International Journal of Medical Laboratory 2019;6(1):124-130.
International Journal of Medical Laboratory
Shahid Sadoughi University of Medical Sciences

Original Article

LB Broth-lyophilized Rabbit Anti-sheep Cell Haemolysin as a Simple Culture Medium for Cultivation of *Leishmania Major* Promastigotes

Vahid Nasiri[1*] Ph.D., Farnoosh Jameie[1]Ph.D, Habibollah Paykari[1] Ph.D.

[1]Protozoology Laboratory, Parasitology Department, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Alborz, Iran.

Article history
Received 3 Jun 2018
Accepted 14 Nov 2018
Available online 31 May 2019

Key words
Culture medium
Haemolysin
Leishmania major
Promastigote

A B S T R A C T

Background and Aims: The protozoan parasites of the genus *Leishmania* are the causative agents of various clinical diseases. Different methods of cultivation of *Leishmania* parasites are available. In the present study, the efficacy of the LB broth with rabbit lyophilized anti-sheep red blood cell haemolysin was evaluated in the cultivation of promastigotes of *Leishmania major*.

Materials and Methods: Conventional LB broth medium was prepared and autoclaved for 15 min at 121°C and then lyophilized rabbit anti-sheep cell haemolysin was added at 1-10% final concentrations. The efficacy of the medium was evaluated by assessing the growth ability and replication patterns of the promastigotes of *Leishmania major*.

Results: Medium with 1-10% lyophilized rabbit haemolysin supported the growth of the parasites and can be used for cultivation of *Leishmania* parasites with acceptable *In vivo* infectivity for research purpose.

Conclusions: The ability of the parasites to survive and proliferate in the presence of lyophilized rabbit haemolysin indicates that this material is a good nutritional source. This study opens a new way to make low-cost medium that can be used in cultivation of *Leishmania* parasites.

[*]**Corresponding Author:** Protozoology Laboratory, Parasitology Department, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Alborz, Iran.
Tel: +989127688144**, Email:** v.nasiri@rvsri.ac.ir

Introduction

Leishmaniasis is an ancient disease and indications as to craving facial infections can be found on ceramic containers excavated in Central America dating as far back as 1,000 years and on pictures of the Spanish conquerors in the 16th century [1]. Cutaneous leishmaniasis has been known since ancient times and it is truly a neglected disease. Bray referred to a tablet in the library of King Ashurbanipal of Assyria in Neinava (Mosul, Iraq), with the description of a painless ulcer most likely cutaneous leishmaniasis, translated from a tablet of the old Akkadian period in the second or third millennium BC [2].

The cell culture technique is an approach to make efforts to prepare complex habitat conditions of living organisms to developing our knowledge about their behavior and finding suitable ways (like effective vaccine) in order to prevent their negative side effects. On the other hand, routine commercial culture media for cultivation of *Leishmania* such as Roswell Park Memorial Institute (RPMI)-1640, medium 199, and Schneider's *Drosophila*, enriched by fetal calf serum (FCS) or blood lysate [3, 4], are many expensive. FCS is highly expensive, and its reliable supply is very difficult to obtain especially in the developing countries [5].

Many attempts have been made to replace FCS in *Leishmania* culture media with different kinds of sera, bovine serum albumin, a mixture of purine bases, vitamins, large concentrations of certain amino acids, hormones, hemin, hemoglobulin, human and animal urine and, more recently chicken serum [6-15] the later of which introduced an alternative low-cost serum that can be used in culture media for primary isolation, routine cultivation and mass cultivation of *Leishmania* parasites [6, 7].

Some investigations indicate that the Iberian hare (*Lepus granatensis*) has been described as a new competent reservoir of the visceral leishmaniasis and its ability to transmit the parasite to sandflies was demonstrated by xenodiagnostic studies which confirmed that Iberian hares can contribute to the outbreak of disease [16-18]. A study found theprevalence of *L.infantum* infection to be 43.6% from principally two hare species (*L.granatensis* and *L.europaeus*) [19] and in another a seroprevalence of 46% was also reported in rabbits [16].

In regard with the above result on the susceptibility of rabbit to leishmaniasis and potential of suitability of rabbit serum for cultivation of *Leishmania* parasites, and because rabbit blood is an essential component of conventional NNN medium [20], we describe a formulation for cultivation of promastigotes of *Leishmania major*.

