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Real-Time PCR for Detection and Quantitation of *Leishmania* in Mouse Tissues

Luc Nicolas,* Eric Prina, Thierry Lang, and Geneviève Milon

Unité d'Immunophysiologie et Parasitisme Intracellulaire, Institut Pasteur, 75724 Paris Cedex 15, France

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***Leishmania* spp. are intracellular protozoan parasites that cause a wide spectrum of diseases in humans and dogs worldwide. However, monitoring of the *Leishmania* burden in its different hosts is still based on cumbersome and poorly sensitive methods. Here we have developed a highly accurate real-time PCR assay with which to reproducibly detect and quantify the relative *Leishmania major* burden in mouse tissue samples. The assay is performed with the LightCycler system using SYBR Green I and primers amplifying a ca. 120-bp fragment from minicircles of the kinetoplast DNA (kDNA). The assay was able to detect as little as 100 fg of *L. major* DNA per reaction, which is equivalent to 0.1 parasite. The standard curve designed for quantitation of parasites showed linearity over an at least 6-log DNA concentration range, corresponding to 0.1 to 10^4 parasites per reaction, with a correlation coefficient of 0.979. The assay also proved to have a detection range of the same magnitude as that used for detection of *L. donovani* and *L. amazonensis*, but it was 100-fold less sensitive for *L. mexicana*. When applied to tissues from experimentally infected mice, the real-time PCR assay is not only as sensitive as a conventional PCR assay for detection of *Leishmania* kDNA but also more rapid. Results indicate that this assay is compatible with the clinical diagnosis of leishmaniasis and will be a great help to scientists who use animals to monitor the efficacy of antileishmanial drugs or vaccines or decipher the unique properties of the life cycle of *Leishmania* spp .**

Leishmania spp. are intracellular protozoa that affect humans and dogs worldwide and are transmitted by the bite of hematophagous sand flies. They cause a large spectrum of diseases, ranging from spontaneously healing skin lesions to fatal visceral symptoms, if left untreated. Two million new human cases arise every year, and at least 350 million people are exposed to the risk of the *Leishmania* parasite infection (2, 9). Experimental hosts, such as laboratory mice, are largely used to study the immunobiology of these parasites and to screen the efficacy of newly developed drugs and vaccines (4, 11, 20, 27). Most of those studies require detection and quantitation of the *Leishmania* burdens in different mouse tissues. This is still routinely performed by culture-based techniques (6, 28), which have several limitations, in particular, the amount of time required and the putative presence of viable but nonculturable parasites, such as persistent parasites (1). PCR-based methods for detecting *Leishmania* species have been developed to amplify rRNA genes, miniexon genes, kinetoplast DNA (kDNA), and repetitive nuclear sequences (15, 18, 24, 26). Recently, we have developed a PCR-based assay with which to quantify the parasite load in mice infected with *Leishmania major* (23) by using primers from the conserved sequences of kDNA. However, this technique is still cumbersome as it requests agarose gel image analysis.

A more rapid alternative is real-time quantitative PCR, which quantifies DNA (12, 29) and therefore has the potential for accurate microorganism enumeration in medical (14, 16, 17), environmental (25), or food samples (13). Here we describe a highly sensitive and specific method by which to detect and/or quantify *L. major* in mouse tissues by using the LightCycler (LC) system (30), which was adapted from a previous conventional PCR assay (23). This system combines an air thermocycler and a fluorimeter, enabling rapid-cycle PCR and monitoring of incorporation of the fluorescent dye SYBR Green I in double-stranded DNA. We show also that the assay can be used for detection of *L. donovani*, *L. infantum*, *L. amazonensis*, and *L. mexicana*.

