Chapter 11

Polymerase Chain Reaction Diagnosis of Leishmaniasis: A Species-Specific Approach

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Abstract

Leishmaniasis is an infectious disease caused by protozoan parasites of the genus *Leishmania* which are transmitted to humans through bites of infected sand flies. The variable clinical manifestations and the evolution of the disease are determined by the infecting species. Recognition at a species level is of utmost importance since this greatly impacts therapy decision making as well as predicts outcome for the disease. This chapter describes the application of polymerase chain reaction (PCR) in the detection of *Leishmania* parasites across the disease spectrum, including protocols for sample collection and transportation, genomic material extraction, and target amplification methods with special emphasis on PCR amplification of the cytochrome *b* gene for *Leishmania spp.* species identification.

Key words Leishmaniasis, Diagnosis, PCR

1 Introduction

The standard methods for diagnosis of cutaneous and visceral Leishmaniasis have traditionally relied on the direct identification of amastigotes by histology, direct microscopy (Giemsa-stained smears), or by the growth of promastigotes in axenic culture [1] and/or laboratory animals. Recent clinical and laboratory research trials have confirmed that not only the immune response of the host but also the species of infecting parasite are responsible in determining the clinical manifestations of leishmaniasis and affect the response to treatment [2, 3]. In this sense, polymerase chain reaction (PCR) has emerged as a powerful tool for diagnosis of this parasitic disease by providing a higher sensitivity, the ability to detect low levels of parasitemia, and the capacity of detecting mixed infections. Most importantly, PCR allows accurate molecular characterization of the causative agent, which is essential to evaluate the efficacy of anti-leishmanial drugs against different circulating strains. In the following chapter, we discuss the general aspects of DNA

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extraction methods, tissue processing, and the wide array of PCR leishmanial targets with special emphasis on the PCR amplification of the cytochrome *b* gene for *Leishmania spp.* species identification [4, 5]. This protocol offers the advantage of collecting and extracting the samples from FTA Classic Cards, making it an ideal and practical method to work with on the field while offering a robust target for identification and phylogenetics of *Leishmania* parasites.

2 Materials

2.1 Equipment

1. Safety cabinet.
2. Water bath.
3. Centrifuge/Micro-centrifuge.
4. Vortex.
5. Pipettes for PCR.
6. Filter tips.
7. DNA quantifier equipment.
8. Thermocycler.
9. Agarose gel electrophoresis equipment.
10. Sequence Detection System (real-time PCR).

2.2 Reagents

1. AxyPrep™ Blood Genomic DNA Kit (Axygen).
2. Phenol:Chloroform:Isoamyl Alcohol (Invitrogen).
3. PCR kit.
4. Primers.
5. Nuclease-free water.
6. Agarose.
7. SYBR® Safe DNA Gel Stain/Ethidium bromide.
8. Restriction enzymes and buffers.
9. FTA Classic Cards.
10. Ampdirect Plus reagent (Shimadzu Biotech, Tsukuba, Japan).

3 Methods

Herein we describe a number of protocols for the diagnosis of visceral (VL), cutaneous (CL), and mucocutaneous leishmaniasis (MCL); therefore, it is important to highlight the differences and adequate type of sample to be used for retrieving DNA templates in each case scenario. For VL the most commonly used sample is whole blood, sometimes used in combination with buffy coat in order to obtain a better yield of DNA concentration. However bone marrow or/and spleen aspirates are also acceptable specimens.

For CL and MCL samples used vary from tissue aspirates and skin scrapings taken from the active lesion to skin biopsies.

3.1 Sample Selection and Storage

Cutaneous (CL) or Mucocutaneous Leishmaniasis (MCL) should be rapidly treated to avoid the potentially disfiguring consequences of parasite spread. Visceral Leishmaniasis (VL), on the other hand, must be promptly diagnosed and treated due to its fulminating and usually rapidly fatal outcome. Therefore early and reliable methods are needed to diagnose this important spectrum of parasitic diseases.

In recent years, molecular techniques for the detection of leishmanial DNA or RNA have emerged as a promising resource to be used not only in diagnosis but also in species identification while providing a higher sensitivity than microscopy and culture. In this context, sample preparation and DNA extraction methods can greatly influence the outcome and reliability of the PCR. In this type of infectious diseases, this process is complicated due to the abundance of host DNA in the samples, which can compete with the parasites' DNA (generally much less abundant) which may interfere with the reaction.

