

Evaluation of DNA Extraction Methods for Detection of *Leishmania* by Polymerase Chain Reaction

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Abstract

There are several methods used to obtain DNA from cells; however, the quantity, integrity, and purity of DNA vary among the methods, which may interfere with the polymerase chain reaction (PCR) results. The objective was to determine the most efficient and cost-effective method that provides the best DNA yield and PCR results. Three methods of DNA isolation were compared: 20% sodium dodecyl sulfate (SDS), guanidine isothiocyanate-phenol-chloroform (GTPC), and DNA extraction using a commercial kit (GE Healthcare GenomicPrep Blood DNA Isolation KitTM). Human peripheral blood samples were inoculated with 10⁴ promastigotes of *Leishmania infantum*. DNA was quantified and PCR was performed with 13A/13B primers. The results showed that a higher DNA yield was obtained using the GTPC technique (214.51 ng/μL), followed by SDS (26.16 ng/μL) and the commercial kit (10.99 ng/μL). We concluded that while all of the techniques were effective for obtaining DNA, the GTPC method provided the best yield and the brightest bands.

Keywords

Leishmania, DNA, Purification, PCR, Diagnosis

1. Introduction

Leishmaniasis is a disease caused by protozoa from more than 20 species of *Leishmania*. The diagnosis of leishmaniasis requires clinical, epidemiological, and laboratory analyses, which may vary according to the different clinical forms

and species of *Leishmania* [1]. Diagnosis is important due to the severity of the disease, treatment difficulties, and high mortality rate. While serological and molecular tests are most commonly used, a definitive diagnosis requires the visualization of the parasite [2].

The advent of polymerase chain reaction (PCR) technology has advanced the study of leishmaniasis. For diagnostic and genotype analysis, it is essential that DNA is pure, intact, and free of cellular debris [3] [4]. Thus, the DNA extraction and purification method determines the success of the molecular diagnosis of leishmaniasis via PCR [5]. DNA can be obtained by various methods, each of which shows variability in the quality and yield of the nucleic acids obtained, the execution time, and the cost/benefit ratio, and every laboratory chooses a technique that best suits their needs [6].

Because PCR is one of the most effective techniques for the diagnosis of leishmaniasis, it is important to be able to extract high quality DNA from samples. Therefore, we compared three methods for obtaining DNA from blood cells according to efficiency, cost, DNA yield, and PCR results.

2. Material and Methods

2.1. Sample Size

This work was approved by the Human Research Ethics Committee (protocol number 3,214,315) of the Universidade Federal da Grande Dourados. The volunteers were informed about the purpose and importance of the study as well as confidentiality. Informed consent was obtained from all individual participants included in the study.

Inclusion criteria: Thirty samples of peripheral blood from volunteers with negative serology for leishmaniasis. Exclusion criteria: volunteers with positive serology for leishmaniasis. The samples were collected by venipuncture, mixed with EDTA, and stored in tubes at -20°C . Two hundred microliters of blood per sample was inoculated with 10^4 promastigotes of *Leishmania infantum*.

2.2. DNA Extraction

The 20% sodium dodecyl sulfate protocol [7] with some modification. Three hundred microliters of 20% SDS (Dinâmica, Indaiatuba, Brazil) was added to 200 μL each sample, mixed by vortexing, and incubated at 65°C for 6 min. The samples were then removed from incubation and 400 μL of chloroform was added. The samples were vortexed vigorously until completely homogenized. Three hundred microliters of protein precipitation solution (3 M potassium acetate, 11% glacial acetic acid) was added to each sample and mixed by vortexing. The samples were then centrifuged at $10,000\times g$ for 10 min at room temperature, after which the aqueous phases were transferred to a new tube. Eight hundred microliters of 96% ethanol was added to each tube, and the samples were mixed by inversion. The samples were centrifuged at $10,000\times g$ for 5 min at room temperature. The supernatants were discarded and 1 mL of 70% ethanol

was added to the pellet. The samples were centrifuged again for 2 min at 10,000× g at room temperature and the supernatants were discarded. The pellets were washed once more with 1 mL of 70% ethanol and centrifuged again for 2 min at 10,000× g at room temperature. Ethanol residue was removed with filter paper. The pellet was dried in a dry block at 65°C for 5 min. Fifty microliters TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) was added to each sample and incubated at 65°C for 5 min for complete solubilization of the pellet. Finally, the samples were frozen at -20°C until amplification.

