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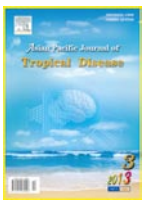

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Use of chicken (Gallus gallus) serum as a costly replacement for the fetal calf serum in cultivation of promastigotes of *Leishmania infantum*

Vahid Nasiri, Abdolhossein Dalimi*, Fatemeh Ghaffarifar

Department of Parasitology and Entomology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

PEER REVIEW

Peer reviewer

Majid Pirestani, PhD of Medical Parasitology, Department of Parasitology and Entomology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.
Tel: +98935966434
E-mail: m.pirestani@modares.ac.ir

Comments

Totally, finding new methods for changing the availability of new tools of vaccine preparation had been a main road in many researches and it seems that this article pointed to one of this beginners methods.
(Details on Page 172)

ABSTRACT

Objective: To evaluate the effectiveness of chicken serum as an alternative to fetal calf serum (FCS) in the culture of *Leishmania*. **Methods:** In the present work, the efficacy of chicken serum was evaluated in the cultivation of promastigotes of *Leishmania infantum* (*L. infantum*), by assessing the parasite growth and replication pattern after adding 5%-10% chicken serum to the RPMI-1640 medium. **Results:** The 10% chicken serum enriched culture medium, supported the growth of *L. infantum*. **Conclusions:** According to our finding, the chicken serum can be used for primary and mass cultivation of *L. infantum* is an effective, easy available and low-cost replacement for FCS.

KEYWORDS

Chicken serum, Fetal calf serum, *Leishmania infantum*, Culture media, Promastigote

1. Introduction

The members of genus *Leishmania* are protozoan parasites that cause severe and debilitating cutaneous, as well as fatal visceral, diseases in sub-tropical/tropical regions of the Old and New Worlds[1]. This parasite was reported endemic in 88 countries, of which 82% are low-income countries and is the cause of one of the 6 primary tropical diseases[2,3]. 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 causes an estimated 2.4 million disability-adjusted life-years[4].

In vitro cultivation of parasitic protozoa provides information that can be used to develop new vaccines to 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[5]. It is estimated that about 500 000 L of serum are produced on an annual basis[6]. For this purpose, more than 1 000 000 bovine fetuses have to be harvested annually[7]. However, the use of FCS may involve both moral and scientific points like suffering to

*Corresponding author: Abdolhossein Dalimi, PhD, Professor, Department of Parasitology and Entomology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.
Tel: +982182883838
Fax: +982182884555
E-mail: dalimi_a@modares.ac.ir
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the animals, in particular to fetuses[7-9], and with regard to scientific problems that the complete composition of FCS is unknown, varies between batches, or may be contaminated with viruses, mycoplasm, prions or antibodies[10-14]. Although several attempts have been made to replace FCS with different kinds of animal sera, 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[15-23]. None of them is widely in practice and some of them need ingredients that are more expensive than FCS. In a study, three strains of *Leishmania* have been cultivated in the yolk sac of the developing chick embryo[24], and in another practice, it has been shown that the capacity of bovine or simian rotaviruses to plaque in the presence of chicken serum was enhanced compared to virus grown in the presence of FCS[25].

In the present work, we investigated the efficacy of chicken serum as an easy available replacement for FCS in the preparation of media for the primary and mass cultivation, as well as, maintenance of *Leishmania infantum* (*L. infantum*) promastigotes was investigated.

2. Materials and methods

2.1. Serum collection

The bloods of chicken were collected from an industrial chicken slaughterhouse in Karaj city, Alborz Province, Iran. The blood was kept at 4 °C for overnight, then the serum was separated by centrifugation at 5000 rpm for about 10 min. All collected sera was inactivated at 56 °C for 30 min and stored at −20 °C until the use of medium preparation.

2.2. Medium preparation

RPMI-1640 was used as a standard base medium and was prepared by dissolving 1.04 g of RPMI-1640 (Sigma, USA) in 80 mL of distilled water and then 10 mL (10%) heat inactivated chicken serum was added. The pH was adjusted to 7 and the total volume was adjusted to 100 mL. Four dilutions of chicken serum were prepared as follows: 1.0%, 2.5%, 5.0% and 10.0%. No antibiotic was used in culture media. The media was sterilized by pressure passage through 0.22 μm membrane filter (Millipore, Germany) and the complete media was stored at 2-6 °C. Above procedures were used for preparation of RPMI-1640 enriched with heat-inactivated fetal calf serum (10%) for positive control.

