The Use of Fluorescent Fragment Length Analysis (PCR-FFL) in the Direct Diagnosis and Identification of Cutaneous *Leishmania* Species

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Abstract. Leishmaniasis is a disease caused by different species belonging to the genus *Leishmania*. It presents different epidemiological and clinical features and requires the development of rapid, sensitive techniques to improve specific diagnosis. In this study, we compared the traditional technique of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) with PCR-fluorescent fragment length analysis (PCR-FFL). Fluorescently tagged primers, designed in the rRNA fragment ITS-1 and 7SL region, were used to amplify fragments, which were later digested and whose sizes were accurately determined using an automated DNA sequencer. We validated the technique using 19 *Leishmania* strains from five cutaneous *Leishmania* species before testing 36 clinical samples: 23 skin biopsies and 13 skin scrapings/lesion exudates on filter paper. In real diagnostic, PCR-FFL has proved to be quick, accurate, and more sensitive (83.3% testing the ITS-1 fragment and 94.4% testing the 7SL) than PCR-RFLP analysis (75% and 80.6%). Filter papers improved the specific diagnosis in both techniques using non-invasive samples.

INTRODUCTION

Leishmaniasis is a vector-borne disease caused by a protozoan parasite of the *Leishmania* genus that is transmitted by the bite of species of the *Phlebotomus* genus in the Old World and the *Lutzomyia* genus in the New World. It is endemic in 88 countries across four continents and it has four known forms: cutaneous, diffuse cutaneous, mucocutaneous, and visceral.¹ In Mediterranean countries, the major etiological agents of the cutaneous forms are *Leishmania infantum*, *Leishmania major*, and *Leishmania tropica*.² The presence of multiple *Leishmania* species that cause similar clinical features requires the development of accurate and sensitive techniques for the diagnosis of *Leishmania* infections and for species identification. This is important for the selection of a suitable treatment and appropriate public health control measures, because each species has its own epidemiological pattern.³ Classical laboratory methods such as the examination of skin lesions using smears and cultures and histopathological examinations are specific but they have a low and variable sensitivity.⁴ Methods based on the observation and identification of the amastigote and promastigote forms through morphology do not distinguish different species, therefore other techniques are needed to correctly diagnose the disease. Isoenzyme analysis is the gold standard technique for the characterization and classification of *Leishmania* parasites.⁵ However, this assay is culture dependent, time consuming, and requires the examination of 15 different enzymatic profiles. It is therefore not available in routine diagnosis.⁵ To overcome these problems, DNA-based methods have been widely used for the identification and classification of *Leishmania* spp. with a variety of targets such as protein-coding genes, non-coding segments, microsatellites, and extrachromosomal DNA such as repetitive kinetoplast DNA (kDNA) minicircles.^{5–7} Several polymerase chain reaction (PCR)-methods, such as PCR-restriction fragment length polymorphism (PCR-RFLP), DNA sequencing, hybridization probes, high resolution melting analysis, and real-time PCR, are used.⁶

Sequence data of the ribosomal RNA gene, the two highly variable internal transcribed spacer regions (ITS-1 and ITS-2), have been successfully used to resolve taxonomic and phylogenetic affinities among related *Leishmania* species.⁵ The PCR-RFLP of ITS-1 has been used as an alternative to conventional methods for the identification of parasites from clinical samples. It is increasingly used for routine diagnosis because it is simple, sensitive, and does not require cultivation.⁸ More recently, targeting another ribosomal gene, the 7 spliced leader (7SL), has proven to be useful for detecting and identifying leishmanial parasites from samples. A comparative PCR-RFLP assay using ITS-1 and 7SL sequences showed more sensitivity in the 7SL assay, which detected a lower number of parasites in the clinical samples.⁹ However, the possibility of false negative results when applying PCR-RFLP and the time-consuming nature of the nested-PCR that is required to solve it made it necessary to develop faster, more sensitive techniques.^{10,11}

The PCR-fluorescent fragment length analysis (PCR-FFL) is a method for discriminating species by gene size polymorphisms.¹² In previous studies, it has proved to be quick and accurate in the identification and classification of African trypanosomes, and has also been useful with other organisms such as anisakids and seaweeds.^{12–15} Therefore, in the current study we analyzed the ITS-1 and 7SL regions using this technique, first applying it to the identification and classification of the different Old World *Leishmania* species from skin samples. To show the advantages of PCR-FFL, we compared it with the widely used PCR-RFLP method and tested the same PCR products from *Leishmania* strains and clinical skin samples.