The guanidine isothiocyanate-phenol-chloroform protocol was according [8] with some modification. Samples were centrifuged at 3500× g for 15 min at room temperature. The supernatants were discarded and the pellets were resuspended in 1 mL of sterile 1× PBS. The samples were centrifuged again at 3500× g for 15 min at room temperature. The supernatants were discarded and any excess was removed with filter paper. The pellet was resuspended in 300 µL of GTPC solution (5 M guanidine isothiocyanate: phenol; v/v). The samples were mixed by inversion for 1 min or until the pellets were completely dissolved. Fifty microliters of cold chloroform was added to each sample and mixed gently. The samples were centrifuged at 10,000× g for 10 min at room temperature. The supernatants were transferred to a new tube containing 300 µL of cold 96% ethanol and mixed by inversion for 1 min. The samples were centrifuged again at 10,000× g for 15 min at room temperature. The supernatants were discarded and 300 µL of 70% ethanol was added to the pellets, which was mixed by inversion until the pellets were dissolved. The samples were centrifuged at 10,000× g for 10 min at room temperature and the supernatants were discarded. The samples were washed again with 70% ethanol and centrifuged at 10,000× g for 10 minutes at room temperature, after which the supernatant was removed. The pellet was dried at 65°C for 10 min. Fifty microliters of TE buffer was added to the dried pellet and incubated at 65°C for 5 min. The samples were stored at -20°C until amplification.

The GE Healthcare GenomicPrep Blood DNA Isolation Kit™ (Buckinghamshire, UK) was used. Extraction using the commercial kit was performed according to the manufacturer's instructions.

A Biodrop™ spectrophotometer was used to quantify the DNA concentration and degree of purity (A260/A230 and A260/A280 ratio) in each sample.

2.3. Polymerase Chain Reaction (PCR)

PCR was performed using the 13A (5'-GTG GGG GAG GGG CGT TCT-3') and 13B (5'-ATT TTA CAC CAA CCC CCA GTT-3') primers [3] that amplify 120-bp (base pairs) of DNA from *Leishmania* spp. The final reaction volume (25 µL) contained 0.4 µM of each primer (Millipore Sigma, Burlington, MA, USA), 1.5 mM of MgCl₂, 0.2 mM of dNTP (ThermoFisher Scientific, MA, USA), 1.5 U of Taq DNA Polymerase (Phoneutria, Belo Horizonte, BR), 1× enzyme buffer, and 2 µL of DNA. Amplification was performed in a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) at 95°C for 5 min, followed by 35 cycles consisting of three

stages: denaturation (95°C for 30 s), annealing (61°C for 30 s), and elongation (72°C for 30 s). After the reaction, the samples were kept at 72°C for 10 min. The products were stored at 4°C until electrophoresis. Eight microliters of the amplified products was subjected to electrophoresis on a 2% agarose gel stained with ethidium bromide (10 mg/mL). A positive control (*L. infantum* DNA) and a negative control (water) were included. Bands were detected using a transilluminator (Loccus Biotecnologia, Cotia, BR).

3. Results

The highest average yield of DNA was obtained using GTPC-based DNA extraction. The A₂₆₀/A₂₈₀ ratio indicated that DNA isolated using the commercial kit had a higher degree of purity, *i.e.* had little RNA or protein contamination. The chemical contamination (indicated by ratio A₂₆₀/A₂₃₀) was lowest in the samples extracted using SDS. The commercial kit was faster at extracting DNA, as the protocol only takes 90 min; GTPC and SDS-based methods take 160 and 180 min, respectively. The SDS-based technique was the most cost-effective (Table 1). All of the samples showed adequate and well-defined bands in the gel following PCR (intensity and size) (Figure 1).

4. Discussion

One of the biggest challenges of diagnosing leishmaniasis is not having a gold standard of diagnosis [9]. PCR-based tests are the main tools used for the identification of *Leishmania* spp. [10]. The success of PCR is highly influenced by the quality of the biological sample and of DNA, which highlights the importance of proper storage and the technique of obtaining nucleic acids. The first step in performing PCR is extracting DNA from a sample, which requires accuracy and precision, as errors will negatively affect the results. While there are a myriad of efficient commercial kits, several in-house protocols are also available that can provide excellent results for a low price.

The extracted DNA must be abundant and have a high degree of purity. Thus, the processes of disrupting the cell membranes and separating DNA from organelles, proteins, and chemical compounds must avoid damaging the DNA and eliminate any PCR inhibitor. Based on our comparison of the methods, differences in DNA yield were observed. The GTPC technique showed a higher yield

Table 1. Parameters analyzed when comparing different DNA extraction methods.

Parameters	GTPC	SDS	Kit
Concentration (ng/μl)	214.51 ± 270.94	26.16 ± 43.18	10.99 ± 8.56
A ₂₆₀ /A ₂₈₀ ratio (nm)	1.311 ± 0.12	2.491 ± 2.07	1.940 ± 0.44
A ₂₆₀ /A ₂₃₀ ratio (nm)	0.174 ± 0.170	1.265 ± 1.60	-2.347 ± 10.27
Time (min)	160	180	90
Cost (US\$) ^a	13.6	8.2	589.7

^aCalculated value for obtaining DNA in 100 samples with reagent budget made in July 2018.

