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Axenic Procyclic Culture of *L. tropica* and *L. donovani* in Culture of FBS-free Medium

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Abstract

Leishmania causes disease ranging from self-healing cutaneous to fatal visceral leishmaniasis (VL). Leishmaniasis is reported endemic in 88 countries, including Iraq, in which 82% in low-income countries. The diseases develop following the bite of sand flies injecting *Leishmania* promastigotes into skin. Promastigotes transform into amastigotes *in vivo* multiplying within macrophages. In this study we have investigated the ability of axenic procyclic promastigotes of cutaneous *Leishmania tropica* and visceral *Leishmania donovani* survive in M199 media with or without Fetal Bovine Serum (FBS) added. Three time incubation periods were adopted (24, 48 and 72) hours and the results showed that *L. tropica* was able to survive and multiply in both FBS-medium and FBS-free medium, while *L. donovani* showed significant decrease in surviving in the FBS-free medium at day three of follow up. Such results may indicate the difference in *Leishmania* species requirements in growth culture *in vitro.*

Keywords: *Leishmania tropica*, *Leishmania donovani*, M199 media, fetal bovine serum (FBS).

المزروع المختبري النقي لطفيلي اللشمانيا الجلديه والاحشائية في الوسط الزرعي الخالي من مصل جنين العجل

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الخلاصة

طفيلي اللشمانيا يسبب داء اللشمانيات والذي يتراوح ما بين الشفاء الجلدي الذاتي لداء اللشمانيات الحشوي القاتل.تفيد التقارير ان داء اللشمانيات متوطن في 88 دولة من ضمنها العراق، ونسبة 82٪ من الإصابات في البلدان ذات الدخل المنخفض. داء اللشمانيات يتطور بعد نقل ذبابة الرمل للطور السوطي للشمانيا في الجلد.الطور السوطي يتحول إلى طور عديم السوط في المضيف الفقري ويتكاثر داخل البلاعم العملاقة. في هذه الدراسة قمنا بدراسة مقاومة الاشكال المسوطة خارج الجسم الحي للشمانيا الجلدية واللشمانيا الحشوية في الوسط الزرعيM199 بوجود او عدم وجود مصل البقر الجنيني (FBS). اذ اعتمدت ثلاثة أوقات للمتابعة (24، 48، 72) ساعة، وأظهرت النتائج أن اللشمانيا الجلدية كانت قادرة على البقاء على قيد الحياة والتكاثر بوجود او عدم وجود FBS على مدى أوقات المتابعة الثلاثة في حين أظهرت اللشمانيا الاحشائية القابلية على النمو و التكاثر في الوسط الزرعي المزود بال FBS بينما انحسرت قابليتها في اول يومين فقط في الوسط الزرعي الخالي من FBS اذ كان هناك انخفاض معنوي في تعداد الطفيلي في اليوم الثالث.تشير هذه النتائج الى وجود فروقات في متطلبات النمو لنوعي اللشمانيا الجلدي والحشوي في النمو في المختبر.

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Introduction

Leishmaniasis is a group of parasitic diseases transmitted by blood-sucking sand flies infected with *Leishmania* parasites [1]. The World Health Organization has declared leishmaniasis among one of the six major tropical diseases, and it is a major human and animal disease in the tropic and subtropical areas of world [2]. Depending on parasite species and host immune status, *Leishmania* infection can cause a wide range of symptoms including localized cutaneous lesions, diffuse cutaneous lesions, destruction of mucocutaneous membranes, and visceral diseases of the hematopoietic organs [3, 4].
These pathogens exist in two distinct parasitic stages, extracellular promastigotes, the infective stage which reside in the gut of the sand-fly and transmitted via the bite of this sand-fly into the human, which then, transform into intracellular amastigotes stage which multiply within the endo-lysosomal vacuoles of the macrophage of the vertebrate host [5].

About 12 million people worldwide suffer from leishmaniasis with an increase numbers in overseas travelers and/or troops. With absence of active vaccine, new treatments are needed to be found and many researches focus on the host-parasite relationship between the intra-cellular form of the parasite, amastigotes, and the hostile macrophages, where the parasite grow and proliferate[5, 6].