MATERIAL AND METHODS

Leishmania strains. The PCR-FFL analysis was validated using 19 *Leishmania* strains belonging to the three cutaneous species of the Old World (*L. infantum*, *L. major*, and *L. tropica*) and the *Leishmania braziliensis* complex from the New World (Table 1). Strains were taken from the *Leishmania* Cryobank at the Universitat de Barcelona and the DNA of five strains was kindly donated by Dr. M. Hide, MIVEGEC, Montpellier, France and Dr. L. García, Centro Universitario de Medicina Tropical, Cochabamba, Bolivia.

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TABLE 1
Control strains examined

Species	Control strains
<i>Leishmania infantum</i>	MCRI/ES/2002/BCN-503 MHOM/ES/2007/BCN-741 MHOM/ES/2007/BCN-742 MHOM/ES/2007/BCN-763 MHOM/ES/2008/BCN-779
<i>Leishmania major</i>	MHOM/ES/2010/BCN-815 MHOM/ES/2006/BCN-876 MHOM/ES/2007/BCN-750 MHOM/OO/2008/BCN-766 MHOM/ES/2010/BCN-822 MHOM/ES/2011/BCN-827 MHOM/IL/81/FRIEDLIN MHOM/SU/73/5ASKH
<i>Leishmania tropica</i>	MHOM/ES/2010/BCN-809 MHOM/SU/74/K27
<i>Leishmania braziliensis</i>	MHOM/BR/86/BCN-19 MHOM/BR/75/M-2901
<i>Leishmania guyanensis</i>	MHOM/BO/09/CUM1000 MHOM/PE/91/LC1447

Patient and sample collection. Twenty-three skin biopsies and 13 skin scrapings or lesion impressions on filter papers (Whatman 3M, Whatman International Ltd., Maidstone, England) were obtained from 36 patients with cutaneous leishmaniasis who were attending different dermatologist services in hospitals in Barcelona and Majorca. The criteria used to confirm cutaneous leishmaniasis and include subjects in the study were microscopic and/or *Leishmania* spp. DNA detection by highly sensitive real-time PCR.¹⁶ We examined 28 skin samples (including all 13 filter papers) from patients from the local endemic area, six patients from other Mediterranean-endemic areas (Morocco, Israel, and Jordan) and two from Mauritania, or people who had traveled to those areas.

Ethical considerations. The ethics committee of each participating center approved the study. All patients gave their written informed consent to participate in the study.

Statistical analysis. Between-test agreement was determined by the concordance coefficient, measured by Cohen's kappa statistic ($k > 0.75$ substantial agreement; $k = 0.4-0.75$, fair to good agreement; $k < 0.40$, poor agreement).

DNA extractions. With control strains, the DNA was extracted from cultured parasites using a chelex resin protocol: 100 μ L of sterile water and 400 μ L of chelex solution [1% Tween 20 (Panreac Química S.A. Barcelona, Spain), 1% Nonidet P-40 (Amresco, Solon, OH), and 20% of chelex 100 resin (Bio-Rad Laboratories, Hercules, CA)] were added to the promastigote sediment. It was heated at 100°C for 20 min and then vortexed. Finally, the mixture was centrifuged for 10 min at 12,000 $\times g$ to separate the resin from the supernatant, which contains the genomic DNA that was used as template for the PCR.

