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*Full Article*

# Use of chicken serum as a good replacement for the fetal calf serum in cultivation of promastigotes of *Leishmania major*

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**ABSTRACT**

Genus *Leishmania* is a protozoan parasite that causes different severe diseases. Fetal Calf Serum (FCS) is the major part of the *Leishmania* culture media, for mass cultivation and the most expensive ingredient in these media. In the present work, the efficacy of chicken serum was evaluated in *Leishmania* culture media. The results indicated that, the (10%) chicken serum enriched culture medium supported the growth of large scale cultures of the parasites and can be used for primary and mass cultivation of *Leishmania* parasites. In conclusion, the chicken serum was effective, easy available and cheap replacement for FCS.

***Keywords:*** Chicken serum, *Leishmania* promastigotes cultivation, Fetal Calf Serum



## INTRODUCTION

The members of genus *Leishmania* are protozoan parasites that cause severe and debilitating cutaneous, as well as fatal visceral, disease in sub-tropical/tropical regions of old and new worlds (Greenblatt 1988). This parasite is reported in 88 countries, of which 82% are low-income countries (Desjeux 1999) and is the cause of one of the 6 primary tropical diseases (WHO 1990). Globally, there are an estimated 1.5-2 million new cases and 70 000 deaths each year, and 350 million people are at risk of infection and disease. It cause an estimated 2.4 million disability-adjusted life-years (WHO 2004).

*In vitro* cultivation of parasitic protozoa provides information that can be used to develop our knowledge to preparing effective tools like vaccines for eradication of the parasitic disease. The media used for the cultivation of *Leishmania* parasites, the basic requirement for vaccine preparation, require fetal calf serum (FCS) as one of their essential ingredients. FCS is highly expensive, and reliable supply is very difficult to obtain, especially in developing countries (Newman 2003). It is estimated that about 500,000 liters of serum are produced on an annual basis (Hodgson 1995). For this purpose more than 1,000,000 bovine fetuses have to be harvested annually (Jochems *et al* 2002). However, the use of FCS may involve both moral and scientific points like suffering to the animals, in particular to fetuses (Jochems *et al* 2002, Jochems 1997, Kohlpoth & Rusche 1997) and with regard to scientific problems that the composition of FCS is unknown, varies between batches, or may be contaminated with viruses, mycoplasm, prions or antibodies(Eloit 1999, Shah 1999, Zabal 2000, Torres

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& Ortega 2006). Hence, several attempts have been made to replace FCS with different materials like animal serums, bovine serum albumin, a mixture of purine bases, vitamins, large concentrations of certain amino acids, hormones, hemin, hemoglobulin, and, more recently, even human and animal urine (Ali 1998, Armstrong & Patterson 1994, Chaudhuri *et al* 1986, Merlen et al 1999, Pal 2000, Schuster *et al* 2002, Shamsuzzaman *et al* 1999, Herman 1966, Trager 1953), but none of them is widely functional in practice and some of them need ingredients that are more expensive than FCS.

In the present work, the use of chicken serum as an available replacement for FCS in the preparation of media for the primary and mass cultivation, as well as maintenance of *Leishmania major* promastigotes were investigated.

## MATERIALS AND METHODS

**Serum collection.** The bloods of chicken were collected from an industrial chicken slaughterhouse in Alborz Province. The blood was kept at 4 °C for overnight then the serum was separated by centrifugation at 3,000 g for about 10 minutes. All collected sera inactivated at 56 °C for 30 minutes and after inactivation stored at -20 °C until the use for medium preparation.

**Medium preparation.** RPMI-1640 was used as a standard base medium for growth. Chicken serum enriched medium was prepared by dissolving10.4g of RPMI-1640(sigma) in 800 ml of distilled water and then 100 ml (10%) heat inactivated chicken serum was added. The pH was adjusted to 7 and the total volume was adjusted to one liter. Four dilutions of chicken serum were prepared as follows: 1 %, 2.5%, 5% and 10%. No any antibiotic in culture media was used. The media were sterilized by pressure passage through 0.22 µm millipore filter and the complete media were stored at 2-6 °C. Above procedures were used for preparation of RPMI-1640 enriched with (10%) heat-inactivated fetal calf serum instead of chicken serum for positive control.