2.3. Parasite cultivation

Mid-log phase promastigotes of *L. infantum* (MCAN/IR/07/Moheb-gh.) that previously had been grown in RPMI-1640 medium supplemented with 10% FCS, were concentrated by centrifugation at 5000 rpm for 10 min and washed twice with sterile phosphate-buffered saline solution to remove any traces of FCS. Parasites were counted with invert microscopy in a Neubauer chamber (Haemocytometer) slide and diluted in phosphate-buffered saline solution to a final concentration of 10^8 parasites per millilimL. Subcultures from each dilution of chicken serum were prepared in 5 series, alongside of the positive control with 10% FCS enriched medium, and at each series was inoculated with 10^6 mid-log phase promastigotes/mL in 25 cm^2 plastic culture flasks that every flask totally contained 15 mL of parasites and complete media mixture. The flasks were placed in incubator at 26 °C and the number of parasites was counted every day using haemocytometer slide. Long-term continuous cultivation of the parasites were done by successful passages every week (Day 5-6 after every passage) into fresh medium. After ten sub-passages, according to primary passages, sub-culture performed in larger scale passages of 175 cm^2 plastic culture flasks that contained 100 mL of culture medium. In all sub-cultures, the parasites growth pattern was assessed qualitatively and quantitatively by microscopic observations and Giemsa slide preparation, the number of parasites was counted every day using Haemocytometer slide.

2.4. Statistical evaluation

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.

3. Results

3.1. Assessment of the parasite growth and replication pattern

Results indicated that the addition of 5%-10% chicken serum to the RPMI-1640 medium significantly stimulated the growth of the promastigotes of *L. infantum* and it was equivalent to the growth observed in RPMI-1640 supplemented with FCS ($P<0.05$). Fine grown parasites and typical morphology of the promastigotes were observed in Giemsa-stained smears prepared from culture media of all serum dilutions. Under invert microscopy, it was observed that the promastigotes were elongate and had rapid motility, rosette forms which is an indication of the appropriate culture conditions were made. Parasites inoculated into the all culture media took about 5-6 d to reach to the late log-phase.

3.2. Effect of the chicken serum on the growth quantity

The culture medium containing 10% chicken serum showed a maximum count of 3×10^6 parasites/mL at about Day 7, and then the numbers of promastigotes decreased. The effect of

various concentrations of the chicken serum in the culture media on the growth quantity of the promastigotes and a typical growth curve is shown in Figure 1. It is observed that the culture medium supported the large scale cultures of the parasites and also established that continuity of the parasites in successive passages was achieved in both kind of sera (FCS & chicken serum). Replication of the parasite in FCS enriched culture medium and in chicken serum enriched culture medium appeared very close and parallel to each other.

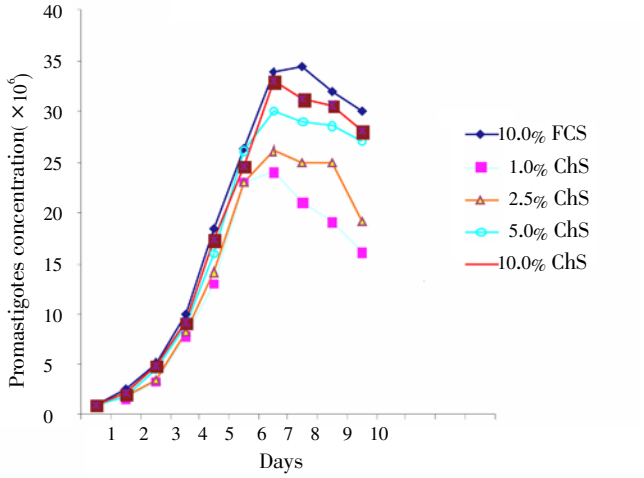

Figure 1. Growth curve of *L. infantum* promastigotes in RPMI-1640 medium plus 10% FCS and different concentrations of chicken serum. FCS: fetal calf serum; ChS: chicken serum.