MATERIALS AND METHODS

***Leishmania* strains and DNA extraction.** *L. major* strain NIH173 (MHOM/IR/−/173), *L. donovani* LV9 (MHOM/ET/1967/Hu3:LV9), *L. infantum* 2176 (MHOM/FR/1991/LEM 2176), *L. amazonensis* LV79 (MPRO/BR/1972/M1841), and *L. mexicana* M379 (MNYC/BZ/1962/M379) were cultured at 26°C in Hosmem-II medium (3) supplemented with 10% heat-inactivated fetal calf serum (Dutscher, Brumath, France), 100 U of penicillin per ml, and 100 μg of streptomycin per ml (Seromed, Berlin, Germany). Stationary-phase promastigotes of the different strains were harvested by centrifugation, washed twice with phosphate-buffered saline, enumerated with a Malassez hemacytometer, pelleted, and stored at −80°C until DNA extraction.

Genomic DNA was extracted from approximately 2×10^7 promastigotes with a DNeasy Tissue Kit (Qiagen, Courtaboeuf, France) in accordance with the manufacturer's protocol. The DNA concentration was estimated by spectrophotometric determination of A_{260}.

Source of mouse tissue DNA. Tissues were collected from BALB/c mice chronically infected in other studies in our laboratory or from naive mice as controls. Mice were infected intradermally at the ear with 10^4 metacyclic promastigotes of *L. major* strain NIH173 and killed at 6 or 12 months postinfection (23). Other mice were infected in the footpad with 2×10^6 amastigotes of *L. amazonensis* strain LV79 or *L. mexicana* strain M379 and killed at 12 or 32 weeks postinfection, respectively. Bone marrow and spleens were also collected from mice infected with *L. donovani* strain LV9 (19). Briefly, mice were inoculated by the intravenous route in the tail vein with 2×10^7 stationary-phase promastigotes and killed 30 days postinoculation.

The following tissues were sampled from infected or uninfected BALB/c mice: ears, retromaxillary or popliteal draining lymph nodes, spleen, liver, femoral bone marrow, blood, footpad, and tail skin (Table 3). Homogenates were pre-

* Corresponding author. Mailing address: Institut Pasteur, Unité d'Immunophysiologie et Parasitisme Intracellulaire, 25 rue du Dr Roux, 75724 Paris Cedex 15, France. Phone: (33) 1 45 68 81 70. Fax: (33) 1 40 61 31 69. E-mail: lnicolas@pasteur.fr.

TABLE 1. Times and temperatures used for the PCR configuration with SYBR Green I and the LC

Parameter	Temp (°C)	Time (s)	Slope (°C/s)	Acquisition mode
Denaturation	95	8	20	None
Amplification (40 cycles)	95	10	20	None
	56	10	20	None
	72	8	20	Single
Melting	95	10	20	None
	67	30	20	None
	95	10	0.1	Continue
Cooling	40	60	20	None

pared as previously described (23). Tissues were removed by using different scissors or scalpels to avoid contamination and were minced with Potter grinders and then carefully homogenized in 1.5-ml microtubes with single-use blue pellet pestles (Polylabo, Paris, France) in phosphate-buffered saline. Aliquots of the homogenates were stored at −20°C until DNA extraction. DNA was extracted from aliquots of homogenates with a DNeasy Tissue Kit.

Primers. Detection of *Leishmania* DNA was carried out with previously described primers (23) (forward, 5′-CCTATTTTACACCAACCCCCAGT-3′ [JW11]; reverse, 5′-GGGTAGGGGCGTTC TGCGAAA-3′ [JW12]) that amplify a ca. 120-bp fragment of the minicircle kDNA of *L. major*, ca. 10,000 copies of which are present in each parasite. These primers match the conserved sequences of the kinetoplast minicircle but do not match human or mouse frequent nucleic acid sequences according to the PCR-Rare software (10). Primers were provided by Genset (Paris, France) as EasyOligos.