1. Blood Samples. Blood must be collected into ethylenediaminetetraacetic acid (EDTA) tubes and should be processed as soon as possible. Otherwise, keep blood samples refrigerated at 4 °C for no more than 2 weeks. For longer periods of preservation, addition of Guanidine-HCl buffer could be used (in a relation 1:1); this treatment will help preserve the DNA in blood samples for as long as 1 year at 4 °C, or 3 months at ambient temperature [6].

   Mononuclear cells purification. In order to attain a higher yield of DNA, buffy coat or mononuclear cells may be used as samples for DNA extraction. For diagnostic purpose we have purified mononuclear cells using the Hystopaque 1077 as follows:

   (a) Transfer carefully 3 ml of whole blood onto 3 ml of Hystopaque 1077 (it must be at ambient temperature before adding the blood). Centrifuge at 400 × *g* for 30 min at ambient temperature.

   (b) Carefully aspirate and discard the upper layer (plasma), taking extra care not to disturb the opaque interface. Then transfer the mononuclear layer (the opaque interface) to a clean, labeled, 15 ml centrifuge tube.

   (c) Wash twice with 10 ml of isotonic phosphate buffered saline solution (PBS) and centrifuge at 1000 × *g* for 10 min each time (at this point the process could be done at 4 °C).

   (d) Resuspend pelleted cells in 1 ml of PBS and transfer to a clean 1.5 ml centrifuge tube. Centrifuge at Vmax for 5 min.

   (e) Discard the supernatant and at this point you may store the sample at −20 or −70 °C until its use, or you could directly extract total DNA.

2. Bone Marrow Samples. Bone marrow samples should be stored in tubes containing 0.5 % EDTA as anticoagulant (must be properly collected by a physician) and processed as soon as possible. If stored, samples should be preserved at –20 °C until its use. Also, bone marrow aspirates can be collected on filter paper (Whatman no. 3 or FTA Cards), and then stored in separate plastic bags at –20 °C for further analysis. Bone marrow aspirates, as well as whole blood and spleen aspirates are commonly used samples, either for demonstration of parasites by smear evaluation or for molecular diagnosis. Bone marrow and spleen aspirates are obtained by invasive methodologies and may carry possible complications. Hence, to avoid invasive sampling methods, among other reasons, blood samples and molecular approaches have been more widely used lately for VL diagnosis [7].
3. Tissue Samples. Tissue aspirates from active lesions should be collected from the outer (infiltrated) border and base of the lesion, into syringes containing 0.5 ml of saline solution, 1000 U/ml penicillin, and 0.3 mg/ml streptomycin. Sample should be processed immediately or stored at –20 °C until further analysis [8]. Biopsy specimens are obtained from the active edge of the lesion with a sterile 4-mm diameter biopsy puncher, while for lesion scraping simple sterile lancets should be used. Samples should be stored in absolute ethanol at –20 °C until further use. For field work sampling, we recommend that tissue material is spotted onto FTA Classic Cards (Whatman Inc., Newton, MA, USA) [5], and samples be stored separately in plastic bags at ambient temperature for 18 months, or with silica gel at 4 °C for longer time.

When selecting whole blood (WB) versus buffy coat (BC) for DNA extraction, one should balance advantages and disadvantages between both methods. The use of WB is easier particularly in field studies and it has been reported to provide good sensitivity [9]. On the other hand, as *Leishmania* are obligate intracellular parasites (in the vertebrate host), they are expected to be more concentrated in the BC, thus this sample should yield a better sensitivity. Lachaud and coworkers in 2001 [10] performed a study comparing both samples for PCR diagnosis of VL, finding that BC develops a tenfold increase in sensitivity over that of WB. Therefore, as indicated by this study and our experience, we highly recommend using buffy coat or mononuclear purification strategies for Leishmaniasis diagnosis.

Because most areas of the world where Leishmaniasis is endemic are low-income rural areas with limited resources for adequate collection, storage, and transport of specimens, we have employed and highly recommend the use of FTA Cards, which are a filter paper that readily lyses the spotted material and fixes nucleic acids, making it a fast, simple, and reliable method for direct sampling [4, 5]. A similar sample collection technique has been

described using filter paper (Whatman No. 3), with noticeably differences in the DNA extraction process and in diagnostic sensitivity [11]. The filter paper sampling technique has been widely used for Leishmaniasis diagnosis, recognized as a versatile and sensitive methodology, useful for field studies and to archive samples for very long periods of time and it is compatible with the use of many biological materials (blood, bone marrow, lymph aspirates and tissue aspirates or scraping as samples).