Materials and Methods

Media preparation

For the Luria-Bertani (LB) broth, 1 g peptone, 0.5 g yeast extract and 1 g NaCl were suspended in 100 ml of distilled water and complete medium autoclaved for 15 min at 121°C and were left to cool down and then 5 ml of it was added to lyophilized rabbit anti-sheep cell haemolysin vial (Razi Vaccine and

Serum Research Institute, Islamic Republic of Iran) according to the manufacturer's protocol for reconstitution of it and were all mixed thoroughly. After reconstitution of lyophilized rabbit serum, dilutions of it in LB broth were prepared as follows: 1%, 2.5%, 5% and 10%. RPMI-1640 supplemented with heat-inactivated (56°C for 30 min) fetal calf serum (10%) was used as a standard medium for proliferation pattern comparison. No antibiotic was added to the media; they were kept at 4°C.

Continuous cultivation of the promastigotes

The procedures were performed according to the our previous experiences [21]. The Mid-log phase promastigotes of *Leishmania major* (MRHO/ IR/76/ER) which had been previously grown in RPMI-1640 supplemented with 10% FCS were concentrated by centrifugation at 2500 g for 10 min and washed twice with sterile phosphate-buffered saline solution (PBS) to remove any FCS traces. Parasites were counted using a Neubauer chamber (Haemocytometer) slide and diluted in PBS to a final concentration of 10^8 parasites per milliliter. Subcultures from each dilution of rabbit serum were performed in 3 repeated series and in each one, triplicate cultures prepared, alongside of the positive control (RPMI-10% FCS), and all the experimented media were inoculated with mid-log phase promastigotes at the final concentration of 5×10^6 promastigotes/ml in 25 cm^2 plastic culture flasks. Every flask totally contained 10 ml of parasites and complete media mixture. The flasks were placed in an incubator at 26˚C. The continuous cultures of the parasites were made weekly by sub-culturing into fresh medium until they reached the 10^{th} sub-culture. In all the sub-cultures, parasites proliferation was assessed both qualitatively and quantitatively by microscopic observations and Giemsa slide preparation. The number of parasites was counted daily by using the Neubauer chamber slide.

***In vivo* infectivity determination of cultivated parasites**

Stationary-phase promastigotes of *Leishmania major* (2×10^6 parasites) from the 4^{th} subculture in new formulation medium were washed twice in sterile PBS and were inoculated subcutaneously into the tail base of 10 female BALB/c mice (6-7 weeks) with 10 mice serving as negative (inoculated with PBS) and positive (inoculated with parasites grown in RPMI-10% FCS) control. The animals were evaluated macroscopically every day until the appearance of lesions.

This research was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of Razi Vaccine and Serum Research Institute. All animals experiments, including maintenance, handling and blood collection were approved by Institutional Animal Care and Research Advisory Committee of Razi Vaccine and Serum Research Institute based on the Specific National Ethical Guidelines for Biomedical Research issued by the Research and Technology Deputy of Ministry of Health and Medicinal Education of Iran.

Statistical analysis

SPSS version 22 for windows® was used for data evaluation. The differences between the averages of the quantitative variables were

evaluated by Student's T-test and the value of $p<0.05$ was accepted as statistically significant.

Results

Assessment of the parasite growth quantity and replication pattern

It was observed that the addition of 1-10% lyophilized rabbit serum to the LB broth medium significantly stimulated the growth of the promastigotes of *Leishmania major* their effects of which were comparable to the growth pattern of the parasites grown in the RPMI-1640 -10% FCS ($p<0.05$). Under invert microscopy, it was also observed that the promastigotes were elongate and had rapid motility. Fine grown parasites and typical morphology of the promastigotes were observed in Giemsa-stained smears prepared from culture media of all serum dilutions. Parasites inoculated into the culture media took about 7 days to reach to the late log-phase. It was also observed that the new formulation supported the continuity of the parasites in successive passages. The effect of various concentrations of the lyophilized rabbit anti-sheep cell haemolysin in the culture media on the growth quantity of the promastigotes and a typical growth curve of the parasite growth in all dilutions of lyophilized rabbit serum is shown in figure 1.