**Parasite cultivation.** *Leishmania* promastigotes (*Leishmania major*: MRHO/IR/76/ER ) that previously had been grown in RPMI-1640 medium supplemented with 10% FCS, were concentrated by centrifugation at 3,000 g for 10 minutes and washed twice with sterile phosphate-buffered saline solution (PBSS) to remove any traces of FCS. Parasites were counted with invert microscopy in a Neubauer chamber (Hemocytometer) slide and diluted in PBSS to a final concentration of $10^8$ parasites/ ml. Sub-cultures were prepared in 10 series and in each series, triplicate cultures of all media dilution with chicken serum, alongside of the positive control with 10% FCS enriched medium, were inoculated with mid-log phase promastigotes (about five days) in 25$cm^2$ plastic culture flasks at the final concentration of $10^6$ promastigotes /ml that totally every flask contained 15 ml of mixture of parasite seed and media for each dilution of sera. The flasks were placed in incubator at 26 °C and the number of parasites was counted every day using haemocytometer slide. Long-term continuous cultures of the *Leishmania* were maintained by successful passages every week (days 5th -6th after every passages) and sub-culturing into fresh medium. After ten sub-passages, according to the primary passages, sub-culture performed in larger scale passages in 175-$cm^2$ plastic culture flasks that contain 200 ml of culture medium. In all sub-cultures, parasites growth was assessed qualitatively and quantitatively by microscopic observations on the appearance and mobility of promastigotes and by enumeration of parasites in a Haemocytometer slide.

***In vivo study.*** About $3 \times 10^6$ parasites (at the 5th subculture) from stationary-phase promastigotes of *Leishmania major* of two kind of culture media (enriched with chicken serum and FCS) were washed twice in sterile phosphate-buffered saline solution (PBSS) and were inoculated subcutaneously into the base of tail of 10 male Blab/c mice (6-7-weeks old), with five mouse serving as control. The presences of tail lesions were observed macroscopic every day and

after appearing the lesion biopsy performed and inoculated in NNN medium.

## RESULTS

**Replication of *Leishmania major*.** Typical promastigotes morphology was observed in Giemsa-stained smears prepared from culture media of all serum dilutions and fine grown promastigotes appeared elongated and pointed with an anteriorly directed flagellum exceeding the body length. Many of the promastigotes were observed in various stages of division and showed the pattern of rosette shape growth behavior that indicates to the suitability of media conditions. Parasites inoculated into the all culture media took about 6-7 days to reach to the late log-phase. The culture medium containing 10% chicken serum showed a maximum count of 32 x $10^6$ parasites/ml at about 7th day and subsequently, the number of promastigotes decreased but they could survive about 10-14 days (Table.1). A typical growth curve of the parasites growth in all dilution of culture media is shown in figure 1.

Replication of the parasite in FCS enriched culture medium and in chicken serum enriched culture medium appeared very close and parallel to each other.

***In vivo* assessment of pathogenicity.** Two to six weeks following inoculation of the promastigotes to BALB/c mice, the presence of typical cutaneous lesions of *Leishmania* parasites were observed on the base of the tail of all mice. By sampling from the lesions and transferring the exudates to chicken serum enriched culture medium and sub-culturing the resulted promastigotes, confirmed that the medium RPMI-1640 with 10% chicken serum supported transformation of amastigotes of *leishmania* from skin lesion into promastigotes *in vivo*, and indicating that the medium can be used for parasites isolation from infected samples.

## DISCUSSION

Leishmaniasis is a major tropical disease acknowledged by WHO (WHO1990). For many years making an effective vaccine against leishmaniasis remained as a dreamy desire especially for tropical and subtropical countries where the disease is a result of poverty and poor healthy conditions. 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(Berens 1976, Schlein 1983) are very expensive.