Conventional PCR. A conventional PCR was carried out with an automated thermocycler PCR-Express (Hybaid, Ashford, United Kingdom) as already described (23). Extracted DNA (2 μl) was mixed with a solution containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 250 μM each dNTP, 10 pmol of each primer, and 0.5 U of *Taq* polymerase (Promega, Charbonnières, France) in a 40-μl final volume. A hot-start procedure was used to increase specificity. After initial denaturation (4 min at 94°C), 40 cycles of denaturation for 1 min at 64°C, annealing for 30 s at 58°C, and elongation for 30 s at 72°C were carried out and the PCR was terminated by a final extension at 72°C for 10 min. Each sample was tested in duplicate. Negative control tubes that received 2 μl of water instead of DNA extract were included in each PCR run to detect any amplicon contamination. PCR products were visualized after electrophoresis on a 1.5% agarose gel.

Real-time PCR with LC. A real-time hot-start PCR was performed with the LC FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Meylan, France) in an LC (Roche Diagnostics). The 12-μl reaction mixture contained 1× LC FastStart DNA Master SYBR Green I, 2 mM $MgCl_2$, 10 μM each primer, and 1.2 μl of template. Times and temperatures are shown in Table 1. For fluorescence signal acquisition, channel F1 was used and the gain was set at 5. For normalization of fluorescent data, the F1/1 ratio was applied.

Data and statistical analysis. In order to determine the variability of the assays, intraassay and interassay (repeatability) precision was measured. Three replicates of five different concentrations of *L. major* DNA were tested simultaneously in the same run. The precision among four assays was assessed by using the previous *L. major* DNA concentrations run on different days. Variability is shown as the mean ± the standard deviation (SD) and reported as the coefficient of variation. Statistical and regression analyses were carried out with Sigma Plot Software (SPSS Inc., Chicago, Ill.).

RESULTS

LC PCR development. The JW11 and JW12 primers, which amplify a ca 120-bp DNA fragment from *L. major* kinetoplast minicircles, have already been used (23). A 100-pg sample of DNA extracted from in vitro-grown promastigotes of *L. major* strain NIH173 was used as the template for establishment of the LC PCR assay, in particular, determination of the optimal annealing temperature and magnesium chloride concentration. Agarose gel electrophoresis of the PCR product confirmed the amplification of a ca 120-bp DNA fragment (data not shown). The reaction volume was minimized to 12 μl including 10% template DNA.

Sensitivity and reproducibility of the assay for *L. major*. To determine the detection limit of our assay and establish a standard curve that could be used for quantitation, serial dilutions of *L. major* DNA with final concentrations ranging from 10,000 parasites to 0.01 parasite per reaction were subjected to a real-time PCR analysis. We were able to detect as little as 0.1 parasite, corresponding to 100 fg, per reaction in a 12-μl reaction volume. The mean standard curve, calculated from four independent experiments, was linear over an at least 6-log range of DNA concentrations, with a correlation coefficient of 0.979 (Fig. 1). A negative control consisting of the reaction mixture and water instead of template DNA was added in each run. A melting curve analysis of PCR products showed that the melting temperature of the kDNA amplicon was ca. 84°C while that of nonspecific products was ca. 79.5°C.

To analyze the reproducibility and reliability of the real-time PCR assay, we assessed the intraassay and interassay coefficients of variation. Four replicates of five 10-fold DNA concentrations, from 10^3 parasites per reaction to 0.1 parasite per reaction, were assessed in a single run. The intraassay variations of C_T values (the cycle numbers reflecting a positive PCR result differentiated from the background noise) among the replicates were 1.25, 0.22, 0.54, 1.35, and 0.43% for the five different concentrations, respectively. In addition, four replicates of 10-fold *L. major* DNA dilutions were performed on different days. The interassay variations of C_T values for the DNA concentrations ranging from 10^4 to 0.1 were 11.30, 6.69, 6.51, 4.38, 3.49, and 2.57%, respectively (Fig. 1).