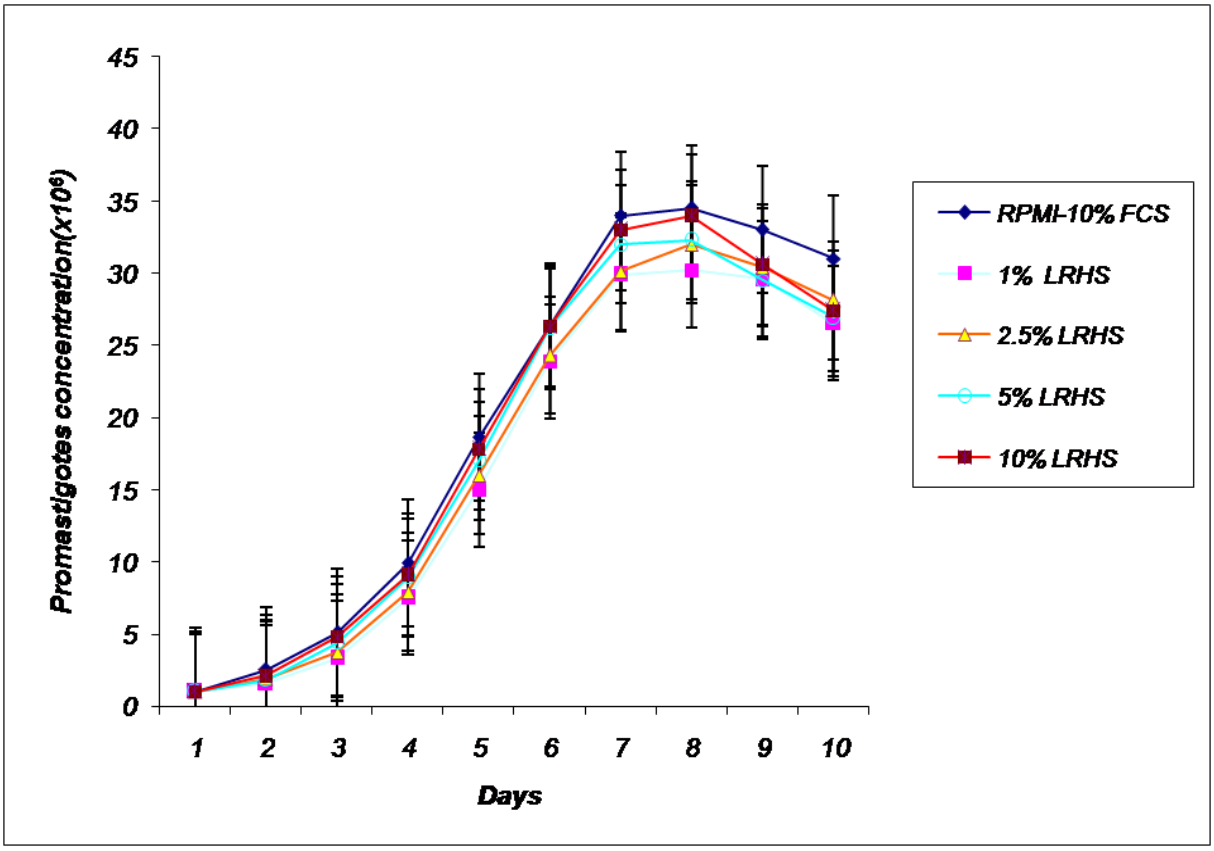

Fig1. Growth curve of promastigotes of *Leishmania major* in RPMI-10% FCS and different concentrations of lyophilized rabbit anti-sheep cell haemolysin.

In vivo assessment of pathogenicity of cultivated parasites

Four weeks after infection of BALB/c mice, typical cutaneous lesions of leishmaniasis were observed in the tail base of all experimentally infected animals. Samples of

the lesions were taken and evaluated by Giemsa stain for parasitological confirmation and the presence of amastigotes was confirmed in all lesions.

Discussion

The leishmaniases are a group of human diseases caused by kinetoplastid protozoa of the genus *Leishmania*. Despite having a long history, dating back from more than 4500 years according to ancient descriptions [22], leishmaniasis still ranks at the top three of the neglected tropical diseases caused by protozoa [23]. There are about 1.5-2 million new cases and 70 000 death reports each year, and 350 million people are at risk of infection and disease with about 2.4 million disability-adjusted life-years [24].

The cultivation of *Leishmania* and other hemoflagellates has been a subject of much interest due to the necessity of performing biochemical and immunological studies with isolated parasites in an effort to develop future therapeutic and preventive tools [25]. *In vitro* cultivation of these parasites have progressed from crude media with undefined ingredients to fully defined media with serum substitutes capable of supporting good to excellent growth of the organisms [11]. Generally, the media used for *Leishmania* cultivation can be divided into two categories: biphasic and monophasic. Handling biphasic media are substantially more technically demanding than handling liquid media, which are more suitable for obtention of large amounts of the parasites [26]. Recently, Nasiri had a new insight out of an old NNN biphasic medium to cultivate *Leishmania* on sheep blood-LB agar base medium (SLM) as a simple and suitable medium for obtaining *Leishmania major* promastigotes [27].