Although serum of many animals like hamster, rabbit, sheep are suitable for cultivation of promastigotes, but, the collection of the serum of rabbit and hamster, is costly. Herman (1966) has studied the effect of serum from rabbit, chicken, man, calf, hamster and cotton rat on the number and morphosis of *L. donovani* as well as on the cells in cultures of hamster-peritoneal macrophages that had been infected in vivo(Schuster 2002). In a cell-free culture system, Trager (1953) found that serum from man or hamsters was relatively beneficial, as compared with that from rabbits, guinea pigs, or ducks, on multiplication at 37 °C of intermediate morphologic stages of *L. donovani*. They mentioned that Duck serum rapidly destroyed both the Leishmania and leptomonad forms at room temperatures 21. In addition, Jones et al. (1944) cultivated three strains of *Leishmania* in the yolk sac of the developing chick embryo successfully [24] and in another research Atif Ali et al. (1997) had maintained in vitro *Leishmania* promastigotes in an

**Table 1.** Replication of *L.major* promastigotes in RPMI-1640 medium plus 10% Fetal Calf Serum (FCS) and RPMI-1640 medium plus 1%, 2.5%, 5% , 10% Chicken Serum (ChS).

| Sera | No. of promastigotes (x $10^6$ ) on days | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** |
| 10% FCS | 1 | 2.2 | 5.6 | 12 | 20 | 28 | 33 | 32.5 | 32 | 25 | 17.5 | 7.5 | 2.5 | 1.3 |
| 1% ChS | 1 | 1.9 | 3.5 | 8 | 15 | 23 | 24 | 18 | 18 | 14 | 12 | 4 | 1 | 1 |
| 2.5% ChS | 1 | 2 | 3.6 | 8.7 | 14.7 | 23 | 26 | 25 | 25 | 19 | 14 | 7 | 2.5 | 1 |
| 5% ChS | 1 | 2 | 4.7 | 9.5 | 17 | 26 | 30 | 29 | 29 | 27 | 17 | 8 | 3.4 | 1.2 |
| 10% ChS | 1 | 1.8 | 4.9 | 10 | 18 | 26 | 32 | 31 | 31 | 28 | 18 | 8.8 | 3 | 1.5 |

egg based biphasic culture medium(Atif Ali *et al*1997). In addition, in another research had been shown that many sera from different adult animals have been used for the cultivation of *Leishmania* promastigotes, but they have exhibited deleterious effects on growth pattern(Evans1986, Pearson & Steigbigel 1980, Schumunis & Herman 1970).

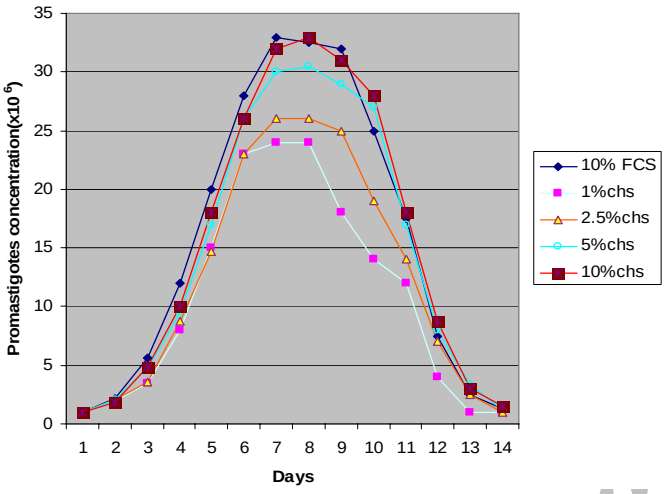


**Figure 1.** Growth curve of *Leishmania major* promastigotes in RPMI-1640 medium plus 10% Fetal Calf Serum (FCS) and RPMI-1640 medium plus 1%, 2.5%, 5%, 10% Chicken Serum (ChS).

