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In Vitro Cultivation and Cryopreservation of *Leishmania Tropica* Promastigotes

Abstract

Leishmaniasis is a parasitic disease caused by protozoans of the genus Leishmania and is a member of the group of neglected diseases. The lack of an effective vaccine and the increasing resistance of the parasites to drugs and of the vectors to insecticides are causing the disease to spread worldwide and remain a serious public health problem in many countries. In recent years, the significant weakening of entomological measures in the endemic regions of our country has led to outbreaks of leishmaniasis in various areas, posing serious epidemiological threats. Although serious measures were taken in our country before the 1990s, the epidemiological situation has significantly worsened over the last 30 years, and the disease has begun to appear in numerous regions of the republic. To date, the species of Leishmania parasites found in the individual endemic regions have not been identified or molecularly typed. Additionally, because isolation of the parasites has not been possible, the susceptibilities of the drug preparations used against them have also not been determined. To solve the aforementioned problems, the isolation of Leishmania parasites, the establishment of their continuous culture, and the creation of a cryobank are of paramount importance. This research article presents in detail the methodological approaches applied for the in vitro cultivation, cryopreservation, and establishment of a cryobank for Leishmania parasites. These studies, conducted at the Department of Vaccine and Nanovaccine Research of the V. Y. Akhundov Scientific Research Institute of Medical Preventive Medicine, provided information on the isolation of parasites, the establishment of continuous cultures, and the creation of a cryobank.

Keywords: *Leishmania tropica*, *leishmaniasis, non-cultivation, biobank, cryopreservation*

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Introduction

Leishmaniasis is a zoonotic, infectious parasitic disease caused by intracellular protozoans of the genus Leishmania (Allahverdiyev et al., 2023; Ayala et al., 2024). According to the World Health Organization (WHO), approximately 1.5–2 million new infections are recorded annually, making it the second most prevalent parasitic disease after malaria. Characterized by three main forms, the most prevalent form of leishmaniasis in Azerbaijan is visceral leishmaniasis (Kelleci et al., 2023). Viral leishmaniasis, recorded in nearly 50 regions of our country, is primarily found in plain, foothill, and mountainous districts (Goychay, Agdash, Barda, Agdam, Tartar, Shamkir, Agjabadi). Azerbaijan is considered an endemic area for leishmaniasis, and there is a need to develop and implement new strategies to combat this disease (Özbel et al., 2022). The main challenges in determining the true epidemiological scale of the disease include limited diagnostic capabilities, inadequate epidemiological surveillance systems, and the absence of an effective vaccine.

Research

In this regard, the identification of Leishmania species, the determination of resistance to antileishmanial drugs, and the conduct of future vaccine research establish an important scientific basis (Allahverdiyev et al., 2023). After many years, for the first time in Azerbaijan, the V. Y. Akhundov Scientific Research Institute of Medical Prevention, Research in the laboratory of the Vaccine and Nanovaccine Research Division on the propagation of *Leishmania tropica* promastigotes, the *in vitro* cultivation of parasites, and the creation of a Leishmania biobank enables the reliable use of local parasite strains for future scientific and experimental purposes. This work will play a significant role in the future production of new vaccines and other scientific research to combat the disease.

Process and cultivation of parasites' extraction from a liquid nitrogen environment

In this study, *Leishmania tropica* (*L. tropica*) promastigotes (MHOM/TR/99EP39) obtained from the Department of Bioengineering at Yildiz Technical University were used. All procedures were performed in the laboratory of the Vaccine and Nanovaccine Research Department at the Scientific-Research Institute of Medical Prevention, in accordance with aseptic techniques. The frozen parasites were taken from the cryobank (BIOBASE LNC-2-30) and thawed in a water bath (Water Bath VS-310). They are then rapidly thawed by gentle agitation in a 37°C water bath for 1–2 minutes. Once the parasites are fully thawed, they are transferred from the cryotube into culture flasks (25 cm^2) pre-added with culture medium. The flask lids are sealed with Parafilm and incubated at 27°C in an incubator (DSI-300D). All work was performed in a laminar flow hood (HF safe 1800LC).

The RPMI-1640 and DMEM culture media are supplemented with 10% fetal bovine serum (FBS), 1% penicillin, and gentamicin, then divided into portions and stored in a refrigerator. HEPES buffer was added to maintain the pH of the culture medium between 6.8 and 8.0.

