Real-Time PCR as a New Tool for Quantifying *Leishmania infantum* in Liver in Infected Mice

**STÉPHANE BRETAGNE,**¹∗ RÉMY DURAND,**2** MARTINE OLIVI,**3** JEAN-FRANÇOIS GARIN,**4** ANNIE SULAHIAN,**4** DANIÈLE RIVOLLET,**3** MICHEL VIDAUD,**3** AND MICHELE DENIAU**1**

Laboratoire de Parasitologie-Mycologie, Hôpital Henri Mondor-APHP and Université Paris XII, Créteil,¹ and Laboratoire de Parasitologie-Mycologie, Hôpital Bichat-APHP,² Laboratoire de Génétique Moléculaire, Faculté de Pharmacie,³ and Laboratoire de Parasitologie-Mycologie, Hôpital Saint-Louis-APHP and Université Paris VI,⁴ Paris, France.

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The leishmaniases are a group of parasitic diseases of major and growing public health importance (9). Standard therapies include pentavalent antimonials and amphotericin B. These drugs cause secondary side effects, and relapses are frequent. Infections were conducted with 5-week-old BALB/c female mice and the *L. infantum* MON1 (MHOM/FR92/LEM 2385) strain. Comparative studies of three techniques of counting the parasites were performed as part of experimental studies of different drug regimens (antimonial pentavalent compounds versus liposomal amphotericin B). The Guiding Principles for Biomedical Research involving animals, published by the Council for International Organizations of Medical Sciences, were followed for all procedures. Mice were inoculated via the tail vein with 10⁷ *L. infantum* promastigotes in a 0.1-ml volume. The livers of 33 control or treated mice were weighed and used for each titration method.

Imprints from each liver were stained with Giemsa stain, and amastigotes were enumerated against hepatic nuclei at a magnification of ×1,000. At least 100 microscopic fields were examined before an imprint was reported as negative. Each positive result was expressed as the number of amastigotes per 500 hepatic cell nuclei.

Culture microtitration was performed as previously described (2). Briefly, a piece of liver was excised, weighed, and homogenized. Serial fourfold dilutions ranging from 1 to 1/4 10⁻⁶ were distributed into 96-well microtitration plates (Becton Dickinson). After 7 and 15 days at 27°C, the presence or absence of mobile promastigotes was recorded in each well. The final titer was the last dilution for which the well contained at least one parasite.

DNA was extracted from about 200 μg of liver biopsy specimens using the High Pure DNA Extraction kit (Roche, Grenoble, France) according to the manufacturer’s recommendations. Ten microliters of the 50 μl final elution was used for each PCR test, and each test was duplicated.

Two TaqMan systems were developed: the *Leishmania* TaqMan system and the mouse TaqMan system. For the *Leishmania* TaqMan system, the target DNA was the DNA polymerase of *L. infantum* (GenBank accession number AF009147), which method (1). In this system, each sample tested is normalized on the basis of its mouse DNA content, and the result is independent of the quantity of the DNA tested.

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is a single-copy-number gene (4). The Leishmania fluorogenic PCR system consisted of the amplification primers (forward primer, 5'-TGTCGCTTGAGACCAGATG-3'; reverse primer, 5'-GCATCGCAGGTGTGAGCAC-3') designed to amplify a 90-bp fragment and the fluorogenic probe (5'FAM-CAGCAACAAGGACCTGGCACC-3'TAMRA).

For the mouse TaqMan system, the target was the mouse brain-derived neutrophic factor (BDNF) gene (GenBank accession number NM007540), a single-copy-number housekeeping gene (10). The amplification primers (5'-TTGGATGCCGAACATGTC-3' [forward] and 5'-CTGCGGCTGACCACTC-3' [reverse]) were designed to amplify a 196-bp fragment. The fluorogenic probe sequence was 5'FAM-TCACACAGCTCAGCTCCACGG3'TAMRA.

Each amplification was performed in duplicate, in a 50-μl reaction mixture using the components of the TaqMan PCR Core Reagents Kit (Perkin-Elmer, les Ulis, France). The reaction mixture included: 1× PCR TaqMan buffer; 3 mM MgCl₂; 0.2 mM each dATP, dGTP, and dCTP; 0.4 mM dUTP; 20 pmol each of either L. infantum primers or mouse BDNF primers (Perkin-Elmer, les Ulis, France); 0.5 U of uracyl-N-glycosylase (Perkin-Elmer, les Ulis, France), 1.25 U of AmpliTaq Gold (Perkin-Elmer, Roissy, France), and 10 μl of eluted sample. The samples were initially incubated for 2 min at 50°C.
for optimum uracel-N-glycosylase activity. This reaction was followed by a 10-min incubation at 95°C to denature the DNA and to activate the AmpliTaq Gold. The temperature cycling (50 cycles at 95°C for 15 s and 65°C for 1 min each) was performed in a 96-well thermal cycler (Perkin-Elmer Applied Biosystems) in the same run for both the *L. infantum* and the mouse gene amplifications. Each amplification run contained several negative controls (buffer and primers alone). Amplification data collected by the 7700 Sequence Detector and stored in the MacIntosh computer were then analyzed by use of the Sequence Detection System software developed by Perkin-Elmer Applied Biosystems. The threshold of detection was set at 10 times the standard deviation above the mean baseline fluorescence calculated from cycles 1 to 15. The fractional cycle number reflecting a positive PCR result is called the cycle threshold (*Ct*). Both PCR tests were performed on liver biopsy specimens by individuals blinded to the results of the other titration techniques.