Like other birds, chickens do not harbour the infection possibly as a result of their higher natural body temperature of 41°C, or biological differences inherent within the chicken such as complement or nucleated RBCs(Schlein *et al* 1983, McCartry-Burke *et al* 1991). Warren (1958) and Borsos (1959) showed that sera from some germ-free chickens could neither lyse nor agglutinate the flagellates although complement was present. In contrast, normal chicken serum was both lytic and agglutinogenic. When only the agglutinin from normal chicken serum was combined with sera from germ-free chickens, lysis of *T. cruzi* was obtained. The role of the normal flora of chickens as antigen assumes increased importance in the light of this observations(Warren 1958, Borsos 1959).

Sant'Anna et al (2010) mentioned that although birds do not acquire a *Leishmania* infection, it is plausible that an avian bloodmeal could follow a *Leishmania* infected mammalian bloodmeal. Their study showed that chicken blood did not inhibit the development of *Leishmania* parasites in sand flies and that chickens were unlikely to offer any protection from disease but may, on the contrary, promote parasite growth and development in the vector thus increasing transmission potential.

Furthermore, our results showed that chicken serum has various advantages to FCS. It is readily available at low cost and its preparation does not require sophisticated expensive equipment and can be collected from any chicken slaughterhouse even in poor country. Although cow, horse and dog serum have negative effect on growth behavior of promastigotes, but chicken serum is very 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 *Leishmania* promastigote. The study has demonstrated an alternative low- cost serum that be used in culture medium for primary isolation, routine cultivation and mass cultivation of *Leishmania* parasites.

## References

Ali, S. A., Iqbal, J., Ahmad, B., and Masoom, M.(1998) A semi synthetic fetal calf serum free liquid medium for *in vitro* cultivation of *Leishmania* promastigotes. *American Journal of Tropical Medicine and Hygiene* 59:163-165.

Armstrong, T. C. and Patterson, J. L. (1994) Cultivation of *Leishmania braziliensis* in an economical serum-free medium containing human urine. *Journal of Parasitology 80*: 1 030-1032.

Atif Ali, S., Khalil N.Y., Iqbal J., Masoom Yasinzai M. (1997). In vitro maintenance of *Leishmania* promastigote in an egg based biphasic culture medium. *Methods in Cell Science* 19: 107-110.

Berens, R.L., Birun R., and Krassner S.M. (1976). A simple monophasic medium for axenic culture of hemoflagellates. *Journal of Parasitology* 62:360-365.

Borsos, T. (1959).Studies on immune factors occurring in sera of chickens against the crithidia stage of *Trypanosoma cruzi*. *Journal of Immunology* 82: 585-590.

Chaudhuri, G., K. Ghoshal, S. Sen, S. Pal., and A. B. Banerjee(1986). Nutrition of *Leishmania donovani donovani:* growth in new semi defined & completely chemically defined media. *Indian Journal of Medical Research* 84:461-468.

Desjeux, P. (1999). Global control and *Leishmania* HIV co-infection. *Clinical Dermatol*ogy17: 317-25.

Eloit, M.(1999). Risks of virus transmission associated with animal Sera or substitutes and methods of control. *Developments in Biological Standardization* 99: 9-16.

Evans, DA.(1986). An inexpensive easily available replacement for foetal calf serum in media for the in vitro cultivation of *Leishmania* spp. *Zetch Parasitenkd* 72: 567-572.

Greenblatt, C.L. (1988). Cutaneous leishmaniasis: the prospects for a killed vaccine. *Parasitology Today* 4:53-4.

Herman, R. (1966). Studies of the numbers and morphology of the intracellular form of *Leishmania donovani* grown in cell cultures. *Journal of Protozoology* 13:408-418.

Hodgson, J. (1995). To treat or not to treat: that is the question for serum. *Journal of Biotechnology* 13, 333-342.

