



Original Contribution

CRYOPRESERVATION OF LEISHMANIA DONOVANI PROMASTIGOTES AT -80°C

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ABSTRACT

Visceral leishmaniasis (VL) is one of the important parasitic diseases caused by the obligate intracellular protozoan parasite of *Leishmania donovani* complex. The disease is associated with severe morbidity and is fatal if left untreated. Human VL is rare in Bulgaria, but local cases are registered annually in the country. Diagnosis of the disease is based on clinical and epidemiological data and the different laboratory methods for ethiological diagnosis. Isolation of the causative agent through cultural methods, allows to make an extremely accurate diagnosis, but require time and cost of materials. Cryopreservation of isolated parasites allows their keeping for a long time and recovery in need of subsequent immunological and molecular analyzes.

The described and optimized cryopreservation method of *Leishmania* species promastigotes in laboratory conditions is easily feasible and reliable and can be used for long-term storage of these parasites without changing their viability and biological characteristics.

Key words: cryopreservation, *Leishmania* promastigotes, visceral leishmaniasis.

INTRODUCTION

Visceral leishmaniasis is a protozoan, vector borne disease characterized by chronic course, protracted fever, splenohepatomegaly, and anemia to complete pancytopenia and secondary immunosuppression. It is caused by viscerotropic species belonging to the group of *Leishmania donovani* complex (*Leishmania donovani*, *Leishmania infantum* and *Leishmania chagasi*) (1, 2).

The parasite has a dimorphic life cycle: extracellular stage (promastigotes), which multiply and develop in the digestive tract of specific vectors, different species sand flies, and intracellular amastigotes, which reside and multiply in phagolysosomal vacuoles of

mammalian phagocytes (3). The mechanism of infection is blood transmissible – promastigote forms are transmitted through sand flies vectors and are inoculated into the host by blood sucking. Once got in the host, they are rapidly phagocytosed by macrophages and eventually metamorphosed into amastigotes.

The definitive diagnosis of the disease is based on the identification of parasitic amastigote forms in lymph nodes, bone marrow and spleen aspirates, but these procedures are invasive, with high risk and often impossible to perform. Immunological methods (RIF, ELISA, WB, RDT) are also used, and they have good sensitivity and specificity (2, 4), but do not allow reliable diagnosis of recurrence due to the detection of specific antibodies in serum for a long period after treatment. In recent years, molecular biological methods have been applied with high sensitivity and specificity and make it possible to determine the type of causative agent (5, 6).

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Leishmania parasites can be isolated by inoculation of infectious material in laboratory animals (xenodiagnostics) or in nutrient media (culture methods). The most commonly used diagnostic medium is NNN, a culture developed for cultivation by Novy, McNeal and modified by Nicolle. Nicolle – Novy – MacNeal (NNN) media was first used by Nicolle (1908) to isolate the causative agents of cutaneous leishmaniasis and in its traditional formula is a biphasic blood medium very useful for initial isolation of parasites and for maintaining a strain in culture (7). The modified medium consists of two phases - blood agar and liquid coating layer medium (Locke's solution). The blood agar base is a highly nutrient medium supports the growth of fastidious organisms such as *Leishmania* and *Trypanosoma* and is highly recommended for the primary isolation of *Leishmania* parasites (8). Samples were inoculated into the liquid phase of the biphasic medium and incubated, as a consequence amastigotes transforming to the promastigotes in about 24 hours.

However, conventional methods for preserving and maintaining of these protozoa in culture are too expensive, time consuming and possess high risk of contamination (9). It is also possible that the antigenic, biological and chemical capabilities of organisms change in the environment. Therefore, cryopreservation is recommended as an appropriate method, which ensures long time protection of parasites and allows their subsequent cultivation and investigation (10).

The **aim** of the present study is to introduce an optimized method for long-term storage of *Leishmania* by cryopreservation and to determine its ability for long-term preserving of parasites without changing their vital characteristics.

MATERIALS AND METHODS

Parasite cultivation and in vitro maintenance of promastigotes

In the Department of Parasitology and Tropical Medicine (NCIPD) a laboratory strain of *L. donovani donovani* is maintained. The used cultivation medium is blood agar with overlay nutrient medium (NaCl, peptone, 10% NaOH, 20% NaHCO₃, distilled water, buffer solution of Na₂HPO₄ and KH₂PO₄), antibiotic - penicillin, pH - 7.7). A liquid enrichment medium (7 ml)

was placed on the slant agar, which covered the agar layer and was used for culturing *Leishmania* in the laboratory. Tubes containing the parasites were incubated at $24 \pm 1^\circ\text{C}$ for 2 weeks (1 week the cultures were in the early log phase). Promastigote colonies were visible as white, mucoid patches at the boundary between the two phases. Cultivation is carried out at two-week intervals, than some of the parasites are transferred to a new medium.