In vitro cultivation of protozoan parasites is very important for diagnosis, antibody production, assessment of parasite immune modulating capabilities, drug screening tests, improvements in chemotherapy, differentiation of parasites, molecular determination of strains, obtaining of purified antigen for vaccine production, development of attenuated strains, and investigation of host parasite interactions. However, the *in vitro* culture of *Leishmania* parasites involves highly complex procedures because these parasites have very complex life cycles and, depending on the life cycle stage, may require different culture parameters [7]. Amastigote cultures are carried out by using macrophage cells in liquid media, and promastigotes cultures are carried out in liquid, biphasic, and semisolid culture media. *Leishmania* promastigotes were first grown on diphasic blood agar, and these non-defined diphasic media are still used today for adaptation and cultivation of *Leishmania* parasites directly isolated from both vertebrate and invertebrate hosts. Further progress has been made with the use of liquid monophasic media [8]. However, these diphasic and monophasic media for parasite culture should be enriched with large amounts of certain amino acids, vitamins, hormones, and peptides [9-15].

Fetal bovine serum (FBS) is the liquid fraction of clotted blood from fetal calf, depleted of cells, fibrin and clotting factors, but containing a large number of nutritional and macromolecular factors essential for cell growth. The globular protein, bovine serum albumin (BSA), is a major component of fetal bovine serum [16, 17]. Fetal serum contains more growth factors and has low gammaglobulin (immunoglobulin) content. In addition, fetal serum contains lower levels of complements than those from adults or newborns serum. Complements have the undesired effect of lysing cells in culture, and interfere with immunoassays [18, 19]. A common treatment of fetal bovine serum is heat inactivation, where FBS is heated at 56°C for 30 minutes in a water bath with occasional shaking. It is important to make certain that the FBS temperature is right, since it might take a significant amount of time to heat a bottle of frozen FBS to 56°C. The purpose is to inactivate the complement system [20]. Heat-inactivation may also have undesired effects as well [21]. Another original purpose of heat-inactivation was to remove *mycoplasma* contamination, which nowadays is no longer an issue, since all serum products are filtered with much smaller pore size filters to remove *mycoplasma.* [22-25].

Depending on the number as well as the volume of the cultured material, the cost of FBS in a routine laboratory setting can in fact be very high. Especially for large scale cultures, novel and alternative supplementary materials to FBS as well as serum-free methods have been continuously investigated [26, 27].

Materials and Methods:

Leishmania tropica was isolated from a patient in Baghdad Medical City, a pateint diagnosed with cutaneous leishmaniasis and a sample was taken from hand lesion. *Leishmania* donovani strain isolated from MHOM/ IQ/2005/MRU15, were obtained respectively from Medical Research Unit at College of Medicine AL-Nahrain University.

M199 Media Preparation:

This media was purchased from Sigma-Aldrich and prepared according to the manufacturer's procedure [28].

1. 90% of final required volume was measured in room temperature.
2. Powder medium added and stirred until dissolved.
3. The original package with a small amount of water was rinsed to remove all traces of powder.
4. To the solution in step 3, 2.2 g sodium bicarbonate was added for each liter of final volume of medium.
5. While stirring, pH was adjusted using of 1N HCl or 1N NaOH.
6. Additional water added to bring the solution to final volume.
7. Media Sterilized by filtration using a membrane with a porosity of 0.22 microns.
8. Media dispensed into sterile container and stored at 4^{o}C.

Axenic Procyclic Promastigotes

Axenic procyclic promastigotes were cultured in M199 at pH 7.4 containing 10% heated inactivated fetal bovine serum (HIFBS) or without FBS, 50 µg/ml Gentamycin was added to the culture and incubated at 26^{o}C. Here, we have investigated the ability of *in vitro L. tropica* and *L. donovani* to maintain healthy culture in the presence or absence of fetal bovine serum.