Jochems, C.E., van der Valk, J.B., Stafleu, F.R., Baumans, V. (2002). The use of fetal bovine serum: ethical or scientific problem? *Alternatives Laboratory Animals* 30: 219-227

Jochems, C.E. (1997). Use, trade and harvest of livestock sera. Thesis, Utrecht University, Department of Laboratory Animal Science & Wageningen Agricultural University, Department of Animal Sciences - Animal Husbandry / Health and Reproduction Group.

Jones H., Rake G., Hamre D.(1944). Cultivation of *Leishmania* in the Yolk Sac of the Developing Chick Embryo. *American Journal of Tropical Medicine* 24(6):381-38

Kohlpoth, M., Rusche, B. (1997). Kultivierung einer permanenten Fischzellinie in serumfreien Medien: Spezielle Erfahrungen mit Einem Zytotoxizita tstest fur Abwasserproben. *Alternatieven zu Tierexperimenten* 14: 16-20.

McCartry-Burke, C., Bates P. A., and Dwyer D. M. *L.* (1991).*donovani:* use of two different commercially available chemically defined media for the continuous in vitro cultivation of promastigotes. *Experimental Parasitology* 73:385-387.

Merlen, T., Sereno D., Brajon N., Rostand F., and Lemesre L. (1999). *Leishmania* spp.: completely defined medium without serum and macromolecules (CDM/P) for the continuous *in vitro* cultivation of infective promastigote forms. *American Journal of Tropical Medicine and Hygiene* 60:41-50.

Newman, C. (2003). Serum-free cell culture-the ethical, scientific and economic choice. *Biomedical Scientist* 941-942.

Pal, J. K., and Purandare, M. J. (2000). Dose-dependent differential effect of hemin on protein synthesis and cell proliferation in *Leishmania donovani* Promastigotes cultured in vitro. *Journal of Bioscience* 26:225-231.

Schlein Y, Warburg A, Schur LF, Shomai J. (1983). Vector compatibility of *Phlebotomus papatasti* on differentially induced digestion. *Acta Tropica* 40:65-70.

Schumunis, G.A.., Herman, R. (1970). Characteristic of so called natural antibodies in various normal sera against culture forms of *Leishmania. Journal of Parasitology* 56: 889-896.

Schuster, F. L., and J. 1.(2002). Sullivan Cultivation of clinically significant hemoflagellates. *Clinical Microbiology Review* 15:374-389.

Shah, G. (1999). Why do we still use serum in the production of biopharmaceuticals? *Developments in Biological Standardization* 99: 17-22.

Shamsuzzaman, S. M., Furuya M., Korenaga M., Imamura K., and Hashigughi Y. (1999). 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. Annals Tropical Medine and Parasitology* 93:613-620.

Torres, M. P. and Ortega, Y. R. (2006). *Neospora caninum* antibodies in commercial fetal bovine serum. *Veterinary Parasitology*140: 352-355. Warren, L.G.(1958). Biochemical studies on chicken macrophages infected in vitro with *Trypanosoma cruzi*. *Experimental Parasitology* 7: 82-91.

Trager, W. (1953). The development of *Leishmania donovani* in vitro at 37 °C. Effects of the kind of serum. *Journal Experimental Medicine* 97:177-188

Wessman, S.J., Levings, R.L. (1999). Benefits and risks due to animal Serum used in cell culture production. *Developments in Biological Standardization*99: 3-8.

WHO, TDR News (1990). WHO/TDR-CTD/HH., 90, 1, 14-15.4. WHO. The world health report 2004. Changing history. Geneva: WHO, 2004.

Pearson, R.D., Steigbigel, R.T. (1980). Mechanism of lethal effect of human serum upon *Leishmania donovani. Journal of Immunology* 125: 2195-2201.

Zabal, O., Kobrak, A.L., Lager, I.A., Schudel, A., Weber A.

(2000). Contamination of bovine fetal serum with bovine viral diarrhea virus. *Journal of Review Argentina Microbiology* 32(1):27-32.