Cryopreservation

For the *Leishmania* parasites, a medium created by Baltz et al. (1985) for cryopreservation of *Trypanosoma* was used, which consist of 70% fetal calf serum and 30% glycerol (11).

Leishmania promastigotes monitoring was performed under a light microscope with Giemsa stain.

Promastigotes were counted and calculated at day 1, 7 and 14 using a Bürker counting chamber with a x40 objective under standard light microscopy.

RESULTS

Cryopreservation conditions

Freezing

A two-week culture of *Leishmania donovani* promastigotes was carefully collected at the boundary between the two phases of the culture medium used for cultivation and placed on ice. To the obtained material, which contains enrichment medium and parasites (about 2.5 ml) carefully was added 1 ml cryopreservation medium dropwise (70% fetal calf serum + 30% glycerol (11)). The obtained parasite suspension was transferred to cryotubes (about 1 ml of material), labeled and frozen, cooling gradually by placing the cryotubes first at -20°C and then at -80°C in a laboratory freezer.

Thawing conditions

After certain periods of time (1 month, 6 months and 1 year), one of the cryotubes is removed from the low-temperature freezer (-80°C) and thawed quickly under running hot water (for about 3 minutes). The material containing the parasitic forms (1 ml) was placed in a warm culture medium of *Leishmania* - blood agar with enrichment medium (7 ml) and stored in the dark at room temperature.

Microscopic observation of material prepared from the thawed material in a light microscope (x40) showed the movement of the *Leishmania* promastigotes. The count of parasites from the

thawed material in a Bürker chamber shows the amount of starting material - about 4×10^6 parasites/ml (**Figure 1**).

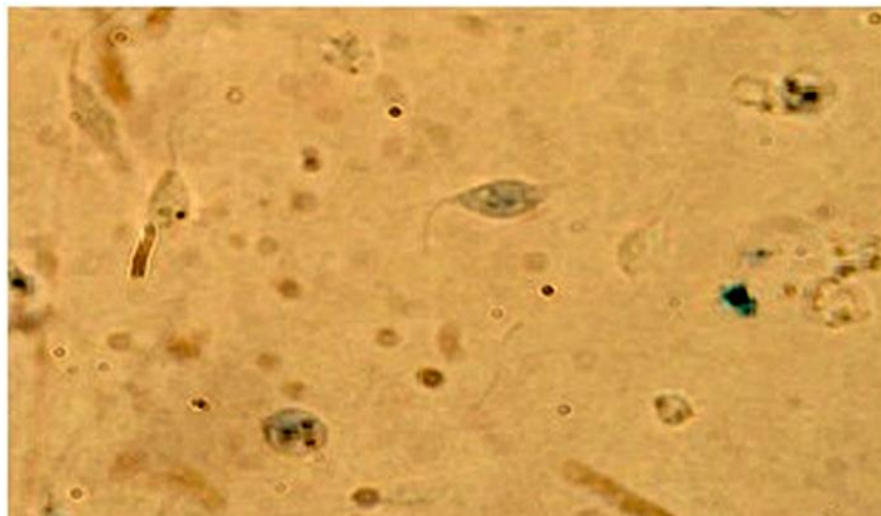


Figure 1. *L. donovani* promastigotes in thawed material (x100).

After inoculation of thawed *Leishmania donovani* material in culture medium appearance of live moving promastigotes were observed still

on the first week after inoculation for all periods of cryopreservation. Two weeks after inoculation a large number of parasites were observed with good motility and viability (**Figure 2**).

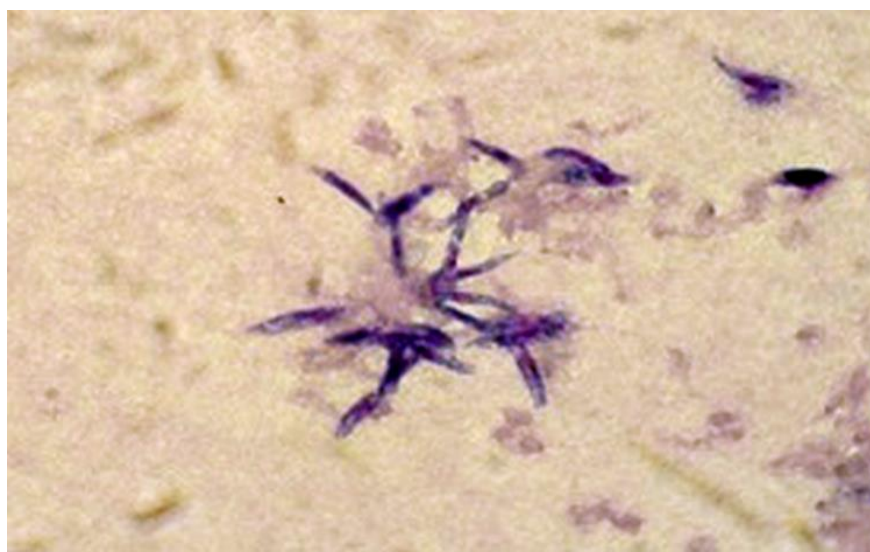


Figure 2. *L. donovani* two-week thawed culture (Giemsa stained, x100).