For the skin samples, DNA was extracted from 25–50 mg of tissue lesion or one filter paper 6 mm in diameter by isolating the nucleic acids using the mammalian tissue protocol of the High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany), in accordance with the manufacturer's instructions.

PCR amplification. For the identification and diagnosis of the *Leishmania* species, we amplified the ribosomal ITS-1 region with the previously described primers LITSR (5'-CTGGATCATTTTCCGATG-3') and L5.8S (5'-TGATACC ACTTATCGCACTT-3'),¹¹ which were fluorescently labeled

with blue and green fluorochromes (6-FAM and VIC), respectively (Applied Biosystems, Warrington, Cheshire, UK). For the 7SL region, we used the primers TRY7SL.For1 (5'-TGCT CTGTAACCTTCGGGGGCT-3') and TRY7SL.Rev1 (5'-GG CTGCTCCGTYNCCGGCCTGACCC-3').^{17,9} These primers were also fluorescently labeled with the same fluorochromes (6-FAM and VIC), respectively (Applied Biosystems).

Amplification reactions were performed in volumes of 50 μ L containing 5 μ L of 10X buffer (BIOTAQ DNA Polymerase, Bioline, London, UK), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μ M of each primer and 1.5 units of *Taq* polymerase (BIOTAQ DNA Polymerase, Bioline, London, UK). We added 3 μ L of isolated DNA to the mixture and incubated it in the thermal cycler (MJ Research PTC-200 DNA Engine, Alameda, CA) under the following conditions: for the ITS-1 PCR, we started with a denaturing step at 95°C for 2 min, followed by 35 cycles of denaturing for 20 sec at 95°C, annealing for 30 sec at 53°C, and extension for 1 min at 72°C, followed by a final extension at 72°C for 1 h. For the 7SL PCR, the denaturing step lasted for 5 min at 95°C, followed by 35 cycles of denaturing for 20 sec at 95°C, annealing for 30 sec at 65°C, and extension for 1 min at 72°C. The final extension consisted of 10 min at 72°C. Previously, the optimal primer annealing temperatures (T_a) for both programs were experimentally determined using the temperature gradient program of the thermal cycler. The concentrations of $MgCl_2$ and the primers were also previously tested to achieve better conditions for the PCR.

PCR-FFL analysis of amplified ITS-1 and 7SL sequences.

The PCR products were digested with the restriction enzyme *BsuRI* (*HaeIII*) (Fermentas, Life Sciences, Germany, EU) without prior purification, in accordance with the manufacturer's recommendations. Digestion products were pooled with the internal lane size standard GeneScan-500 LIZ (Applied Biosystems, Warrington, UK) for the analysis on the DNA sequencer ABI Prism 3730 (Applied Biosystems). Fragment sizes were determined using the fragment analysis program Peak Scanner Software v1.0 (Applied Biosystems).

PCR-RFLP analysis of amplified ITS-1 and 7SL sequences.

The PCR products, previously digested with the restriction enzyme *BsuRI* (*HaeIII*), were separated by electrophoresis in 3% wide-range agarose (Sigma) at 150 V in SGTB 1X buffer (GRISP LDA, Research Solutions, Porto, Portugal). A solution of SYBR safe DNA gel stain (Invitrogen Ltd. Paisley, UK) was used to visualize the separated DNA fragments under UV light. This system was performed in parallel with the same strains and samples we used in the PCR-FFL analysis, which made it possible to compare the two methods.

RESULTS

Validation of PCR-FFL using control strains. Initially, we tested PCR-FFL using control DNAs in parallel with the commonly used PCR-RFLP analysis; in both cases, previous digestion with the restriction enzyme *BsuRI* (*HaeIII*) was required.

For the ITS-1 PCR, all isolates examined with capillary electrophoresis gave two different peaks (Figure 1). Only from the position of these peaks, the method detected and identified the Old World *Leishmania* species without any overlap between them, giving reproducible results and a small degree of intraspecific variation (Table 2). The *L. braziliensis* complex was clearly distinguishable from the Old World species studied.

