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

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The parasitic loads of mouse livers experimentally infected with *Leishmania infantum* were determined using a double real-time quantitative PCR test targeted to the parasite DNA polymerase gene and to the mouse brain-derived neutrophic factor gene. The *Leishmania* DNA copy number was normalized to the number of mouse gene copies in order to quantify the former independently of liver weight. The correlation coefficient with the microtitration method was 0.66. This PCR assay can be considered for experimental pharmaceutical studies.

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. Therefore, other antileishmanial compounds (11) or new formulations of existing ones (14) are needed.

Mouse inoculation is the most used in vivo model of visceral leishmaniasis for the evaluation of anti-*Leishmania* drugs (5–7, 16). Assessment of parasitic burdens is usually based on microscopic enumerations of amastigotes against host cell nuclei on liver imprints (15). This type of assay is time-consuming and subjective and is not reliable when the parasites are not equally dispersed on the slides. More recently, culture microtitrations have been developed (2, 17). These techniques are more sensitive than the imprint method, but the assays remain labor-intensive.

Since recurrences of leishmaniasis are associated with tissue loads of residual, latent parasites after treatment, nonquantitative PCR tests (3, 12, 13) are of little value in indicating a positive or negative result. A recent approach for quantitation of DNA copy number is based on the 5′ nuclease activity of *Taq* polymerase for fragmentation of a dual-labeled fluorogenic hybridization probe (8). A real-time quantitative TaqMan PCR assay for measuring the copy numbers of *Leishmania infantum* DNA in mouse liver was developed. A first possibility was to use absolute quantitation. This requires the design of standards known by independent means. Several critical points must be considered, such as the reliability of the serial dilutions of the parasites, the accuracy of pipetting, and the stability of the diluted standards. For the present purpose, i.e., to quantify *L. infantum* in mouse tissues, very precise weighing of liver biopsy specimens is also necessary. Another possibility was to use relative quantification using the $\Delta\Delta C_t$ 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.

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 10^7 *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 (Boehringer-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

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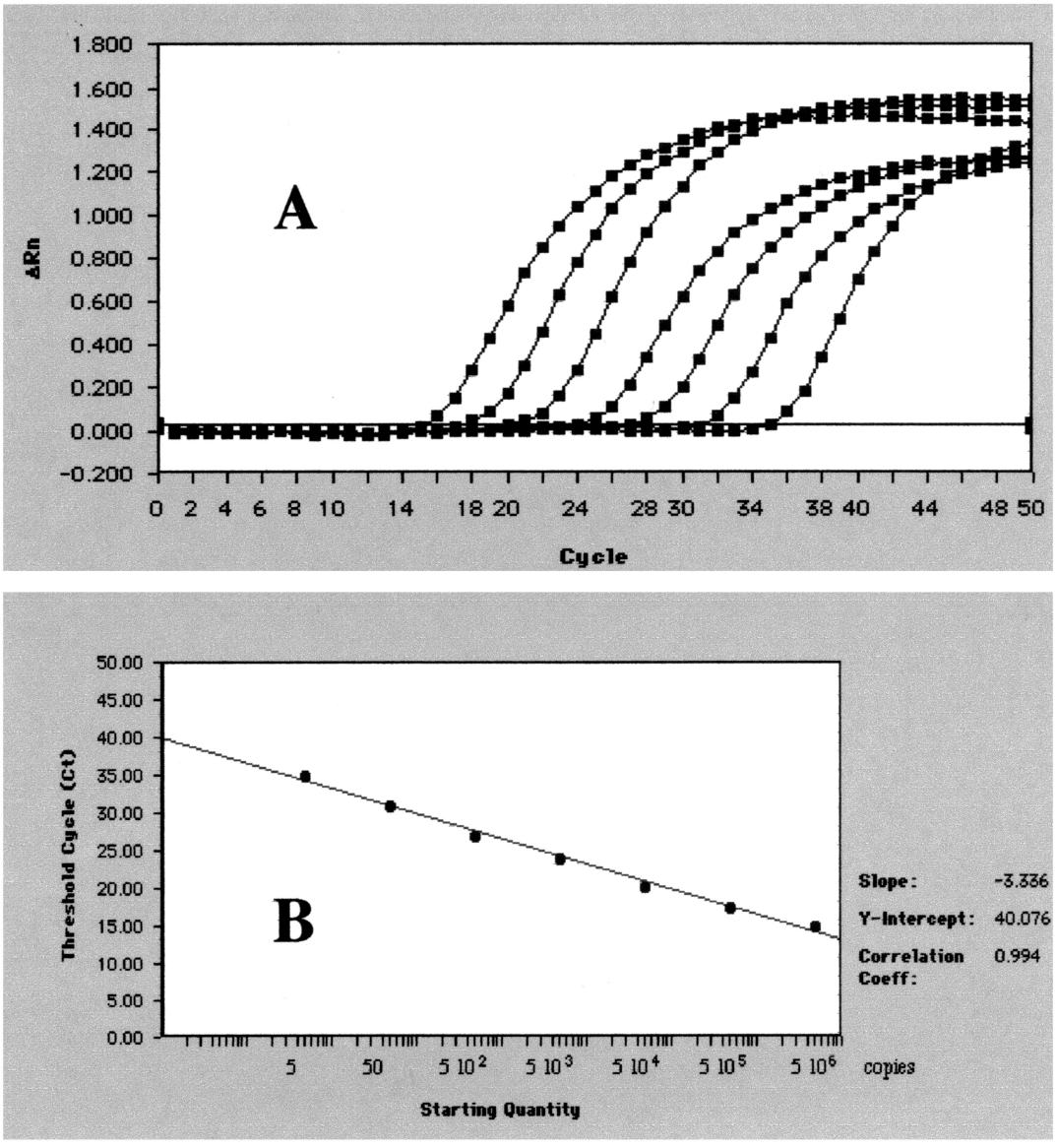

