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Culture Microtitration: a Sensitive Method for Quantifying *Leishmania infantum* in Tissues of Infected Mice

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We developed a microtitration method to determine the parasite burdens in homogenized organs of mice infected with *Leishmania infantum*. This method proved more sensitive than direct enumeration of amastigotes in stained organs, was appropriate for describing the kinetics of infection, and can be considered for physiopathological or pharmaceutical experimental studies.

Rodent models of visceral leishmaniasis have been used for experimental purposes for at least 6 decades (3). Assessment of parasite burdens is usually based on microscopic enumeration of amastigotes against host cell nuclei on tissue imprints (4). However, when parasites are confined to a few infected macrophages, optical quantification is not considered reliable (2). Thus, we adapted the sensitive microtitration method of Titus et al. (5) to develop a culture-based micromethod for quantification of parasite burdens in tissues of mice infected with *Leishmania infantum*, the species responsible for most Leishmania infections in AIDS patients (1).

Adult female BALB/c mice (Iffa Credo, Lyon, France) were infected by intraperitoneal injection of 10^8 promastigotes of *L. infantum* MHOM/FR/91/LEM2259 isolated from an AIDS patient. Groups of mice were killed at intervals after infection for determination of parasite burdens. The spleen and a piece of the left lobe of the liver were excised, weighed, and then homogenized with a tissue grinder (Ultra Turrax, Staunfen, Germany) in 4 ml of Schneider's drosophila medium (Gibco BRL, Eragny, France) supplemented with 20% heat-inactivated fetal calf serum (Gibco BRL), penicillin (100 U/ml), and streptomycin (50 mg/ml) (bioMérieux, Marcy-l'Etoile, France) (SDM/FCS). Under sterile conditions, serial fourfold dilutions ranging from 1 to $1/4 \times 10^{-6}$) were prepared in wells of 96-well microtitration plates (Becton Dickinson) containing 225 μl of culture medium.

After 7 and 15 days of incubation at 26 to 28°C, plates were examined with an inverted microscope at a magnification of ×100 or ×200. 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. The number of parasites per gram (parasite burden) in the corresponding organ was calculated as follows: parasite burden = (geometric mean of reciprocal titers from each duplicate/weight of homogenized cross section) × 400, where 400 is the reciprocal fraction of the homogenized organ inoculated into the first well.

Smears or imprints prepared from liver and spleen tissues were stained with Giemsa (RAL, Paris, France), and then 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 $\log_{10}$ number of amastigotes per 500 hepatic cell nuclei.

To estimate the correlation between the two titration methods, both methods were used to determine the parasite burdens in the livers of 13 mice infected for 63 days, among which 9 had been treated with dexamethasone at 6.65 mg/kg/day intraperitoneally from day 20 to day 42 after inoculation to increase the degree of infection.

In a second experiment, the kinetics of *L. infantum* infection were examined by culture microtitration: liver, spleen, and lung parasite burdens were determined in 20 mice sacrificed in groups of 4 on days 3, 8, 20, 30, and 60 postinfection.

By the culture microtitration method, exponential parasite growth was observed for the first positive dilutions but was unconstant for the last positive dilutions, even after 15 to 20 days of incubation. Occasionally, either a few or even only one promastigote cluster was seen. Hence, careful examination of the whole well was performed after 7 and 15 days of incubation before it was reported as negative. Motility of such clusters allowed easy identification of viable parasites. No difference between investigators was observed when the same plates were read in parallel. The reproducibility of culture microtitration was assessed with five mice. Three titrations, each one performed in duplicate with material from the same organ homogenate, yielded coefficients of variation of 8 and 4% for the liver and spleen, respectively.

A good correlation was found between the two methods of quantification of parasite burdens, with a coefficient of correlation of 0.869 (Fig. 1). However, culture microtitration proved to be more sensitive as the line correlating parasite counts determined by staining organs (x) with parasite counts determined by culture (y) had a y intercept of >0, which means that parasites can be detected by culture when they cannot be seen on stained preparations. The threshold of detection of parasites on stained organs was empirically determined by comparing the results obtained from liver and spleen imprints to the titer obtained by culture microtitration (Table 1). Liver imprints were always negative for titers of $\leq 5 \times 10^4$ parasites per g and always positive for titers of $\geq 5 \times 10^5$ parasites per g. Spleen imprints were always negative for titers of $\leq 10^5$ parasites per g and almost always positive for titers of $\geq 10^6$ parasites per g. No organ with positive imprints had a negative culture. Comparatively, the threshold of detection by culture microtitration was found to be as low as 10^3 parasites per g and we conclude that culture microtitration allows quantification of parasite burdens at least 50- to 100-fold lower than the minimal parasite burdens quantifiable on stained organs.