3.2 Preparation of Template for PCR

As for all infectious diseases, the limitation for diagnosis is the amount of the infecting organism in the host sample. In this specific case, it is the number of *Leishmania* parasites in the sample taken from the mammal host. The sensitivity of the diagnostic method will depend on the enrichment of the parasite in the sample; therefore it is important to select a proper sample and use an effective DNA extraction method.

For a better yield of DNA from blood samples we usually recommend organic extraction with phenol–chloroform–isoamyl alcohol (25:24:1), using either whole blood or buffy coat. However, depending on the number of samples to be processed, a blood DNA extraction kit may be used. The same recommendation stands for tissue DNA extraction, only that it undergoes a previous treatment with Proteinase K. Although the organic DNA extraction method in our experience gives a high yield of DNA, it does show some limitations as it uses hazardous organic solvents, is relatively time-consuming, and is almost impossible or prone to automate, being unsuitable for high-throughput applications [12]. Moreover, when using this method you should be aware of possible residual phenol or chloroform in the DNA, which may interfere with PCR reaction.

Leishmaniasis is a poverty-related disease, distributed mainly in rural areas of endemic countries. Thus, for epidemiologic studies, one must make sure to use a sampling method compatible with the conditions found in these areas, with basically no availability of refrigeration systems. In this regard, for molecular diagnosis of Leishmaniasis we recommend employing FTA Classic Cards which can be used directly for PCR and no previous DNA extraction process is needed.

1. From samples in filter paper (Adapted from Ref. 11).
   (a) Disks of filter papers with spotted biological material are punched out with a paper punch. After each sample is obtained, a clean sheet of paper sprayed with 90 % alcohol is punched ten times in order to prevent DNA contamination from one sample to the next.
   (b) Two disks (approximately 15 µl of aspirate) are placed in 250 µl of lysis buffer (50 mM NaCl, 50 mM EDTA pH 7.4, 1 % v/v-Triton X-l00, and 200 µg of proteinase K per ml) and incubated for 3 h or overnight at 60 °C.
   (c) Samples are subjected to phenol-chloroform extraction, ethanol and Na-Acetate precipitation [13].

(d) DNA pellets are dried using a speed vacuum dryer for 5 min and redissolved in 50 µl of TE buffer (10 mM Tris 1 mM EDTA pH 7.5) or milli-Q water, DNA should be quantified in a spectrophotometer, and the quality of DNA measured at 260/280 nm. DNA samples are kept at −20 °C until its use.

2. From samples in FTA Classic Cards.
   (a) The biological material must be added onto the paper and let it dry completely. Then a 2 mm diameter disk is punched out from the filter paper for further processing.
   (b) Place the disk in a PCR tube and wash three times with the FTA Purification Reagent. Discard the used reagent after each wash.
   (c) Wash twice with TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) and discard the used buffer after each wash.
   (d) The disk is air dried in a PCR tube and subjected directly to PCR amplification.

Filter paper sampling method is a very useful tool for field work and easy to handle especially when working with many samples. When filter paper Whatman No. 3 is used, DNA extraction protocol is laborious, requiring the use of organic solvents, and at some point samples should be stored at 4 °C in order to attain better maintenance. In contrast, the use of FTA Cards provides a rapid and safe method without using any organic solvent or specialized equipment. When the samples are spotted onto an FTA card, the cells are lysed, pathogens are inactivated, and the nucleic acids are instantly fixed on the matrix of the card, resulting in protection from nuclease, oxidative and UV damage, and prevention of growth of bacteria and other microorganisms. This card is also suitable for long-term storage and the transportation is at room temperature [4].

3.3 Detection of Leishmania DNA by PCR

Diagnosis of Leishmaniasis commonly relies on culture methods, direct observation of parasites, or immunological techniques that detect specific antibodies in the sample. However, these techniques do not give any subtyping information of the infecting parasite. Therefore, molecular techniques like DNA-based analysis have been replacing the traditional methods, as they tend to be more specific and stable; and direct detection of molecular markers in host samples is being preferred. Since epidemiological studies normally involve large sample sets, methods that are cost-effective and allow high-throughput analyses are desirable [14].