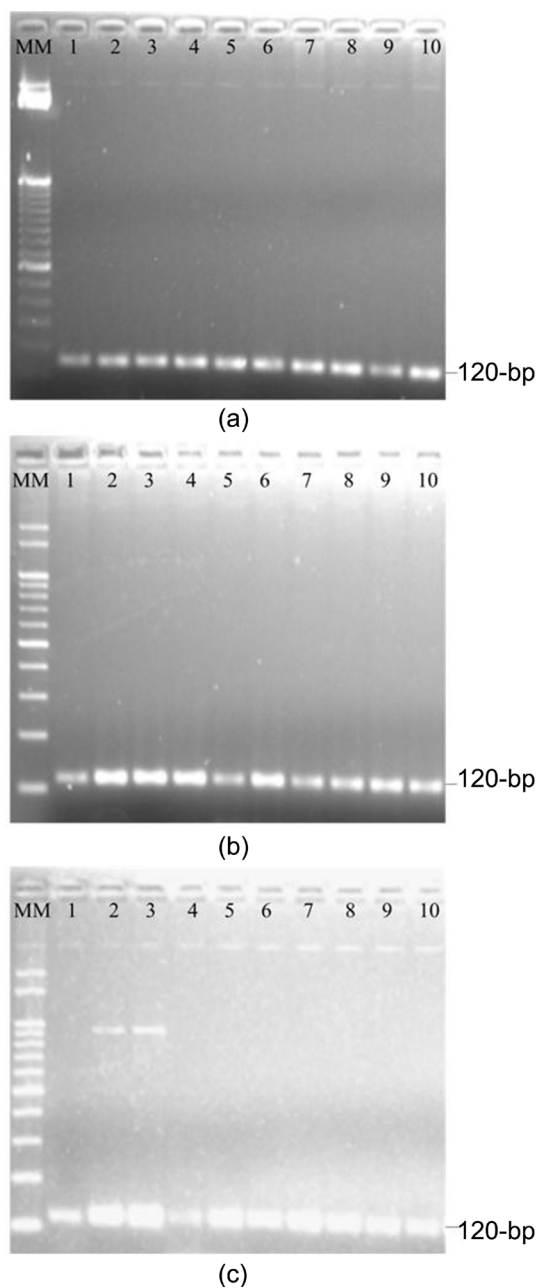


Figure 1. Agarose gel electrophoresis with peripheral blood samples amplified with 13A/13B primers. MM: 100-bp molecular marker; 1 to 10: samples. DNA obtained with (a) SDS, (b) GTPC and (c) commercial kit (GE Healthcare GenomicPrep Blood DNA Isolation KitTM).

of DNA. The use of phenol/chloroform in this technique positively affected DNA concentration and purity because it allowed for more effective deproteinization [11]. However, both the GTPC and SDS techniques require using EDTA, which causes the chelation of cations and cofactors of DNase, which can result in a decrease in DNA yield and viability [12].

It is crucial that the extracted DNA is pure. An A260/A280 ratio of less than 1.6 indicates protein contamination, whereas a ratio of more than 1.9 indicates

RNA contamination. An A260/A230 ratio below 1.8 indicates contamination with chemical compounds. In our results, the DNA produced with the commercial kit demonstrated an excellent degree of purity in relation to in-house kits; however, the yield was low. Although the DNA extracted using the GTPC technique did not have satisfactory purity values, amplification and electrophoresis showed clear bands (**Figure 1**), which indicates that even when spectrophotometric measurements imply contamination, it does not necessarily impair the efficacy of PCR.

When compared to other studies [2] [7], our attempts at the SDS and GTPC techniques showed lower yields. This suggests that the reproducibility of in-house methods may be affected by several factors, such as the brands of reagents, equipment, and physical conditions in the laboratory.

The efficiency of DNA acquisition must also be considered, as efficiency influences a method's practicality. In our study, the commercial kit provided the most time-efficient DNA extraction. The techniques that gave the highest yields (GTPC and SDS) also took the longest, whereas the technique that provided the lowest yield was faster, which may suggest that more steps can extract more DNA.

With regard to biosafety, commercial kits are less harmful, as they prevent exposure to toxic substances (e.g. phenol) because they contain ready-made solutions.

The GTPC and SDS-based methods showed significant cost advantage over the commercial kit. Commercial DNA extraction kits, although safer and tested by the manufacturer, are often not viable due to their high cost.

All of the samples showed adequate and well-defined bands in the gel following PCR (intensity and size), confirming the integrity of the DNA. The buffers used in the extraction should be chosen in such a way as to minimize DNA fragmentation. Additionally, internal reaction controls should be used to confirm that the primers are working properly and that there is no contamination [2]. In addition to evaluating various DNA extraction techniques, it is important to evaluate different types of primers to improve PCR results. Real-time PCR has shown better results compared to conventional PCR [13], but these results depend of the synergy between extraction and PCR.

Although all of the techniques we described were able to detect *L. infantum* in peripheral blood samples, the GTPC-based technique demonstrated the highest efficiency. Thus, based on the higher cost-benefit ratio and the quality and quantity of DNA, we conclude that in-house techniques are a better option than commercial kits. In addition, in-house techniques are more flexible and allow for optimization.

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Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee (Human Research Ethics Committee of the Universidade Federal da Grande Dourados—protocol number 3,214,315) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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