Counting of the total number of the parasites in FBS or FBS-free media:

Healthy log-phase culture of *L. tropica* and *L. donovani* was seeded at 1X10^{4} cell/ml in M199-FBS or FBS- free media and follow up of three periods were adopted, (24, 48 and 72) hours. Parasites were counted by using haemocytometer under microscope [29]. All experiments were done in triplicates.

Statistical Analysis

The Statistical Analysis System- SAS (2012) was used to effect of different factors in study parameters. Least significant difference –LSD test was used to significant compare between meansin this study [30].

Results and Discussion

In this study we have studied the ability of two Iraqi strains of *Leishmania* to survive and multiply *in vitro*, in the presence or absence of FBS in media. The media we have used in this study is M199, which is a rich cell culture media. Three periods of follow-up were adopted (24, 48 and 72) hours.
Counting results showed that there was an increase in the total number of parasites of procyclic promastigotes for species, *L. tropica* and *L. donovani*, cultured in M199-FBS medium along the three days of follow- up. Moreover, the parasite cultured in M199-free media, *L. tropica* grown normally and was no significant decrease in parasite number; while *L. donovani* procyclic promastigotes were grown normally at the first two days of follow-up, but at the day three of follow-up (72 hours) there was a significant decrease in the total number of parasite Tables-1,-2.

Table 1-Counting of the number of the L. tropica in FBS or FBS-free media:

	Cell number in serum-media	Cell number in free-media
24h	5.67 X10^{4}	1.33 X10^{4}
48h	9.10 X10^{4}	1.81 X10^{4}
72h	31.05 X10^{4}	1.69 X10^{4}

Table 2- Counting of the number of the *L. donovani* in FBS or FBS-free media:

	Cell number in serum-media	Cell number in free-media
24h	4.88 X10^{4}	1.12 X10^{4}
48h	60.09 X10^{4}	1.92 X10^{4}
72h	70.33 X10^{4}	0.87 X10^{4}

The results showed that the ability of axenic procyclic promastigotes of cutaneous *Leishmania tropica* and visceral *Leishmania donovani* survive in M199 media with Fetal bovine serum (FBS) higher than FBS- free- media, and the results showed that *L. tropica* was able to survive and multiply in both FBS-media and FBS-free media at three time incubation periods (24, 48 and 72) hours.

Fetal bovine serum (FBS) is a common supplement of *in vitro* and *ex vivo* cell, tissue, organ, and *Leishmania* parasite cultures with concentrations varying between 10% and 30%. The rich variety of proteins in fetal bovine serum maintains cultured cells in a medium in which they can survive, grow, and divide [8].

M199 media was used for its efficient and rich constituents in comparison with the old-fashioned NNN-media. Due to its low price and easy preparation, NNN culture medium is especially used in the production of parasites obtained through bone marrow aspiration, spleen puncture biopsy, and skin biopsy. However, for many studies of *Leishmania* isolates *in vitro*, liquid culture media producing a large number of promastigotes in a short time are needed; those are RPMI 1640 medium, medium 199, and Schneider's *Drosophila* medium [27, 15].

Another study using medium M199, supplemented with 10% FBS and 2%urine, which is the most widely used liquid medium for the large-scale culture of *Leishmania* promastigotes, found that *Leishmania* promastigotes, under optimum conditions, reach the log phase within 72–84 hours post inoculation but in M199 media without FBS the parasites survived for 48–60 hr, and no replication took place [31, 32]. These findings are similar to our results, in which the *L. tropica* and *L. donovani* promastigotes numbers were increasing throughout the incubation periods (24, 48 and 72) hours, excluding the 72 hours culture of *L. donovani* without FBS at day three, where significant increase was noticed.

The variations in the present study are due to many effected factors, such as the strains, *in vitro* conditions, FBS (type, concentrations), and other conditions of the test that used. And this may conclude the significant decrease in cell number that happened in the third day of follow up of *L. donovani*, in this study.

Another study showed that during infection with *L. donovani*, the visceral species of leishmaniasis, the parasite stimulates the host macrophages to up-regulate the production of ceramide and endogenous ceramide in the host macrophage, this elevated level of ceramide produced by the host is important for the parasite survival as it down-regulates the Nitric Oxide production and the classical activity of Protein Kinase-C (PKC), which both have a major anti-leishmanial activity within the host cell, indicating that the parasite may scavenge host sphingolipid for survival and proliferation inside the macrophage [33, 34].