FIGURE 1. Example ITS-1 electropherograms of different *Leishmania* spp. reference strains.

FIGURE 2. Example 7SL electropherograms of different *Leishmania* spp. reference strains.

The 7SL PCR gave one peak in the capillary electrophoresis, corresponding to the fluorescent labeled primer TRY7SL. For1, for *L. infantum* and *L. major*. Two fragments were visible on the *L. braziliensis* complex species and *L. tropica* (Figure 2). From the position of these peaks, the method detected and identified the Old World *Leishmania* species without any overlap between them, with a small degree of intraspecific variation (Table 2). The *L. braziliensis* complex

TABLE 2

Experimental ITS-1 and 7SL fragment length base pair (bp) profiles obtained by PCR-FFL from the 19 *Leishmania* control strains studied*

Species	ITS-1 fragment (bp)		7SL fragment (bp)	
	LitSR-FAM	Lit5.8S-VIC	TRY7SL.For1-FAM	TRY7SL.Rev1-VIC
<i>L. infantum</i> (5)	68–69	186–193	129–134	ND
<i>L. major</i> (8)	129–136	196–205	160–168	ND
<i>L. tropica</i> (2)	53–54	185–193	103–109	76
<i>L. braziliensis</i> (3)	134–140	157–164	102–104	76
<i>L. guyanensis</i> (1)	144	164	104	76

*PCR-FFL = polymerase chain reaction-fluorescent fragment length; () = number of *Leishmania* strains analyzed; ND = not detected.

species could not be distinguished from *L. tropica* because the peak position obtained for both was the same.

Analysis of biological samples by PCR-FFL. The 36 clinical samples were tested in triplicate, with a week of difference between the analyses, by PCR-FFL with ITS-1 and 7SL sequences obtaining a reproducibility of 98.6%. With the ITS-1 sequence, the analysis was positive for 30 of the samples, with three different patterns corresponding to *L. infantum*, *L. tropica*, and *L. major* having a product length variation within that previously observed in the analysis of the control strains (*L. infantum* 68–69, 186–193; *L. major* 129–135, 196–205; *L. tropica* 53, 185–187). When we analyzed the 7SL region, we obtained 34 positives, with three different patterns corresponding to *L. infantum*, *L. tropica*, and *L. major* and the fragment lengths within those previously predicted (*L. infantum* 129–134; *L. major* 160–168; *L. tropica* 103–108, 76). Between the samples, we analyzed 13 filter papers and obtained 12 positives for both sequences tested (ITS-1 and 7SL) (Table 3).

PCR-RFLP analysis and comparison with the PCR-FFL system. The PCR-RFLP method is the most widely used assay for the identification of *Leishmania* species. We tested it in

TABLE 3

Leishmania species identified by PCR-FFL (ITS-1 and 7SL sequences) from 36 CL patients biological samples*

Species	Biopsies (23)		Filter paper† (13)	
	ITS-1	7SL	ITS-1	7SL
<i>L. infantum</i>	11	14	12	12
<i>L. major</i>	5	5	—	—
<i>L. tropica</i>	2	3	—	—
NI	5	1	1	1

*PCR-FFL = polymerase chain reaction-fluorescent fragment length; ITS-1 = internal transcribed spacer region; 7SL = 7 spliced leader; CL = cutaneous leishmaniasis; NI = non-identified.