4. Discussion

In the cultivation of promastigotes of *Leishmania*, the routine commercially culture media like RPMI-1640 that is usually enriched by FCS is expensive[26,27]. In addition, it is not manufactured in many countries, especially most of the poor tropical countries where leishmaniasis is one of the most important health problems for them. Like other birds, chickens do not support *Leishmania* infections and appear to have no direct role in the transmission cycle of zoonotic visceral leishmaniasis and possibly do not harbor the infection 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[28,29].

Herman *et al.* has studied the effects of rabbit, chicken, man, calf, hamster and cotton rat sera on the number and morphology of *Leishmania donovani* (*L. donovani*) as well as cells in cultures of hamster-peritoneal macrophages that had been infected *in vivo*[22]. In a cell-free culture system, Trager *et al.* 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 morphological stages of *L. donovani*. He mentioned that duck serum rapidly destroyed both the *Leishmania* and leptomonad forms at room temperatures[23]. Warren *et al.* and Borsos *et al.*, by using culture forms of *Trypanosoma cruzi* (*T. cruzi*), showed that sera from some germ-free chickens could neither lyse nor agglutinate the flagellates. In contrast, normal chicken serum was found 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 these observations[30,31].

The chicken houses is acknowledged as a possible environmental risk factor for *L. infantum* infection[32-34]. For instance, in Brazil, it has been reported that the dwellings of persons affected by visceral leishmaniasis were about four times more likely to have chicken houses in their yards when compared to people living in the same area but not affected by the disease[35]. This could be explained by the fact that chickens constitute a "natural lure" for phlebotomine sand flies, including *Lutzomyia longipalpis*, the main vector of *L. infantum* in Brazil[36]. Otrantoa *et al.* provided definitive evidence that chickens are refractory to *L. infantum* infection[37].

In our previous researches about finding suitable replacements for FCS, we found that the serum and the urine of many animals like hamster, rabbit, sheep are suitable for cultivation of promastigotes, but collecting of the serum of some of them, is not feasible. Jones *et al.* mentioned that *Leishmania* have been cultivated in the yolk sac of the developing chick embryo successfully[24], and other researches mentioned that they were able to *in vitro* maintenance of *Leishmania* promastigotes in an egg based biphasic culture medium[38].

In previous researches, many sera from different adult animals have been used for the cultivation of *Leishmania* promastigotes, but they exhibited deleterious effects on a growth pattern[39-41]. Sant'Anna *et al.* 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 are unlikely to offer any protection from disease but may, on the contrary, promote parasite growth and development in the vector thus increasing transmission potential[42].

Furthermore, our results show that chicken serum in many aspects is superior to FCS. It is readily available at low cost and its preparation does not require sophisticated expensive equipments and can be collected from any chicken at slaughter house. Unlike the cow, horse and dog whose sera have negative effects on growth of promastigotes, chicken serum is very suitable for the nutritional requirements of parasites and is a comparatively simply available that could be replaced in the media that require FCS enhancement and indicate to a potent new medium that could be used in long-term *in vitro* cultivation of *L. infantum* promastigotes. This study has demonstrated an alternative low-cost serum that

could be used in culture media for primary isolation, routine cultivation and mass cultivation of *Leishmania* parasites.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

The cell culture technique is an approach to prepare complex conditions of beside human living organism's habitat for developing our knowledge about their behavior and finding an effective vaccine to prevent negative side effects of them.

Research frontiers

The new aspects of cell culture and changing pattern of their procedures need many attempts and details, and this article will open a new insight into future works on different cell culture.

Related reports

The authors of article have made very good historical background about the basic knowledge of *Leishmania* cultivation and I think that this is the first report on the successive use of chicken serum for cultivation of *L. infantum*.

Innovations & breakthroughs

Omitting the depending of research to killing a fetus for collecting of their serum and saving money of the tropical poor countries by using this easy available serum is the main result and innovation of this research.

Applications

In the cell biology, we use FCS for cultivation of many different cells and more studies will show whether we can use chicken serum in cultivation of other cells like *Leishmania*.

Peer review

Totally, finding new methods for changing the availability of new tools of vaccine preparation had been a main road in many researches and it seems that this article pointed to one of this beginners methods. The authors did not refer to problems of using the new medium and obviously it has its technical problems.

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