FIG. 1. Amplification plots and a standard curve obtained with the TaqMan *Leishmania infantum* PCR test. (A) Serial 10-fold dilution of *L. infantum* DNA from 1 to 10^6 ng per reaction (5 to 5×10^6 copies/reaction); the amplification curves shift to the right as the input target quantity is reduced, since reactions with fewer target molecules require more amplification cycles to produce a detectable quantity of reporter molecules than do reactions with more target molecules. (B) Standard curve obtained by plotting the Ct against the input target quantity, with the latter plotted on a common log scale. Ct represents the fractional cycle number reflecting a positive PCR result differentiated from the background noise.

is a single-copy-number gene (4). The *Leishmania* fluorogenic PCR system consisted of the amplification primers (forward primer, 5′-TGTCGCTTGCAGACCAGATG-3′; reverse primer, 5′-GCATCGCAGGTGTGAGCAC-3′) designed to amplify a 90-bp fragment and the fluorogenic probe (5′FAM-CAGCAA CAACTTCGAGCCTGGCACC-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′-TTGGATGCCG CAAACATGTC-3′ [forward] and 5′-CTGCCGCTGTGACC CACTC-3′ [reverse]) were designed to amplify a 196-bp fragment. The fluorogenic probe sequence was 5′FAM-TCAC ACACGCTCAGCTCCCCACGG-3′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 $MgCl_2$; 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

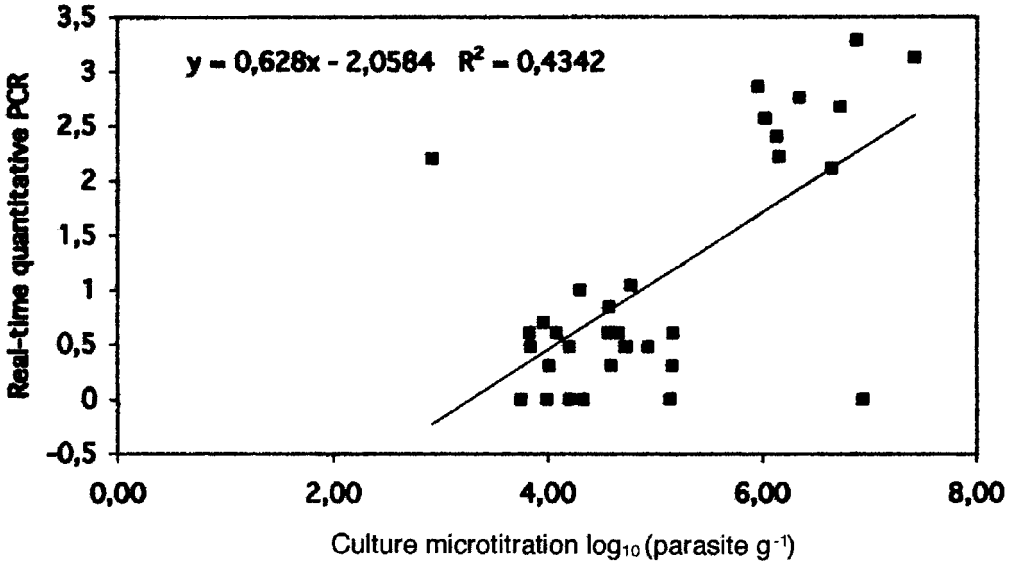

FIG. 2. Correlation between log-transformed individual values of liver parasite burden determined by in vitro micotitration and quantitative PCR test.

for optimum uracyl-*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 (C_t). 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 5×10^6 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 $\Delta\Delta C_t$ 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 $\text{N } L.\ infantum = 2^{\Delta\Delta C_t} = 2^{(\Delta C_t \text{ sample} - \Delta C_t \text{ calibrator})}$, where ΔC_t of the sample and the calibrator is the difference, in threshold cycle number, between the average of the duplicate C_t value of the *L. infantum* gene and the average of the duplicate C_t 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 $\Delta\Delta C_t$ 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 $\log_{10}$ 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 $\leq 10^4$ 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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REFERENCES