Several studies have investigated the presence of *Leishmania* in livestock animals [28-33]. Herman studied the effect of rabbit, chicken, man, calf, hamster and cotton rat sera on the number and morphosis of *L.donovani* as well as cells in cultures of hamster-peritoneal macrophages that had been infected *In vivo* [9]. A research on finding a replacement for FCS in cultivation of promastigotes of *Leishmania major* and *Leishmania infantum* indicated that chicken serum is suitable for the nutritional requirement of parasites and indicates a potentiallty new medium to be used in long-term *In vitro* cultivation of *Leishmanian* promastigotes [6, 7]. In previous studies it was found that the serum and the urine of many animals like hamster, rabbit and sheep are suitable for cultivation of promastigotes. However, collecting of the serum of some of these, like hamster is expensive and using of some of them, like sheep serum, is accompanied with many challenges about adaptation problems of promastigotes to new serum [34].

The LB broth-lyophilized rabbit anti-sheep cell haemolysin medium has some advantages, including the readily availability of chemical compounds, simple storage conditions and low cost preparation. LB broth and its ingredients are readily available in every laboratory at low-cost and its preparation does not require sophisticated, expensive equipment like filtering system and can be prepared by

autoclavation [21]. It has been shown that unlike the cow, horse and dog serum that have negative effects on growth behavior of promastigotes, lyophilized rabbit anti-sheep cell haemolysin, like its blood, is suitable for the nutritional requirement of parasites. According to the manufactures instructor, we could storage lyophilized rabbit anti-sheep cell haemolysin at 4˚C prior to restoration and this is one of the main advantages of this medium.

Conclusion

This study has introduced a potent low-cost medium that can be used in cultivation of promastigotes of *Leishmania major*.

Conflict of Interest

We declare that we have no conflict of interest.

Acknowledgment

The authors are thankful to the director of RVSRI for providing the facilities for the research.

References

[1]. Cox FEG. History of Human Parasitology. Clin Microbiol Rev. 2002; 15(4): 595-612.
[2]. Bray RS, Rahim GF, Taj-Eldin S. The present state of leishmaniasis in Iraq. Protozool. 1967; 2: 171-86.
[3]. Berens RL, Brun R, Krassner SM. A simple monophasic medium for axenic culture of hemoflagellates. J Parasitol. 1976; 360-65.
[4]. B Adler S, Leishmania BD. Advances in parasitology. Adv Parasitol. Academic Press New York; 1964; p. 2.
[5]. Newman C. Serum-free cell culture- The ethical, scientist and economic choice. Biomed Sci. 2003; (9):941-42 .
[6]. Nasiri V, Dalimi A, Habibi GH, Esmailnia K, Article F, Nasiri V, et al. Use of chicken serum as a good replacement for the fetal calf serum in cultivation of promastigotes of Leishmania major. Arch Razi. 2011; 66(1): 59-64.
[7]. Nasiri V, Dalimi A, Ghaffarifar F. Use of chicken (Gallus gallus) serum as a costly replacement for the fetal calf serum in cultivation of promastigotes of Leishmania infantum. Asian Pacific J Trop Dis. 2013; 3(3): 169-73.
[8]. Trager W. The development of Leishmania donovani in vitro at 37° c effects of the kind of serum. J Exp Med. 1953; 97(2): 177-88.
[9]. Herman R. Studies of the numbers and morphology of the intracellular form of Leishmania donovani grown in cell culture. J Protozool. 1966; 13(3): 408-18.
[10]. Shamsuzzaman SM, Furuya M, Korenaga M, Imamura K, Hashiguchi Y. Use of urine samples from healthy humans, nephritis patients or other animals as an alternative to foetal calf serum in the culture of Leishmania (L.) donovani in vitro. Ann Trop Med Parasitol. 1999; 93(6): 613-20.
[11]. Schuster FL, Schuster FL, Sullivan JJ, Sullivan JJ. Cultivation of clinically signi cant hemo agellates. Society 2002; 15(3): 374-89.
[12]. Pal JK, Joshi-Purandare M. Dose-dependent differential effect of hemin on protein synthesis and cell proliferation in Leishmania donovani promastigotes cultured in vitro. J Biosci. 2001; 26(2): 225-31.
[13]. Merlen T, Sereno D, Brajon N, Rostand F, Lemesre J-LL. Leishmania spp.: Completely defined medium without serum and macromolecules (CDM/LP) for the continuous in vitro cultivation of infective promastigote forms. Am J Trop Med Hyg. 1999; 60(1): 41-50.
[14]. Ghoshal K, Sen S, Pal S, Banerjee AB. Nutrition of Leishmania donovani donovani: growth in new semidefined & completely chemically defined media. Indian J Med Res. 1986; 84: 461.
[15]. Ali SA, Iqbal J, Ahmad B, Masoom M. A semisynthetic fetal calf serum-free liquid medium for in vitro cultivation of Leishmania promastigotes. Am J Trop Med Hyg. 1998; 59(1): 163-5.
[16]. Moreno I, Álvarez J, García N, de la Fuente S, Martínez I, Marino E, et al. Detection of anti-Leishmania infantum antibodies in sylvatic lagomorphs from an epidemic area of Madrid using the indirect immunofluorescence antibody test. Vet Parasitol. 2014; 199(3): 264-67.
[17]. Molina R, Jiménez MI, Cruz I, Iriso A, Martín-Martín I, Sevillano O, et al. The hare (Lepus granatensis) as potential sylvatic reservoir of Leishmania infantum in Spain. Vet Parasitol. 2012; 190(1): 268-71.
[18]. García N, Moreno I, Alvarez J, de la Cruz ML, Navarro A, Pérez-Sancho M, et al. Evidence of Leishmania infantum infection in Rabbits (Oryctolagus cuniculus) in a natural area in Madrid, Spain. Biomed Res Int. 2014; 2014: 318254.