Detection of kDNAs from other *Leishmania* species. Primers JW11 and JW12 were also able to amplify a ca 120-bp DNA fragment from promastigotes of *L. donovani* LV9, *L. infantum* 2176, *L. amazonensis* LV79, and *L. mexicana* M379 by conventional PCR (data shown). Therefore, they were also assessed

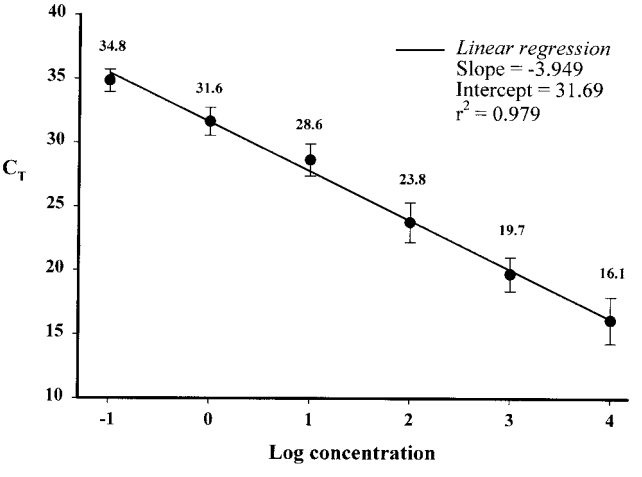

FIG. 1. Sensitivity of the LC PCR assay with 10-fold dilutions of *L. major* DNA. A plot of mean C_T values from four replicates tested on different days against the logarithmic concentration of parasite DNA (ranging from 0.1 to 10^4 parasite per reaction) is shown. Variability is shown as the mean C_T value ± 1 SD.

TABLE 2. Mean C_T values and intraassay SDs of dilution series of promastigote DNAs from different *Leishmania* species obtained with the LC PCR assay

Leishmania species	Mean C_T value ± SD with following no. of parasites/reaction:				
	1,000	100	10	1	0.1
L. major NIH173	21.06 ± 0.260	25.51 ± 0.057	29.46 ± 0.160	33.05 ± 0.446	35.85 ± 0.153
L. donovani LV9	17.71 ± 0.017	21.75 ± 0.070	24.57 ± 0.055	28.83 ± 0.228	32.29 ± 0.224
L. infantum 2176	19.95 ± 0.011	24.36 ± 0.272	28.90 ± 0.006	33.39 ± 0.051	ND[a]
L. amazonensis LV79	19.37 ± 0.121	23.42 ± 0.266	27.48 ± 0.250	32.14 ± 0.075	34.18 ± 0.122
L. mexicana M379	25.84 ± 0.095	30.09 ± 0.121	35.01 ± 0.627	ND	ND

[a] ND, inconsistent quantification data.

for amplification of kDNA in our LC assay. The sensitivity of the LC PCR assay for detection of these strains was similar to that of *L. major* NIH173, except for *L. mexicana* strain M379, for which the assay was approximately 100 times less sensitive (Table 2). The intraassay variation coefficient was always <1.2%, showing good reproducibility of the assay for those *Leishmania* species as well.

***Leishmania* detection in mouse tissues: comparison of real-time PCR and conventional PCR.** DNA was extracted from various tissues of BALB/c mice infected with various *Leishmania* strains (Table 3) and assayed with either a conventional PCR or the LC PCR. In the latter, the standard curve of the respective species was used to generate a relative *Leishmania* burden based on C_T values. In all of the assays, the C_T values of negative controls were always >36. Whatever the tissue and the strain were, all of the samples positive by the conventional PCR were also positive by the LC assay and most of the C_T values were far below the negative C_T value threshold of 36. In addition, a few samples negative by the conventional PCR were positive by the real-time PCR

DISCUSSION

A new molecular real-time PCR assay for detection and quantification of *L. major* and several other *Leishmania* species of medical or veterinary importance is described. This assay is based on the LC system with SYBR Green I. This quantitative LC PCR assay allows highly sensitive and reproducible detection and quantitation of the parasite burden over a wide range, at least 6 logs, of parasite concentrations. The very high sensitivity (less than 0.1 parasite per reaction) is partly due to the high copy number of the target minicircle kDNA, which is present at ca. 10,000 copies per parasite. This avoids the use of internal molecular probes and therefore limits the cost of the assay. Including the DNA extraction step, the assay can be performed within 4 to 5 h without risk of contamination, as the reaction capillary remained closed. This is much more rapid than microtitration assays or even a conventional PCR. Using another real-time PCR with the TaqMan system, Bretagne et al. (5) have compared the real-time PCR and culture microtitration for quantification of *L. infantum* in mouse tissues and shown a good correlation between the two techniques.