1. **Bièche, I., B. Parfait, V. Le Doussal, M. Olivi, M.-C. Rio, R. Lidereau, and M. Vidaud.** 2001. Identification of CGA as a novel estrogen receptor-responsive gene in breast cancer: an outstanding candidate marker to predict the response to endocrine therapy. Cancer Res. **61:**1652–1658.
2. **Buffet, P. A., A. Sulahian, Y. J. Garin, N. Nassar, and F. Derouin.** 1995. Culture microtitration: a sensitive method for quantifying *Leishmania infantum* in tissues of infected mice. Antimicrob. Agents Chemother. **39:**2167–2168.
3. **Costa, J.-M., R. Durand, M. Deniau, D. Rivollet, M. Izri, R. Houin, M. Vidaud, and S. Bretagne.** 1996. PCR enzyme-linked immunosorbent assay for diagnosis of leishmaniasis in human immunodeficiency virus-infected patients. J. Clin. Microbiol. **34:**1831–1833.
4. **Croan, D. G., D. A. Morrison, and J. T. Ellis.** 1997. Evolution of the genus *Leishmania* revealed by comparison of DNA and RNA polymerase gene sequences. Mol. Biochem. Parasitol. **89:**149–159.
5. **Durand, R., M. Paul, D. Rivollet, H. Fessi, R. Houin, A. Astier, and M. Deniau.** 1997. Activity of pentamidine-loaded poly(D,L-lactide) nanoparticles against *Leishmania infantum* in a murine model. Parasite **4:**331–336.
6. **Durand, R., M. Paul, D. Rivollet, R. Houin, A. Astier, and M. Deniau.** 1997. Activity of pentamidine-loaded methacrylate nanoparticles against *Leishmania infantum* in a mouse model. Int. J. Parasitol. **27:**1361–1367.
7. **Gangneux, J. P., A. Sulahian, Y. J. Garin, and F. Derouin.** 1997. Efficacy of aminosidine administered alone or in combination with meglumine antimoniate for the treatment of experimental visceral leishmaniasis caused by *Leishmania infantum*. J. Antimicrob. Chemother. **40:**287–289.
8. **Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams.** 1996. Real-time quantitative PCR. Genome Res. **6:**986–994.
9. **Herwaldt, B. L.** 1999. Leishmaniasis. Lancet **354:**1191–1199.
10. **Hofer, M., S. R. Pagliusi, A. Hohn, J. Leibrock, and Y. A. Barde.** 1990. Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. EMBO J. **9:**2459–2464.
11. **Jha, T. K., S. Sundar, C. P. Thakur, P. Bachmann, J. Karbwang, C. Fischer, A. Voss, and J. Berman.** 1999. Miltefosine, an oral agent, for the treatment of Indian visceral leishmaniasis. N. Engl. J. Med. **341:**1795–1800.
12. **Lachaud, L., J. Dereure, E. Chabbert, J. Reynes, J.-M. Mauboussin, E. Oziol, J.-P. Dedet, and P. Bastien.** 2000. Optimized PCR using patient blood samples for diagnosis and follow-up of visceral leishmaniasis, with special reference to AIDS patients. J. Clin. Microbiol. **38:**236–240.
13. **Osman, O. F., L. Oskam, N. C. M. Kroon, G. J. Schoone, E. T. Khalil, A. M. El-Hassan, E. E. Zijlstra, and P. A. Kager.** 1998. Use of PCR for diagnosis of post-kala-azar dermal leishmaniasis. J. Clin. Microbiol. **36:**1621–1624.
14. **Paul, M., R. Durand, H. Fessi, D. Rivollet, R. Houin, A. Astier, and M. Deniau.** 1997. Activity of a new liposomal formulation of amphotericin B against two strains of *Leishmania infantum* in a murine model. Antimicrob. Agents Chemother. **41:**1731–1734.
15. **Stauber, L. A., E. M. Franchino, and J. Grun.** 1958. An eight-day method for screening compounds against *Leishmania donovani* in golden hamster. J. Protozool. **5:**269–273.
16. **Sulahian, A., Y. J. Garin, F. Pratlong, J.-P. Dedet, and F. Derouin.** 1997. Experimental pathogenicity of viscerotropic and dermotropic isolates of *Leishmania infantum* from immunocompromised and immunocompetent patients in a murine model. FEMS Immunol. Med. Microbiol. **17:**131–138.
17. **Titus, R. G., M. Marchand, T. Boon, and J. A. Louis.** 1985. A limiting dilution assay for quantifying *Leishmania major* in tissues of infected mice. Parasite Immunol. **7:**545–555.