†Scraping or lesion impression on filter paper.

parallel with PCR-FFL for the ITS-1 and 7SL regions after digestion with the restriction enzyme *BsuRI* (*HaeIII*). As a result, we found the same speciation results for the two analysis systems in both sequences studied. The 19 control strains were identified in all tests. However, in real diagnosis situations, PCR-FFL was more sensitive (83.3% when testing the ITS-1 fragment and 94.4% when testing the 7SL sequence, versus 75% and 80.6% with PCR-RFLP, respectively). The 7SL fragment sensitivities for PCR-FFL were higher than 7SL PCR-RFLP (fair agreement $k = 0.392$), although the ITS-1 fragment sensitivities by PCR-FFL were higher than PCR-RFLP but not significantly (good agreement $k = 0.750$). These differences were clearly observed with the skin biopsies in the 7SL sequence (82.6% in PCR-RFLP and 95.7% with PCR-FFL, fair agreement $k = 0.355$) but not in the ITS-1 sequence (73.9% in PCR-RFLP and 78.3% with PCR-FFL, very good agreement $k = 0.881$). However, filter paper PCR-FFL was more sensitive (92.3% in ITS-1 and 7SL) than the PCR-RFLP (76.9% for ITS-1 and 7SL) in both techniques (moderate agreement $k = 0.435$) (Table 4).

DISCUSSION

Molecular techniques using PCR technology are useful for the identification and classification of *Leishmania* species found in human skin samples. The ITS-1 region, lying between the genes coding for the 18S and the 5.8S rRNA, allows classification of the parasites studied because of the differences in interspecific sequences and the low level of intraspecific variation.¹⁸ This region does not encode any product and has less impact on organism viability, which permits it to evolve at a faster rate than the ribosomal coding regions.¹⁹ Recently, the evaluation of the 7SL rRNA gene for the identification of *Leishmania* spp. has produced satisfactory results.^{20,21} Its advantages include being very abundant in cells; having a sequence that is not particularly similar to the mammalian

7SL, although they have the same secondary structure; and having a divergent central domain flanked by other conserved domains.^{22,23} These characteristics make both rRNA fragments useful as genetic markers because of the length polymorphism and the nucleotide differences among species.

The PCR-RFLP method has been widely used because it is simple and cheaper than other techniques such as sequencing, making it useful for routine analysis.²⁴ The digestion of the PCR product with the restriction enzyme *BsuRI* (*HaeIII*) makes it possible to identify the most clinically important species by their RFLP patterns.¹¹ However, the problems encountered in the identification of some species and the false negative results with a sensitivity rate of about 85%¹⁰ led to the development of a nested PCR to increase sensitivity, but this is more time consuming and has greater potential for contamination.¹²

The study described here compares PCR-RFLP with the new PCR-FFL technique using both rRNA fragments described, ITS-1 and 7SL, for the identification and classification of the *Leishmania* species. The prior use of control strains as standards makes subsequent diagnosis in real skin samples (biopsies and filter papers) possible by comparing the combination of fragment sizes obtained. When we tested ITS-1, all control strains were positive with both techniques, and two different peaks were obtained for each fluorescent-labeled primer in the PCR-FFL technique. The new method differentiated the Old World *Leishmania* species without overlap between them, but the New World species (*L. braziliensis* complex) could not be distinguished from each other, as occurs in PCR-RFLP analysis. In the 7SL analysis, the results were also satisfactory, with 19 positives, and only one peak that differentiated the Old World species without overlap between them. Previous studies indicated that 7SL assay cannot be used to distinguish between *L. donovani* and *L. infantum*.¹⁷ When we tested the New World species, we obtained two different peaks. However, as with the ITS-1 fragment and PCR-RFLP technique, they could not be differentiated from each other or from the Old World species *L. tropica*. In prior studies with the 7SL sequence, *L. tropica* could not be differentiated from *Leishmania aethiopica*.^{9,17} In this respect, the ITS-1 has the advantage of being more specific and could be more useful for identifying species.