In conclusion, the promastigotes of *L. tropica* and *L. donovani* were able to survive in FBS-free M199 for at least 48 hours and it is recommended to use this condition to maintain the parasite when no FBS is available.

References

1. Datta, A. K., Datta, R. and Sen, B. **2008**. Antiparasitic chemotherapy: tinkering with the purine salvage pathway. *Adv. Exp. Med. Biol*. 625: 116-132.
2. Wheeler, R. J., Gluenz, E. and Gull, K. **2011**. The cell cycle of *Leishmania*: morphogenetic events and their implications for parasite biology. *Mol. Microbiol.* 79: 647-662.
3. Berman, J. **2003**. Current treatment approaches to leishmaniasis. *Curr. Opin. Infect. Dis*. 16: 397–401.
4. Castillo, E., Dea-Ayuela, M. A., Bolas-Fernandez, F., Rangel, M. and Gonzalez-Rosende, M. E. **2010.**The kinetoplastid chemotherapy revisited: current drugs, recent. Advances and future perspectives. *Curr Med.Chem.*17: 4027–4051.
5. Myler, P. J. and Fasel, N. **2008**. *Leishmania after the genome*. First Edition, Caister Academic. Press. Norfolk.UK.
6. Pal, S., Ravindran, R. and Ali, N. **2004**. Combination therapy using sodium antimony gluconate in stearylamine-bearing liposomes against established and chronic *Leishmania donovani* infection in BALB/c mice. *Antimicrob. Agents. Chempther*. 48(9): 3591-3593.
7. Visvesvara, G. S. and Garcia, L. S. **2002**. Culture of protozoan parasites. *Clin. Microbiol. Rev*. 15: 327-328.
8. Merlen, T., Sereno, D. and Brajon, N.**1999**. *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*. 60: 41-50.
9. Iovannisci, D. M. and Ullman, B. **1983**. High efficiency plating method for *Leishmania* promastigotes in semi defined or completely defined medium. *J. Parasitol* 69: 633-636.
10. Melo, N. M., De Azevedo, W. and Roitman I *et al.*, **1985**. A new defined medium for cultivating *Leishmania* promastigotes. *Acta. Trop*. 42: 137-141.
11. O'Daly, J. A. and Rodriguez, M. B. **1988**. Differential growth requirements of several *Leishmania* spp. in chemically defined culture media. *Acta. Trop.* 45: 109-126.