With the microtitration culture method, the kinetics of in-

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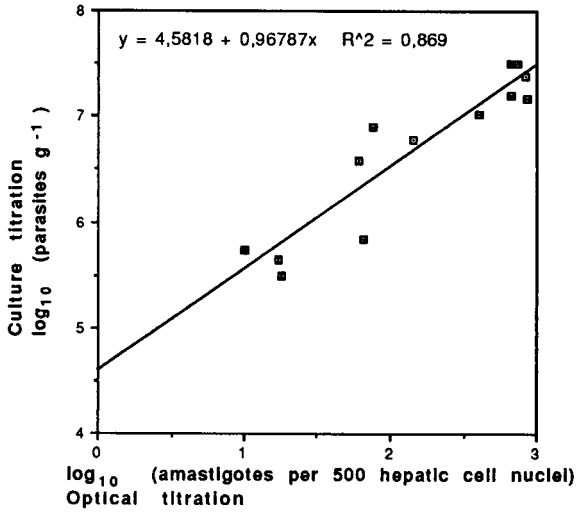

FIG. 1. Correlation between log-transformed individual values of liver parasite burdens determined either by culturing serial dilutions of tissue homogenates or by enumerating amastigotes on tissue imprints.

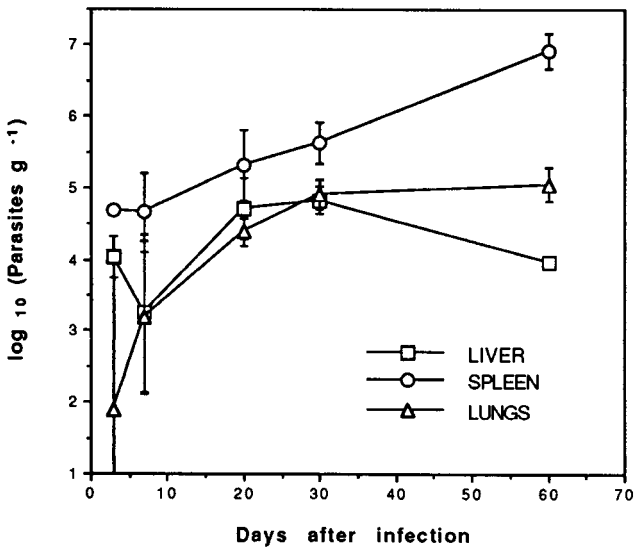

FIG. 2. Kinetics of liver, spleen, and lung parasite burdens of mice infected on day 0 with 10^8 promastigotes of *L. infantum*. Each point represents the mean ± the standard error of the mean for four mice.

fection could be determined in the livers, spleens, and lungs of infected mice. The results from one representative experiment are presented in Fig. 2. Parasite burdens in livers and spleens decreased from day 3 to day 8 and then increased from day 8 postinfection to day 20, when parasites became detectable on tissue imprints. At days 30 and 60, liver parasite burdens had decreased to 10^5 and 10^4 parasites per g, respectively, and were again optically undetectable at day 60. In spleens, parasite burdens increased and achieved levels of $\geq 5 \times 10^6$ parasites per g at day 60. In lungs, the kinetics of parasite burdens were grossly similar to those of liver parasite burdens, except that a plateau phase was observed between days 30 and 60.

We conclude that culture microtitration of *L. infantum* in homogenized organs of infected mice seems reliable for determining the kinetics of infection in various organs. Because of its high sensitivity, this method may be particularly well suited for experimental situations in which parasites cannot be visualized in stained organs: e.g., kinetics of relapses after therapeutic intervention or low-level parasite persistence in mice.

TABLE 1. Parasite detection on tissue imprints compared to parasite burden levels determined by culture microtitration

Tissue tested and degree of infection (no. of parasites/g of tissue)	No. of mice tested	Mean no. of parasites/g of culture (range) determined by titration[a]	No. of mice positive by imprint method
Liver			
$<5 \times 10^4$	9	8.9×10^3 (0–2.5×10^4)	0
5×10^4–5×10^5	7	1.5×10^5 (5.1×10^4–3.6×10^5)	3
$>5 \times 10^5$	9	1.4×10^7 (2.3×10^6–3.3×10^7)	9
Spleen			
$<10^5$	6	2.7×10^4 (0–8.7×10^4)	0
10^5–10^6	10	4.5×10^5 (1.5×10^5–8.1×10^5)	2
$>10^6$	9	3.5×10^6 (1.2×10^6–1.1×10^7)	8

[a] Means and ranges of 25 randomly selected mice used in two experiments are shown.

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