LB broth-lyophilized Rabbit serum (LLR) as a new and suitable culture medium for cultivation of promastigotes of *Leishmania major*

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Abstract Fetal calf serum is the major part and the most expensive ingredient of the *Leishmania* culture media. Here, the efficacy of the LB broth-lyophilized Rabbit serum medium (LLR) was evaluated in cultivation of *Leishmania major*. Conventional Luria–Bertani (LB) broth medium was prepared and autoclaved for 15 min at 121 °C and then lyophilized Rabbit serum was added at the 1, 2.5, 5 and 10 % final concentrations. The efficacy of medium was evaluated by assessing the growth ability and replication pattern of the promastigotes of *L. major*. According to our finding, the LLR medium with 5–10 % lyophilized Rabbit serum supported the growth of the parasites and can be used for cultivation of Leishmanian parasites with acceptable *In vivo* infectivity for research purpose. The ability of the parasites to survive and proliferating in the presence of lyophilized Rabbit serum indicating that this serum is a good nutritional source. This study opens a new way to make low-cost medium that could be used in cultivation of Leishmanian parasites.

Keywords *Leishmania major* · Lyophilized Rabbit serum · Fetal calf serum · Promastigote

Introduction

Leishmaniasis is a major neglected tropical disease of poverty acknowledged by WHO (The World Health Organization 2010) as its victims are among the poorest and according to ranking after malaria it is a second most prevalent parasitic disease (Kumar 2013). 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 (The World Health Organization 2004).

Cutaneous leishmaniasis (CL), also known as button d’orient, chiclero’s ulcer, Aleppo sore, Delhi’s boils, etc., is known since ancient times and 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 CL, translated from a tablet of old Akkadian period in the second or third millennium BC (Bray et al. 1967).

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) to prevent negative side effects of them. The many of different kinds of media that have been using for cultivation of genus *Leishmania* require expensive and batch to batch different quality fetal calf serum (FCS), that is not manufactured in many countries specially most of the poor tropical countries, as one of their essential ingredients. FCS is highly expensive, and reliable supply is very difficult to obtain, especially in developing countries (Newman 2003) and using it in cell culture is problematic for several reasons as complexity, highly variable, and difficult to characterizing (Armstrong and Patterson 1994). Several attempts have been made to replace FCS in leishmania culture media with

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different kinds of sera, bovine serum albumin, a mixture of purine bases, vitamins, large concentrations of certain amino acids, hormones, hemin, hemoglobin, human and animal urine and, more recently chicken serum (Trager 1953; Herman 1966; Ghoshal et al. 1986; Ali et al. 1998; Merlen et al. 1999; Shamsuzzaman et al. 1999; Pal and Joshi-Purandare 2001; Schuster et al. 2002; Nasiri et al. 2013a, b; Nasiri 2013) that the later introduced an alternative low-cost serum that can be used in culture medium for primary isolation, routine cultivation and mass cultivation of *Leishmania* parasites (Nasiri et al. 2011, 2013a).

Results of recent investigations indicates 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 and confirmed that Iberian hares were contributing to the outbreak of disease (Molina et al. 2012; García et al. 2014; Moreno et al. 2014). In a study found a prevalence of *L. infantum* infection of 43.6 % from principally two hare species (*L. granatensis* and *L. europaeus*) (Ruiz-Fons et al. 2013) and in another a seroprevalence of 46 % was also reported in rabbits (Moreno et al. 2014). These results demonstrate that *L. infantum* infection is present in the rabbit population (García et al. 2014).

With consideration of the above result about the susceptibility of rabbit to leishmaniasis and potential of suitability of rabbit serum for cultivation of Leishmanian parasites, because rabbit blood is an essential component of conventional NNN medium (Taylor and Baker 1987), we describe a new formulation as a simple and suitable medium 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 left to cool down and then added to lyophilized Rabbit serum (Razi Vaccine and Serum Research Institute, Islamic Republic of Iran) according to manufacturer's protocol for reconstitution of it and 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, and they were kept at 4 °C.