In initial experiments, we determined the dynamic range of the real-time quantitative *Leishmania* TaqMan PCR test by making serial dilutions of *L. infantum* DNA in water, consisting of the DNA equivalent from $5 \times 10^6$ to 5 cells. The dilutions were subjected to analysis by the *Leishmania* TaqMan system (Fig. 1). The efficiency of the amplification was close to 1. The intra-assay coefficient of variation was below 1% for the high-concentration DNA and 1.6% for the low-concentration DNA. Reproducibility was estimated by testing the 10-fold dilution 10 times in independent runs. The interassay coefficient of variation was 6.4, 12.3, 13.8, and 36% for $10^3$, $10^2$, 10, and 1 parasite, respectively. Similar results were obtained with the mouse BDNF TaqMan system (data not shown).

The *L. infantum* DNA copies were quantitated using the \( \Delta \Delta Ct \) method, which has been described in detail elsewhere (1). Briefly, as the precise amount of genomic DNA added to each reaction (based on optical density) is difficult to assess, the *L. infantum* DNA copies were normalized on the basis of their mouse gene copy content. The *L. infantum* DNA copies were also normalized to a calibrator, or 1× sample, consisting of the sample among our tested series which contained the fewest *L. infantum* DNA copies. Final results, expressed as fold differences in *L. infantum* gene copies relative to the mouse gene copies and the calibrator, termed \( N_{L. infantum} \), were calculated by the equation \( N_{L. infantum} = 2^{\Delta \Delta Ct} = 2^{(\Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}})} \), where \( \Delta Ct \) of the sample and the calibrator is the difference, in threshold cycle number, between the average of the duplicate \( Ct \) value of the *L. infantum* gene and the average of the duplicate \( Ct \) value of the mouse gene. Although the absolute number of *L. infantum* gene copies in the calibrator is not known, this method allows one to ascertain that a sample with an \( N_{L. infantum} \) of \( x \) has \( x \)-fold more DNA copies than the calibrator. Since we have initially checked that the efficiencies of the *L. infantum* and mouse gene amplifications were approximately equal and close to 1, and since, in testing 1/10-diluted DNA liver samples, the relative quantification was similar, the comparative \( \Delta \Delta Ct \) method was valid for our PCR assays.

The range of enumeration of amastigotes against hepatic nuclei was 0 to 500 parasites per 500 hepatic cells. A high rate (11 of 33; 33%) of negative results was observed. With the other two methods, negative results were not observed in infected mice, and no organ with positive imprints had a negative culture or a negative PCR result. The TaqMan PCR assay results showed a normalized *Leishmania* gene copy number between 1 and 1,968. The correlation between the TaqMan PCR assay and the microtitration was calculated with the data expressed as log10 units to assume a normal distribution of the results. The correlation coefficient was 0.66 with a *P* of <0.01 (Fig. 2). Using the nonparametric Spearman test, the correlation coefficient was 0.52 with a *P* of <0.01.

To search for any unequal distribution of the parasites in the liver which could explain discrepancies between the techniques, five different liver biopsy specimens from two mice were tested using the TaqMan PCR test. The different liver
biopsy specimens gave similar results, showing that the parasites were equally distributed in the liver.

The present work is the first development of TaqMan probes for L. infantum. Instead of quantifying the copy number with a standard curve, we chose a relative quantification according to the liver biopsy. In addition, we determined that liver infestation with Leishmania microorganisms is homogenous. Therefore, the biopsy can be performed anywhere in the liver, and very precise weighing is not necessary in using the double real-time PCR system.

Among the three quantitative techniques tested, the imprints had an extremely high rate of negative results whereas the other techniques gave positive results. Previous studies have shown that the liver imprints were always negative for titers of $\approx 10^4$ parasites per g (2). Therefore, the imprint technique cannot be used alone in a mouse model.

The correlation coefficient between TaqMan and microtitration was 0.66 (Fig. 2). One could have expected a better figure. However, the techniques could be complementary rather than redundant. Indeed, PCR is unable to distinguish between dead and live parasites. The DNA can come from the liver but also from circulating DNA originating in other cells or organs. The addition of a quantitative PCR test targeted at the mRNA of a housekeeping gene specific to Leishmania should discriminate between live and dead parasites. In contrast to the present TaqMan test, microtitration culture tests only the capability of live amastigotes to transform in vitro into mobile promastigotes. The microtitration technique gives functional information which cannot be directly linked to the number of parasites in the initial tissue. This reasoning may explain some of the aberrant points observed in comparing the microtitration culture method and the real-time quantitative tests. Keeping these limitations in mind, the double PCR TaqMan test developed in this study can give reliable results with a low workload compared with in vitro cultivation to assess the leishmanicidal effect of a given drug in mice.

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