Parasitology International xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

An experimental challenge model of visceral leishmaniasis by *Leishmania donovani* promastigotes in mice

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ARTICLE INFO

Article history:
Received 27 December 2015
Received in revised form 24 February 2016
Accepted 13 March 2016
Available online xxxx

Keywords:
Leishmania donovani
Leishman-Donovan units
Mouse model
Parasite burden
Promastigotes
Real-time PCR
Stabilates

ABSTRACT

Although visceral leishmaniasis is a fatal disease in humans and dogs, the use of mouse models is important for obtaining a better understanding of the pathogenesis, immunity, and host–parasite interactions of this disease. Such models are also useful for the evaluation of vaccines and chemotherapies for treatment of visceral leishmaniasis. Here, we present our method of experimental inoculation of mice with *Leishmania donovani* promastigotes. Nutrient-enriched undefined media may be beneficial for laboratory maintenance of promastigotes for maintaining their virulence or infectivity in mice. With this method, we could preserve the infectivity of promastigote lines recovered from inoculated animals and use these lines for further *in vivo* experiments. Furthermore, the use of cryopreserved stabilates is highly recommended for the reproducibility of experiments. To assess a newly developed method for determination of parasite burden in infected animal tissues, initial comparison of parasite burden in the liver obtained in the classic Leishman-Donovan units (LDU) with values obtained from the new method is recommended. As an example, the association between parasite burden determined by LDU and real-time PCR assay targeting the leishmanial *gp63* gene in the liver of mice is presented.

1. Background

Mouse models for visceral leishmaniasis, produced by inoculation with *Leishmania donovani* or *Leishmania infantum* (synonym: *Leishmania chagasi*), have been extensively utilized for the study of the immunopathology [1] and genetic regulation [2], as well as for the development of vaccines [3] and immunochemotherapies [4] for this neglected tropical disease. However, the implication of using data obtained from different mouse models should be considered due to the variety of factors of such models, including mouse strain, age and immunity of the mice, stage of parasite, number of inoculated parasites, and route of inoculation. Organ-specific immunity has been described in various experimental mouse models [5]. The liver is often the site of acute but resolving infection, while the spleen can become a site of parasitic persistence with associated immunopathological changes. However, natural or artificially immunodeficient mice, including cytokine gene knockout mice, have demonstrated the persistence of parasites in the liver [6,7]. Thus, the classical method of determining the parasite load as Leishman-Donovani units (LDU) [8] in the liver remains valuable and worthwhile.

Selection of the culture media for growing and maintaining *Leishmania* promastigotes depends on the research objective. The major problem in *in vitro* promastigote culturing is the possible loss of virulence or infectivity of the parasite in laboratory animals [9]. Although the underlying mechanism of the loss of *in vivo* infectivity of cultured promastigotes remains unknown, nutrient-enriched undefined blood-agar based media remains commonly used for the isolation and maintenance of parasites [10].

2. Objectives

The present study aimed to establish an experimental challenge model of mouse visceral leishmaniasis using a mouse-passaged line of *L. donovani* promastigotes. Procedures for the maintenance of the parasites, preparation of the cryopreserved stabilates, and molecular determination of parasite burden in the organs of the mice are described.

3. Methods

3.1. Culture medium

Undefined blood-agar-based media are suggested to be more suitable for the maintenance of parasite infectivity in experimental animals than defined media [9,10] (Fig. 1A). The NNN medium is suitable for primary isolation of any *Leishmania* species, [10], but it is a biphasic medium and contains particulate matter. Semi-defined liquid media, however, such as modified Mansour's liquid medium [9,11] and MEM:

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http://dx.doi.org/10.1016/j.parint.2016.03.008
1383-5769/

