishmania) infantum* DNA (145bp) using the four extraction protocols to test urine.

Sample	Protocol 2	Protocol 3	Protocol 4
1	+	+	-
2	+	-	+
3	+	-	-
4	+	+	+
5	-	+	+
6	+	-	-
7	+	-	-
8	-	+	-
9	+	-	-
10	-	-	+
11	+	-	-

kDNA: kinetoplast DNA; PCR: polymerase chain reaction; DNA: deoxyribonucleic acid; +: amplification present; -: amplification absent.

volumes and the fact that this kit is not indicated for DNA extraction from urine. It is worth noting that the manufacturer's recommendations do not mention performing the assay using urine. However, because the kit has been widely used to obtain DNA from various types of biological samples with good results (unpublished observations), we decided to evaluate the performance of this kit for testing urine. The differences between the kits in terms of the detection limits and numbers of samples testing positive indicate that attempts to replicate conditions in the body do not always simulate natural conditions. Evaluation using a larger number of positive samples is required to establish the robustness of the protocols.

PCR is still regarded as an expensive technique by several services and health professionals. In the present study, however, the best method for extraction was also the least expensive, with a cost of US\$1.75 per sample. Dourado et al.<sup>28</sup> evaluated the cost per diagnosis and found that immunochromatography cost \$2.71, whereas the cost of parasitological diagnosis exceeded US\$72.10 because it required a qualified medical practitioner, anesthesia, and specific materials to obtain the bone marrow or visceral aspirate. However, the cost of parasitological diagnosis inputs was also higher (US\$25.00). Serology via immunofluorescence assay (IFA) costs US\$8.56. These data suggest that the cost of PCR may not be high relative to many of the alternatives.

Our results therefore provide further support for the use of PCR as a noninvasive diagnostic tool for VL, using urine as a source of DNA. To increase the sensitivity and reduce the cost of this test, phenol:chloroform with an ethanol precipitation prior to extraction should be the preferred method for PCR experiments with urine. However, further assays are needed to better characterize the reliability of urinary PCR for the diagnosis of VL.

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

**FINANCIAL SUPPORT**

The authors received financial support for this study from *Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco* (FACEPE).

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