Application of the real-time PCR for research and clinical diagnosis in parasitology is just starting and so far concerns mainly *Toxoplasma gondii* (8, 14, 21) and *L. infantum* (5). With primers common to several *Leishmania* species, our assay was also used to determine the relative parasite burdens in mouse tissues infected with *L. amazonensis* and *L. donovani* and, to a lesser extent, in mouse tissues infected with *L. mexicana* based on C_T values. Preliminary assays in our laboratory have shown that the PCR yield may be influenced by a tissue DNA concentration above a threshold. Therefore, we are now develop-

TABLE 3. Results of real-time and conventional PCR assays for *Leishmania* kDNA in mouse tissue samples

Sample(s)	Species	Source	Result of conventional PCR	Real-time PCR	
				C_T	Qualitative data
1–6	*L. major*	Ear	−	>36	−
7	*L. major*	Ear	+	28.61	+
8	*L. major*	Ear	+	22.66	+
9	*L. major*	Ear	+	24.64	+
10	*L. major*	Ear	+	34.94	+
11	*L. major*	Ear	−	35.14	+
12	*L. major*	Ear	−	>36	−
13	*L. major*	Lymph node	+	29.68	+
14	*L. major*	Lymph node	+	30.68	+
15	*L. major*	Lymph node	+	23.77	+
16–18	*L. major*	Tail skin	−	>36	−
19	*L. mexicana*	Blood	−	>36	−
20	*L. mexicana*	Blood	+	31.42	+
21	*L. mexicana*	Blood	−	33.05	+
22	*L. mexicana*	Bone marrow	+	28.80	+
23	*L. mexicana*	Bone marrow	+	21.44	+
24	*L. mexicana*	Lesion	+	17.94	+
25	*L. mexicana*	Lesion	+	18.16	+
26	*L. mexicana*	Lesion	+	16.46	+
27	*L. mexicana*	Liver	+	23.66	+
28	*L. mexicana*	Liver	+	24.46	+
29	*L. mexicana*	Lymph node	+	19.81	+
30	*L. mexicana*	Lymph node	+	20.70	+
31	*L. mexicana*	Spleen	+	24.61	+
32	*L. mexicana*	Spleen	+	20.96	+
33	*L. mexicana*	Tail skin	+	23.89	+
34	*L. mexicana*	Tail skin	+	15.36	+
35	*L. mexicana*	Tail skin	+	18.67	+
36	*L. amazonensis*	Blood	−	>36	−
37	*L. amazonensis*	Lesion	+	15.81	+
38	*L. amazonensis*	Liver	−	>36	−
39	*L. amazonensis*	Lymph node	+	25.42	+
40	*L. amazonensis*	Spleen	−	>36	−
41	*L. amazonensis*	Tail skin	−	29.97	+
42	*L. donovani*	Bone marrow	+	22.73	+
43	*L. donovani*	Bone marrow	+	27.87	+
44	*L. donovani*	Bone marrow	+	28.44	+
45	*L. donovani*	Spleen	+	19.64	+
46	*L. donovani*	Spleen	+	19.88	+
47	*L. donovani*	Spleen	+	23.56	+

ing internal standards based on housekeeping genes to determine the parasite burden more accurately.

Identification of a *Leishmania* infection for laboratory clinical diagnosis by culture or serological techniques requires a long time and has poor specificity. With the development of a real-time PCR assay that can be improved for identification of *Leishmania* species with internal probes or different primers, as has been done for other pathogenic microorganisms (7, 22, 31), we hope that this assay will replace or supplement the current serology technique. In addition, a large field of application for our assay is monitoring of *Leishmania* infections in research experiments.

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