Continuous cultivation of the promastigotes

The Mid-log phase promastigotes of *L. 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 (PBSS) to remove any FCS traces. Parasites were counted using a Neubauer chamber (Haemocytometer) slide and diluted in PBSS 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 experimented media were inoculated with mid-log phase promastigotes at the final concentration of 5×10^6 promastigotes/ml in 25 cm² plastic culture flasks that every flask totally contained 10 ml of parasites and complete media mixture. The flasks were placed in incubator at 26 °C. The continuous cultures of the parasites were made weekly by sub-culturing into fresh medium until they reached the 10th sub-culture. In all sub-cultures, parasites proliferation was assessed qualitatively and quantitatively by microscopic observations and Giemsa slide preparation. The number of parasites was counted every day using the Neubauer chamber slide.

In vivo infectivity determination of cultivated parasites

Stationary-phase promastigotes of *L. major* (2×10^6 parasites) from the 4th subculture in LLR medium were washed twice in sterile PBSS 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 appearance of lesions.

Ethics statement

This research was carried out accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Razi Vaccine and Serum Research Institute and 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-18 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 found that the addition of 5–10 % lyophilized Rabbit serum to the LB broth medium significantly stimulated the growth of the promastigotes of *L. major* and their effect were similar to the growth pattern of the parasites grown in the RPMI-1640-10 % FCS ($p < 0.05$). Under invert microscopy, it was 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 all culture media took about 6–7 days to reach to the late log-phase. It was also observed that the LLR medium supported the continuity of the parasites in successive passages. The effect of various concentrations of the lyophilized Rabbit serum in the culture media on the growth quantity of the promastigotes and a typical growth curve of the parasites growth in all dilution of lyophilized Rabbit serum is shown in Fig. 1.

In vivo assessment of pathogenicity of LLR 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 from the lesions were taken and evaluated by Giemsa stain for parasitological confirmation that presence of amastigotes confirmed in all lesions.

Discussion

Leishmaniasis is a typical example of zoonosis found on all the continents except Australia and Antarctica and is reported by the World Health Organization as being 1 of the major tropical diseases, and 20 million people in the world are infected each year with 400,000 additional phenomena (Castelli et al. 2014).

In study about different aspects of Leishmanian parasites, the presence of liquid culture media that are able to producing a large amount of promastigotes is one of the essential needs. Of course the routine commercially culture media like RPMI-1640, medium 199, and Schneider's *Drosophila* that are usually enriched by FCS or blood lysate (Adler 1964; Berens et al. 1976) are many expensive. In the mass cultivation of parasites the major problem is that we need to use expensive and batch to batch different quality FCS that is an importing material and is not manufactured in many countries specially most of the poor tropical countries that often leishmaniasis is one of the important health problems of

Fig. 1 Growth curve of promastigotes of *Leishmania major* in RPMI-10 % FCS and different concentrations of lyophilized Rabbit serum

them (Nasiri et al. 2013b). Herman has 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* (Herman 1966). A research about finding replacement for FCS in cultivation of promastigotes of *L. major* and *Leishmania infantum* indicated that chicken serum is suitable for nutritional requirement of parasites and is a comparatively simply available and inexpensive serum that can replace in the media that requires FCS enhancement for promastigote forms and indicate a potentiality of the new medium to be used in long-term *In vitro* cultivation of Leishmanian promastigotes (Nasiri et al. 2011, 2013a). In previous researches, we founded that the serum and the urine of many animals like hamster, rabbit and sheep are suitable for cultivation of promastigotes, but, collecting of the serum of some of them, like Hamster, is expensive and using of some of them, like sheep serum, accompanied with many challenges about adaptation problems of promastigotes to new serum (unpublished work). We should imply that using of this new medium, also accompanied with some little adaptation problems of promastigotes to new serum, but this problem takes place in any medium change and important point is that the final adaptation of parasites to new medium.

The LB broth-lyophilized Rabbit serum medium (LLR) has various 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 could prepare by autoclavation. It have been shown that unlike the cow, horse and dog serum that have negative effects on growth behavior of promastigotes, lyophilized Rabbit serum, like its blood, is suitable for the nutritional requirement of parasites. According to the manufactures instructor, we could storage lyophilized Rabbit serum at 4 °C prior to restoration and this is one of the main advantages of this new medium.

In conclusion, this study has introduced a potent new low-cost medium that could be used in routine cultivation of promastigotes of *L. major*.

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Compliance with ethical standards

Conflict of interest We declare that we have no conflict of interest.

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