12. Jackson, J. E., Tally, J. D. and Tang, D. B.**1989**. An *in vitro* micro method for drug sensitivity testing of *Leishmania*. *Am. J. Trop. Med.* 41: 318-330.
13. Kar, K., Mukerji, K. and Naskar, K. **1990**. *Leishmania donovani*: a chemically defined medium suitable for cultivation and cloning of promastigotes and transformation of amastigotes to promastigotes. *J. Eukaryot. Microbiol.* 37: 277-279.
14. Gupta, A. K. and Saran, R. **1991**. *In vitro* maintenance of *Leishmania donovani* promastigotes in a cheap, serum-free, hemin-based, autoclavable culture medium. *J. Commun. Dis.* 23: 276-277.
15. McCarthy-Burke, C., Bates, P. A. and Dwyer, D. M. **1991**. *Leishmania donovani*: use of two different, commercially available, chemically defined media for the continuous *in vitro* cultivation of promastigotes. *Exp. Parasitol.* 73: 385-387.
16. Shah, G. **1999**. Why do we still use serum in the production of biopharmaceuticals? *Dev. Biol. Stand.* 99: 17-22.
17. Even, M., Sandusky, C. and Barnard, N. **2006**. Serum-free hybridoma culture: ethical, scientific and safety considerations. *Trends. Biotechnol.* 24: 105-108.
18. Issaq, H ., Xiao, Z. and Veenstra, T. **2006**. Serum and plasma proteomics. *Chem. Rev.* 107: 3601-3620.
19. Anderson, N. and Anderson, N. **2002**. The human plasma proteome: history, character, and diagnostic prospects. *Mol. Cell. Proteomics*. 1: 845-867.
20. Giard, D. **1987**. Routine heat inactivation of serum reduces its capacity to promote cell attachment. *In Vitro Cell. Dev. Biol.* 23: 691-697.
21. Triglia, R. and Linscott, W. **1980**. Titers of nine complement components, conglutinin and C3b-inactivator in adult and fetal bovine sera. *Mol. Immunol.* 17: 741-748.
22. Okano, S., Hurley, D., Vandenplas, M. and Moore, J. **2006**. Effect of fetal bovine serum and heat-inactivated fetal bovine serum on microbial cell wall-induced expression of procoagulant activity by equine and canine mononuclear cells *in vitro*. *Am. J. Vet. Res.* 67: 1020-1024.
23. Pinyopummintr, T. and Bavister, B. **1994**. Development of bovine embryos in a cell-free culture medium: Effects of type of serum, timing of its inclusion and heat inactivation. Theriogenology. 41: 1241-1249.
24. Meszaros, K., Aberle, S., White, M. and Parent, J. **1995**. Immunoreactivity and bioactivity of lipopolysaccharide-binding protein in normal and heat-inactivated sera. *Infect. Immun.* 63: 363-365.
25. Kuznetsov, S., Mankani, M., Bianco, P. and Robey, P. **2009**. Enumeration of the colony-forming units-fibroblast from mouse and human bone marrow in normal and pathological conditions. *Stem Cell. Res*. 2: 83-94.
26. van der Valk, J., Mellor, D. and Brands, R. **2004**. The humane collection, of fetal bovine serum and possibilities for serum-free cell and tissue culture. *Toxicol in Vitro*. 18: 1-12.
27. Başalp, A. **2000.** Simple production and purification of monoclonal antibodies in serum-free medium. *Turk. J. Biol.* 24: 189-196.
28. Berens, R. L., Birun, R. and Krassner, S. M. **1976**. A simple monophasic medium for axenic culture of hemoflagellates. *J. Parasitol.* 62: 360–365.
29. Morgan, J. F., Morton, H. J. and Parker, R.C. **1950.** The Nutrition of Animal Cells in Tissue Culture. I. Initial Studies on a Synthetic Medium. *Proc. Soc. Exp. Biol. Med*. 73: 1-8.
30. Denny, P. W., Goulding, D., Ferguson, M. A. J. and Smith, D. F. **2004**. Sphingolipid-free *Leishmania* are defective in membrane trafficking, differentiation and infectivity. *Mol. Microbiol.* 52(2): 313-327.
31. SAS. **2012**. *Statistical Analysis System, Users Guide.* Statistical. Version 9.First Edition. SAS. Inst. Inc.Cary. N. C. USA.
32. Evans,D.A. **1989**. Handbook on Isolation, Characterizatio and Cryopreservation of *Leishmania*. Evans, D. A, ed. *Geneva.World Health Organization* 7: 28–32.
33. Evans, D. A. **1986**. An inexpensive easily available replacement for foetal calf serum in media for the in *vitro* cultivation of *Leishmania* spp. *Z.* Parasitenkd. 72*:* 567–572.
34. Gsosh, S., Bhattacharyya, S., Sirkar, M., Sa, G. S., Das, T., Majumdar, D., Roy and Majumdar, S. **2002**. *Leishmania donovani* suppresses activated protein 1 and NF-kappaB activation in host macrophages via ceramide generation: involvement of extracellular signal-regulated kinase. *Infect. Immun.*, 70(12): 6828-6838.

35. Ghosh, S. H. and Kurban, A. K. **2008**. Leishmaniasis and Other Protozoan Infections. In: Wolff, K., Goldsmith, L. A., Katz, S. I., Gilchrest, B. A., Paller, A. S. and Leffell, D. J. Eds. *Fitzpatrick's Dermatology in General Medicine*. Seventh Edition. McGraw. Hill. 11:2001- 2009.