The experimental PCR product lengths of the control strains, which were accurately determined using an automated DNA sequencer, showed a small degree of intraspecific variation in both the sequences studied (7SL and ITS-1). This may have been caused by the secondary structure or differences in the base composition of the DNA, both of which could affect mobility in the chromatography process.²⁵ In the ITS-1 region studied, there are two known microsatellites that affect the sequence

TABLE 4

Comparison of PCR-RFLP and PCR-FFL techniques applied on the ITS-1 and 7SL sequences for the identification of the 36 CL biological samples (23 skin biopsies and 13 filter papers)

	PCR-RFLP		PCR-FFL	
	ITS-1	7SL	ITS-1	7SL
CL skin biopsies	17/23 (73.9%)	19/23 (82.6%)	18/23 (78.3%)	22/23 (95.7%)
95% CI	53.2–87.7	62.3–93.6	57.7–90.8	77.3–99.9
CL filter papers	10/13 (76.9%)	10/13 (76.9%)	12/13 (92.3%)	12/13 (92.3%)
95% CI	49.1–92.5	49.1–92.5	64.6–99.9	64.6–99.9
TOTAL	27/36 (75%)	29/36 (80.6%)	30/36 (83.3%)	34/36 (94.4%)
95% CI	58.7–86.4	64.7–90.6	67.7–92.5	80.9–99.4

PCR-RFLP = polymerase chain reaction-restriction fragment polymorphism; PCR-FFL = polymerase chain reaction-fluorescent fragment length; ITS-1 = internal transcribed spacer region; 7SL = 7 spliced leader; CL = cutaneous leishmaniasis; CI = confidence interval.

length of the DNA and are also a typical cause of variation in the results of the PCR-FFL technique.⁷ Despite all these possible differences, the results obtained were reproducible.

The two techniques used and the rRNA sequences tested in the analysis of the 36 skin samples presented differences. The ITS-1 gave 27 positive results with the PCR-RFLP method, which obtained 75% sensitivity, compared with 30 positives and 83.3% sensitivity obtained with the PCR-FFL test. This lower sensitivity with the PCR-RFLP technique shows the problem with false negative results observed before with some PCR methods used to analyze clinical samples,⁹ however it is possible to reduce them using PCR-FFL because it increased the number of positive samples in this study. The 7SL analysis gave 29 positives and 80.6% sensitivity with PCR-RFLP and 34 positive samples and 94.4% sensitivity with PCR-FFL. Again, this shows how the PCR-FFL test increases sensitivity obtaining better results with the 7SL sequence, which is in agreement with previous studies of the application of PCR techniques to the 7SL fragment and how it provides better results than the ITS-1 sequence.^{20,21} Additionally, in both sequences, the PCR-FFL showed a small degree of intraspecific variation in the fragment length, which was probably caused by the previously mentioned factors and could be useful for genotyping different lineages in epidemiological studies. This characteristic does not pose a problem for the classification of the *Leishmania* species because of the high level of interspecific variation.

Skin scrapings and aspirates on filter papers are non-invasive clinical specimens and their sensitivity and specificity are similar to the invasive-specimen PCR used in prior studies.²⁶ They offer some advantages over biopsy techniques, including easy transportation from hospitals to diagnostic laboratories and the possibility of storing them for years when frozen and months when kept at room temperature.¹¹ In addition, filter papers are more likely to be favored by patients and clinics because they represent a non-invasive technique for collecting samples. When used in this study, filter papers also resulted in higher sensitivity in PCR-FFL than in PCR-RFLP for both the sequences studied (7SL and ITS-1). This shows how the use of filter papers benefits the analysis, because both methods achieved good, comparable results with both sequences.

In conclusion, PCR-FFL is a simple, quick, and sensitive method based on the amplification of small regions of DNA and fluorescence detection without prior use of *Leishmania* culturing. Using a single pair of primers, it identifies the *Leishmania* species and gives different profiles for each one with a high level of accuracy. The method is easy to standardize, presents a high reproducibility, and can handle a high throughput of samples, typically producing 96 results within 24 hours. Furthermore, the application of this technique on the 7SL ribosomal sequence allowed us to obtain a sensitivity of 94% in the diagnosis of different kinds of skin samples, compared with 83% obtained with ITS-1 using the same analysis method. This makes PCR-FFL on the 7SL sequence the best combination for the diagnosis and identification of *Leishmania* species.

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