The cultures were monitored every other day for the detection of promastigotes. Initially, 10^5 parasites were added to the culture medium; the parasites developed and reached the stationary phase within 3–4 days, at which point the parasite number increased to 10^7 (Fig. 1). To ensure the parasites remained in the logarithmic phase, they were passaged to fresh nutrient medium every 72 hours. Starting from the second day of incubation, the parasites' presence, morphology, viability, and changes in number were assessed every 24 hours using an inverted microscope (Ceti InversoTC-100). Additionally, the promastigote's morphological characteristics were analyzed using a staining method.

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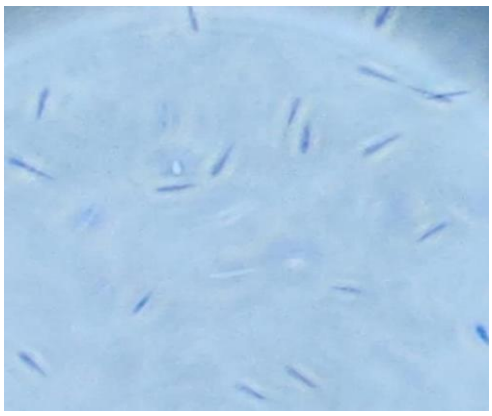
(a)

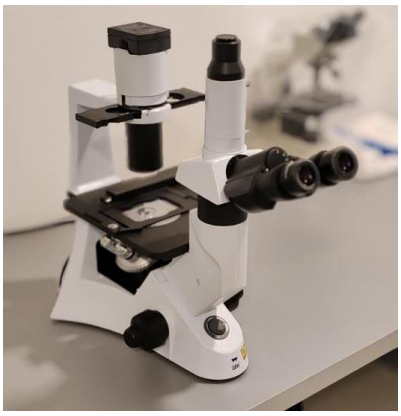
(b)

Figure 1. (a) Appearance of the *Leishmania tropica* parasite under a microscope; (b) Microscope equipment used in the study. (From a culture obtained in the Vaccine and Nanovaccine Laboratory of the ET Institute of Medical Prevention)

Determination of the number of parasites on a Thoma slide

To monitor the parasite multiplication dynamics, counts were performed on a Thoma slide every 24 hours. Due to the motility of promastigotes, direct counting is difficult. Therefore, the parasites were fixed before counting. For this, a sufficient amount of the parasite culture was taken, mixed with a 2% formalin solution in a 1:10 ratio, and incubated at room temperature for 3-5 minutes. During this time, the formalin immobilized the parasites. After fixation, the mixture was homogeneously mixed, and 10 µL was pipetted from the sample onto a Thoma slide and counted using an inverted objective microscope (10×, 20×, 40×) (Fig. 2). The counting results were calculated using the following formula:

Parasite count = Mean cell count x Dilution factor x Thoma slide constant

Parasite count: Number of parasites per 1 ml

Mean cell count: Arithmetic mean of the cells in the 16 squares on the lower and upper halves of the Thoma slide

Dilution factor: Dilution factor used for counting

Thoma blade constant: 10,000


(a)

(b)

Figure 2. (a) Appearance of a slide preparation of the *Leishmania tropica* parasite; (b) The cover slip used in the study. (From a culture obtained in the Vaccine and Nanovaccine Laboratory of the ET Institute of Medical Preventive Medicine)

Morphological Observation

The staining process is performed under sterile conditions in a laminar flow hood. A drop of the cultured *L. tropica* promastigotes is placed on a sterile object slide and spread thinly with another object slide. After the sample is air-dried at room temperature (1–2 minutes), it is fixed with methanol

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for 2–3 minutes. The concentrated Giemsa stain was diluted with distilled water in a 9:1 ratio to prepare a homogeneous solution. Fixed samples are stained with the prepared Giemsa stain and, after a 20 minute wait, are gently washed with distilled water. After the excess stain is removed, the slide is air-dried at room temperature. The morphological characteristics of *L. tropica* promastigotes were observed at 100× magnification (using an oil immersion lens) under a light microscope, and cell forms were evaluated in samples stained with Giemsa stain. Under the light microscope, the parasites are seen as cylindrical in shape, with one end pointed and the other end slightly broader, and the tail portion is clearly visible (Fig. 3).