There are many *Leishmania* species, and the different varieties of diseases are caused by specific species of the parasite. Hence, the ability to distinguish between *Leishmania* species is crucial for the correct diagnosis and prognosis of the disease, as well as for making proper decisions regarding treatment and control measures [14]. This is especially useful for epidemiological studies in areas with

various co-existing *Leishmania* species, and those non-endemic areas were the *Leishmania* parasites have been imported due to the increasing international travel and population migration [15–17]. Hence, to differentiate *Leishmania* parasites at a species and strain levels, reliable, reproducible, and user-friendly tools are required.

Amplification of the parasites' DNA by the polymerase chain reaction (PCR) has evolved as one of the most specific and sensitive substitute methods for parasite detection in diagnosis of many infectious diseases. In the past two decades, many PCR applications for detecting *Leishmania spp.* have been reported, being the target regions usually multi-copy genes or polymorphic regions, in order to increase the probabilities of amplifying the parasites' DNA among a great amount of the host's DNA in the sample [18]. Some of the target regions described so far are the following: (1) small subunit ribosomal RNA gene (SSU-rRNA) [19], (2) kinetoplast DNA [20], (3) internal transcribed spacers (ITS) in the ribosomal operons [21], (4) heat shock protein 70 (hsp70) gene [22], (5) glucose-6-phosphate dehydrogenase gene [23], and (6) cytochrome *b* gene [24], among some others; each with different specificities and approaches in Leishmania diagnosis. Despite there are many PCR assays available, none has become a reference tool in Leishmanias diagnostics; therefore a method that ensures direct diagnosis and identification of pathogenic *Leishmania* species is still required for appropriate therapy programs to be developed [25, 26].

Since the advent of PCR, numerous molecular tools have been described to distinguish species and strains of *Leishmania* parasites. Here we show a review of the most common PCR-related protocols that are used to diagnose Leishmaniasis, identifying the species (Table 1). Depending on your needs and the equipment you have, you may choose the most suitable of these protocols. However, in respect of methods that can be used with samples taken in field work, we have successfully used and recommend the PCR protocol targeting the cytochrome *b* gene, collecting the samples into FTA Classic Cards for a better storage and handling [24, 27].

1. PCR amplification of the cytochrome *b* gene for *Leishmania spp.* species identification.

The cytochrome *b* (Cyt *b*) gene has been considered one of the most useful genes widely used for phylogenetic studies and identification of animals and plants. It is contained in the mitochondrial genome of a wide variety of living forms and encodes the central catalytic subunit of an enzyme present in the respiratory chain of mitochondria [35, 36].

In *L. tarentolae*, the Cyt *b* gene consists of two regions, the edited region (the 5′ region of 23 bp) that undergoes RNA editing, and the non-edited region (the 3′ region of 1056 bp). RNA editing process has been described in mitochondrial genes of kinetoplastid Protozoa [37]. In the RNA editing process, uridylate (U) residues are inserted or deleted to repair a frame-shift present

Table 1
Specifications of different molecular protocols for species-specific diagnosis of Leishmaniasis

Reference	Leishmaniasis	Leishmania species	Target (gene)	Technique	Specificity	Sensitivity
Da Graça et al. 2012 [26]	CL	*L. braziliensis, L. lainsoni, L. naiffi, L. guyanensis, L. shawi*, and *L. amazonensis*	hsp70	PCR–RFLP	72.7 %	73.2 %
Castilho et al, 2008 [23]	CL & MCL	*Leishmania (L.), Leishmania (V.), L braziliensis, L* no *braziliensis*	G6PD	Real-time PCR	NR	NR
Da Silveira Neto et al., 2012 [28]	VL	*L. infantum chagasi*	18S rRNA	PCR	NR	NR
Neitzke-Abreu et al., 2013 [29]	CL	*Leishmania* (Viannia)	MinicirclekDNA	PCR	PCR-L[a]: 100 %; PCR-Bb: 65.6 %	PCR-L[a]: 82.8 %; PCR-B[b]: 92.9 %
Volpini et al. 2004 [30]	ACL	*L.* (V.) *braziliensis* and *L.* (L.) *amazonensis*	Conserved regions of the minicirclekDNA	PCR-RFLP	98.5 %	NR
Adams et al. 2010 [31]	CL & VL	*L. donovani*	18S rRNA	RT-LAMP	VL: RT-LAMP 98 %	VL: RT-LAMP 83 %. CL:98 %
Khan et al. 2012 [32]	VL	*L. donovani*	SSU-rRNA	Nested PCR and LAMP	100 %	PCR: 96 %. LAMP: 90.7 %.
Toz et al. 2013 [33]	VL	*L. donovani complex, L. tropica*, and *L. major*	ITS1	RTq-PCR	NR	94.11 %