[19]. Ruiz-Fons F, Ferroglio E, Gortázar C. Leishmania infantum in free-ranging hares, Spain, 2004-2010. Euro Surveill. 2013; 18(30): 20541.
[20]. Taylor AER, Baker JR. In vitro methods for parasite cultivation. Academic Press Ltd; 1987.
[21]. Nasiri V, Dalimi A, Ghaffarifar F. LB broth-lyophilized Rabbit serum (LLR) as a new and suitable culture medium for cultivation of promastigotes of Leishmania major. J Parasit Dis. 2016; 1-5.
[22]. Akhoundi M, Downing T, Votýpka J, Kuhls K, Lukeš J, Cannet A, et al. Leishmania infections: Molecular targets and diagnosis. Mol Aspects Med. 2017; 57(1):1-29.
[23]. Fenwick A. The global burden of neglected tropical diseases. Public Health. 2012; 126(3): 233-36.
[24]. WHO. Control of the leishmaniases. World Health Organization technical report series. Geneva: World Health Organization; 2010. p. xii.
[25]. Palomino JC. Peptone-yeast autolysate-fetal bovine serum 10, a simple, inexpensive liquid medium for cultivation of Leishmania spp. J Clin Microbiol. Am Soc Microbiol; 1982; 15(5): 949-50.
[26]. Chang KP. Leishmania.In vitro cultivation of protozoan parasites. CRC Press. Boca Raton, Florida; 1983. pp. 111-45.
[27]. Nasiri V. Sheep blood-LB agar base medium (SLM) as a simple and suitable medium for the cultivation of Leishmania major promastigotes. Parasitol Res. 2013; 112(11): 3741-742.
[28]. Solano-Gallego L, Fernández-Bellon H, Serra P, Gállego M, Ramis A, Fondevila D, et al. Cutaneous leishmaniosis in three horses in Spain. Equine Vet J. 2003; 35(3): 320-23.
[29]. Rolão N, Martins MJ, João A, Campino L. Equine infection with Leishmania in Portugal. Parasite. EDP Sciences; 2005;12(2):183-86.
[30]. Ramos-Vara JA, Ortiz-Santiago B, Segalès J, Dunstan RW. Cutaneous leishmaniasis in two horses. Vet Pathol Online. 1996; 33(6): 731-34.
[31]. Muller N, Welle M, Lobsiger L. Identification of an as yet unknown Leishmania genotype causing equine cutaneous leishmaniasis in Central Europe. Vet Parasitol. 2009; 166: 346-51.
[32]. Lobsiger L, Müller N, Schweizer T, Frey CF, Wiederkehr D, Zumkehr B, et al. An autochthonous case of cutaneous bovine leishmaniasis in Switzerland. Vet Parasitol. 2010; 169(3): 408-14.
[33]. Bhattarai NR, Van der Auwera G, Rijal S, Picado A, Speybroeck N, Khanal B, et al. Domestic animals and epidemiology of visceral leishmaniasis, Nepal. Emerg Infect Dis. Centers Dis Control. 2010; 16(2): 231.
[34]. Nasiri V. An overview of the recent findings in the cultivation of Leishmania. Rev Med Microbiol. 2017; 28(1): 34-42.