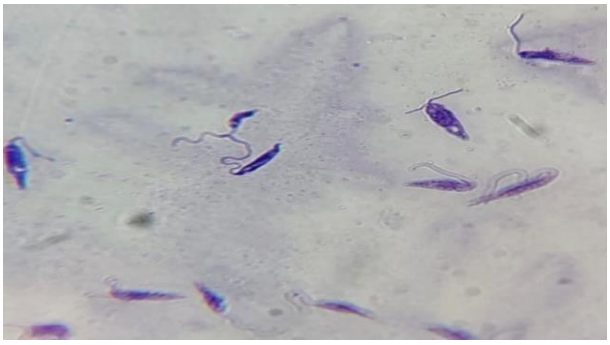

Figure 3. Microscopic appearance of the *Leishmania tropica* parasite, stained with Giemsa stain. (From a culture obtained at the Vaccine and Nanovaccine Laboratory of the ET Institute of Medical Prevention)

Freezing of parasites for long-term preservation

The freezing process is carried out for the storage and long-term preservation of the culture. When the density of *L. tropica* promastigotes reaches approximately 10^7 cells/mL, the samples are frozen under sterile conditions. An appropriate amount of the active promastigote sample is added to each sterile cryotube (LABSERVIS), and 10% dimethyl sulfoxide (DMSO) is slowly added along the inner wall of the tube. The sample and DMSO mixture is then gently pipetted a few times. After the tube caps are tightly sealed, a stepwise freezing procedure is applied: Initially, they are incubated at +4°C for 1 hour, followed by 2 hours in a -20°C freezer (bryusa). After the samples are frozen overnight at -80°C, they are placed in the cryobank system (-196°C, in liquid nitrogen) for long-term storage.

Conclusion

Leishmaniasis, a disease widespread in many tropical and subtropical regions, including Azerbaijan, is considered one of the most important public health problems in 98 countries (Sasidharan & Saudagar, 2021). An epidemiological analysis conducted for the years 2014–2018 determined that the annual average incidence of visceral leishmaniasis in Azerbaijan was approximately 1.7 cases per 100,000 population (Agayev et al., 2020).

The increasing trend in leishmaniosis cases is associated with the emergence of resistance to antileishmanial drugs used in the treatment of the disease (pentavalent antimonials, amphotericin B, miltefosine, etc.) and to insecticides in vectors (Nasiri, 2017). Climate conditions and the dynamics of the vector population also have a significant impact on the spread of the disease. Therefore, studying drug resistance at the molecular level, public awareness, in-depth analysis of the disease's epidemiology, and strengthening vector control measures, as well as the development and implementation of effective vaccine candidates, are considered priority areas in the management of leishmaniasis. The availability of vaccines successfully tested against leishmaniosis in dogs is considered a promising result for the future development of effective vaccines for humans (Morales-Yuste et al., 2022).

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The detection of Leishmania parasites is based on a number of laboratory diagnostic approaches. These methods include culture, cytological, histopathological, serological, and molecular techniques (Allahverdiyev et al., 2023). The *in vitro* cultivation of parasites and their cryopreservation in a biobank is of strategic importance. Various semi-solid, liquid, or two-phase nutrient media are used for the cultivation process (Gow et al., 2022). The most commonly used media include Novy–MacNeal–Nicolle (NNN), Tobie's medium, Schneider's Drosophila medium, RPMI-1640, Brain Heart Infusion (BHI), and blood agar. These media provide different growth patterns depending on the parasite's morphological form and species composition (Ali, 2018). Fetal bovine serum (FBS) used in parasite culture provides growth factors necessary for cell proliferation. However, the high price of FDS and difficulties in its procurement from reliable sources limit the use of this component (de Oliveira et al., 2024). Therefore, in recent years, research has been conducted on the use of various serum replacement formulas and natural extracts that stimulate parasite growth as an alternative to FDS (Allahverdiyev et al., 2012). Parasite in vitro cultivation can be performed using conventional (classic) and microcapillary cultivation methods. The microcapillary method has advantages over classic approaches, such as requiring a smaller sample volume, demonstrating higher sensitivity, providing faster results, and being more cost-effective. The microcapillary cultivation method developed by A. M. Allahverdiyev and colleagues is characterized by high specificity and sensitivity. This method ensures the optimal growth of parasites by creating microaerophilic conditions in capillary tubes (Allahverdiyev et al., 2004).

In the laboratory of the Vaccine and Nanovaccine Research Division, Leishmania parasites are cultivated in vitro, and the resulting samples are cryopreserved and stored in a biobank. Additionally, antigens have been obtained from various species of Leishmania parasites cultivated under in vitro conditions. The results obtained will provide broad opportunities for future research in identifying the cryopreserved Leishmania parasites, determining their susceptibility to drug treatments, and developing nanovaccines.

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