El Tail et al. 2000 [11]	VL and PKDL	*L. donovani*	ITS	PCR-SSCP	100 %	100 %
De Almeida et al. 2011 [34]		*Leishmania spp*	rRNA-ITS	PCR and DNA sequencing	NR	NR
Kato H. et al. 2005 [27]		*Leishmania spp*	Cyt*b*	PCR and DNA sequencing	NR	NR

VL visceral leishmaniasis, *CL* cutaneous lesihmaniasis, *MCL* mucocutaneous lesihmaniasis, *ACL* American cutaneous leishmaniasis, *PKDL* post-kala-azar dermal leishmaniasis, *hsp* heat shock protein, *G6PD* glucose 6-phosphate dehydrogenase, *rRNA* ribosomal RNA, *kDNA* kinetoplast DNA, *SSU-rRNA* small-subunit rRNA, *ITS* Internal transcribed spacer, *Cytb* Cytochrome *b gene*, *PCR* polymerase chain reaction, *RFLP* restriction fragment length polymorphism, *LAMP* loop-mediated isothermal amplification, *SSCP* single-strand conformation polymorphism, *NR* not reported

[a]PCR using DNA extracted from tissue as a template

[b]PCR using DNA extracted from leucocytes (from whole blood) as a template

in the genomic sequence and to create the AUG codon for translation initiation. Therefore, at the 3′ ends, the edited regions of 22 bp have a deletion of one T residue, while those of 24 bp have an insertion of one T residue.

In a study performed by Luyo-Acero et al. [24], they identified sequence variations of the Cyt *b* gene from human-infecting *Leishmania* species/subspecies, which has been widely used to distinguish each one of them in further studies. This approach has allowed exploring the phylogenic relationship among these parasites and has been a suitable and commonly used tool for diagnosis of Leishmaniasis [5, 38].

Cyt b-PCR protocol (Adapted from Refs. 4, 5).

We usually use nested PCR for amplification of *Leishmania* DNA from patient specimens on FTA Card to get maximum sensitivity [4, 7]. The protocol is as follows:

1. For the identification of *Leishmania* species, an analysis of the cytochrome *b* (*cyt* b) gene is performed through a nested PCR amplification with a pair of specific primers, L.cyt-AS (5′·GCGGAGAGRARGAAAAGGC·3′) and L.cyt-AR (5′·CCACTCATAAATATACTATA·3′).
2. Ampdirect Plus reagent (Shimadzu Biotech, Tsukuba, Japan) is to be used to obtain maximum sensitivity of PCR amplification against the possible carry-over of tissue-derived inhibitors for enzymatic reaction in each sample.
3. The suggested thermal profile is as follows: 30 cycles of 95.0 °C for 1 min, 55.0 °C for 1 min, and 72.0 °C for 1 min.
4. One microliter of the PCR product is subjected to the nested PCR with a set of inner primers, L.cyt-S (5′·GGTGTAGGTTTTAGTYTAGG·3′) and L.cyt-R (5′·CTACAATAAACAAATCATAATATRCAATT·3′).

But, same samples such as your FTA samples from infected animals, DNA from parasite culture and infected sand fly specimens, single PCR using L.cyt-S and L.cyt-R primers is enough to amplify the parasite DNA. In many cases of subgenus *Viannia* species infection, parasite number in the lesion is lower in the chronic phase. Therefore, we are using nested PCR for patient specimens. In addition, we are using Ampdirect as a PCR buffer for FTA card and crude DNA as a template. General PCR solution works for FTA cards in most cases, but Ampdirect allows PCR reaction in the presence of enzyme inhibitors and increases the sensitivity.

The advantage of molecular approaches based on PCR or other amplification techniques is that they combine high sensitivity for direct detection of the infecting parasites in various human, animal, and sand fly tissues, with species specificity approaches. In areas where many species are sympatric, different approaches might be necessary, which would increase the cost of diagnostics. Approaches based on initial amplification of genus-specific sequences followed

by subsequent differentiation of *Leishmania* species by RFLP, hybridization with specific probes or sequencing of the amplified sequences have proven most useful. However, the results of PCR diagnosis should always be evaluated in conjunction with clinical diagnosis in order to provide a better and reliable result.

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