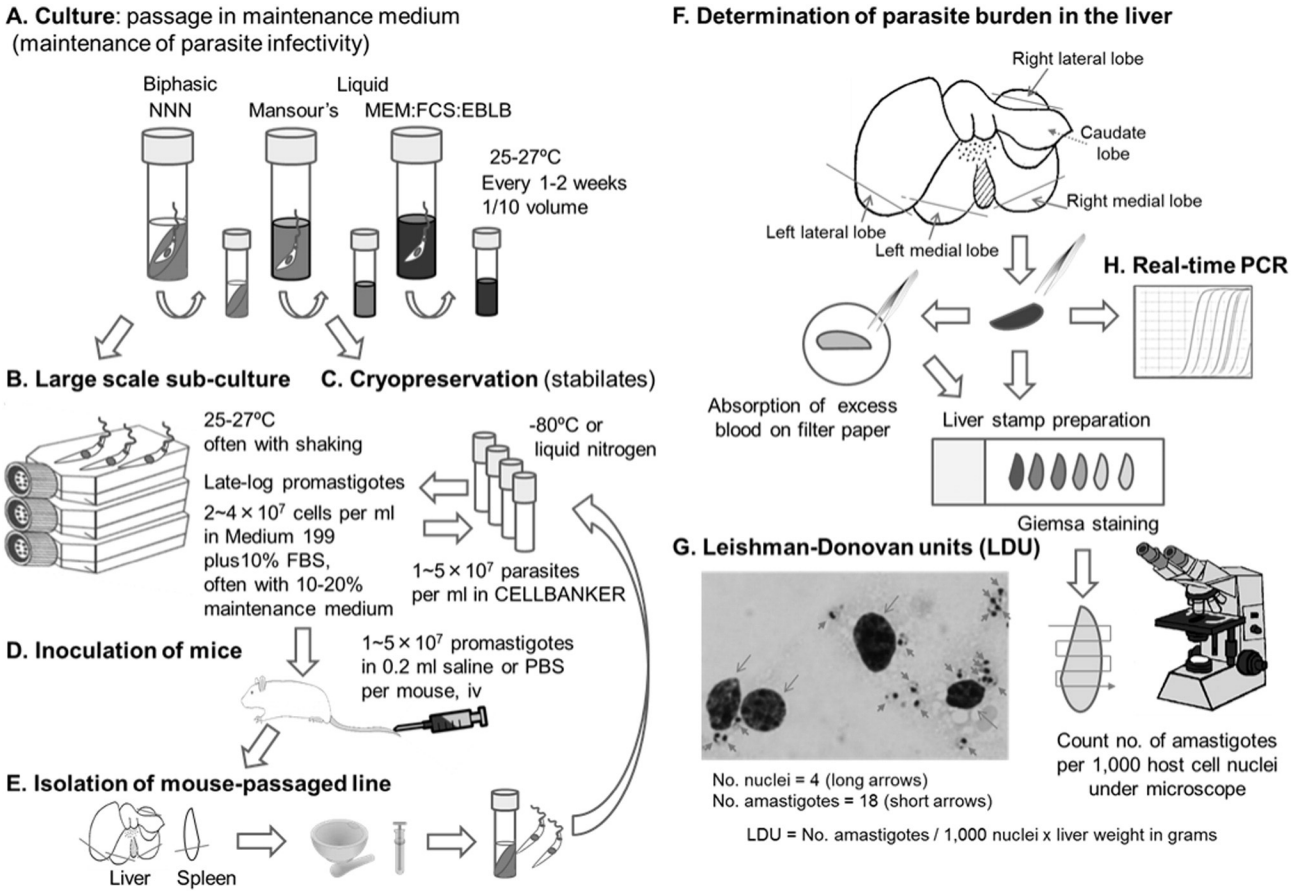

Fig. 1. Procedures of experimental challenge of mice with *L. donovani* promastigotes.

FCS:EBLB medium [10] are free from precipitates and convenient for large-scale culturing of promastigotes.

3.1.1. Novy-MacNeal-Nicolle (NNN) medium (biphasic)

Agar	1 g
NaCl	0.9 g
Distilled water	100 ml

Sterile by autoclaving (121 °C, 15 min) and dispense in 3.75 ml aliquots to screw capped tubes. When it is cooled to about 45 °C, add aseptically 1.25 ml defibrinated whole rabbit blood to each tube and allow solidification of the blood agar medium in a slanted position.

Defibrinated rabbit blood can be frozen in aliquots prior to use. For the liquid phase of NNN medium, usually no additional liquid phase being added for primary isolation of parasite. However, for maintenance of parasites, saline is added to the blood agar, which becomes a reddish color due to the elution of hemoglobin. Growing promastigotes are found predominantly in the upper layer of the liquid. Parasites may survive for 3–4 weeks in the NNN medium at 25 °C. For large scale subcultivation of promastigotes, supplementation of the liquid phase with subculture media is useful for parasite growth (Fig. 1B). Medium 199, supplemented with 25 mM HEPES, 50 μg/ml gentamycin, and 10–15% heat-inactivated fetal bovine serum, can be used for short-term large-scale subcultivation of promastigotes for inoculation of mice, as well as for regular passaging of the parasites.

3.1.2. Modified Mansour's liquid medium

Bacto-beef	50 g
Neopeptone	20 g
NaCl	8 g
Glucose	2.5 g
$CaCl_2$	0.2 g
KCl	0.2 g
KH_2PO_4	0.3 g
Distilled water	1000 ml

Adjust pH to 7.2–7.4 with 1 N NaOH and add defibrinated rabbit blood lysate (or lysate supernatant) at a final concentration of 5–10%.

Infuse bacto-beef with about 1000 ml of distilled water for 1 h at 56 °C, heat in a water bath to 80 °C for 5 min, and filtrate. Add neopeptone, NaCl, glucose, calcium chloride, potassium chloride, and monopotassium phosphate to the resultant filtrate. Adjust pH to 7.2–7.4 with 1 N sodium hydroxide. Adjust the solution to 1 l with distilled water, heat to boiling, left to cool, and then filtrate. Autoclave the filtrate and store at 4 °C until use. Lyse defibrinated rabbit blood by three freeze–thaw cycles. For cultivation of promastigotes, the medium is supplemented with lysed defibrinated rabbit blood at a final concentration of 5–10%, and 50 μg gentamycin (or 100 U penicillin and 100 μg streptomycin) per ml. We have modified this medium using the blood lysate supernatant clarified by centrifugation at 28,000 × *g* for 30 min at 4 °C to remove cellular debris. Promastigotes may be incubated at 25 °C and passaged at intervals of one to two weeks (Fig. 1A).

Please cite this article as: K. Katakura, An experimental challenge model of visceral leishmaniasis by *Leishmania donovani* promastigotes in mice, Parasitology International (2015), http://dx.doi.org/10.1016/j.parint.2016.03.008