The frozen parasitic strain was monitored after 1 month, 6 months and one year cryopreservation and successful recovery of parasites was observed for all study periods.

Detailed conditions of cryopreservation of promastigote forms of laboratory strain *Leishmania donovani* are presented in **Table 1**.

Table 1. *Leishmania donovani* cryopreservation conditions at -80°C.

	Freezing condition		Thawing condition	
<i>Leishmania</i> species	<i>L. donovani</i>		<i>L. donovani</i>	
	a two-week culture of parasites		cryopreserved culture (1 ml) of parasites	
Medium	Fetal calf serum - 70%		Fetal calf serum - 70%	
Cryoprotectant	Glycerol - 30%		Glycerol - 30%	
Medium adding	in the ice; in drops		-	
Terms	gradually	-20°C; -80°C	fast	3 min/hot water
Storage	-80°C	1 year	Blood agar medium	2 weeks/24°C/ in the dark

DISCUSSION

Protozoan parasites of the genus *Leishmania* cause a wide range of human diseases, including cutaneous (CL), mucocutaneous, and visceral leishmaniasis (VL) (12). About 20 *Leishmania* species are known to infect humans (13).

Visceral leishmaniasis is a sporadic disease in Bulgaria, but according to Harizanov et al. (2013) human cases are registered almost every year and were reported in 13 of 28 regions of Bulgaria, mainly in the south part of the country. The data show that there is a transmission of this disease in our country with a high mortality rate, mainly affecting children (14).

For the primary *Leishmania* parasites isolation from patient-derived materials, as well as for their subsequent maintenance, *in vitro* cultivation in laboratory conditions of appropriate culture media is recommended (15, 16). Parasite growth in axenic cultures remains the "gold standard" in definitive diagnosis (17), with *in vitro* cultivation playing an important role in the study and treatment of the disease. However, maintaining these protozoa in appropriate nutrient media requires regular (often weekly) maintaining (18) and these methods are expensive and time consuming (19). Therefore, cryopreservation is applied, and with the help of a suitable cryoprotectant allows long-term preservation of parasites without changing their biological characteristics.

With regard to *Leishmania*, a number cryopreservation techniques have been developed and various protocols have been optimized over

the years. As early as 1973, Callow and Farrant compared methods and conditions for cryopreservation of promastigotic forms of *Leishmania tropica major* at different cooling rates. They showed that parasite survival was higher when 1.5 M dimethyl sulfoxide (DMSO) or 1.0 M glycerol was added as a cooling measure of 0.3°C/min and DMSO provided better protection (20). Bhattacharya et al. (1991) studied the cryopreservation of promastigotes of *L. donovani* in NNN and Tobie's media, as the medium containing the promastigotes forms was placed directly at -80°C and tested for live parasites regularly from 9 to 126 days. In all samples, there were live promastigotes that were used for further growth without obvious morphological changes in subsequent subculturing (21).

In 1997 Pereira and Groggl standardized cryopreservation techniques for human biopsy specimens to facilitate the characterization of *Leishmania* parasites, biopsy specimens were frozen in phosphate-buffered saline plus 3% streptomycin/penicillin (SSP solution) with 10% dimethyl sulfoxide (DMSO) and showed that these methods could be used to cryopreserve human biopsies permitting their transportation to a central laboratory (22). In 2000 Kousha and Mohebbali stored these protozoa for 1 year using dimethyl sulfoxide and concluded that cryopreservation was the most effective of the other available methods and was appropriate for *Leishmania* (10).

To determine the success of cryopreservation performed to protect the genetic material and virulence of *Leishmania* species, Çavuş İ. et al. (2017) washed promastigote cultures of *L. infantum*, *L. major* and *L. donovani* in the logarithmic phase 3 times with phosphate-buffered saline and 15% dimethyl sulfoxide. After thawing, they found that all *Leishmania* isolates saved 60-65% of their vitality, and abnormalities in the structure and movement of promastigotes were not observed (23).

It is believed that the concentration and type of cryoprotectant, as well as freezing, and thawing rates are the factors that determine viability after cryoprotection. The preservation of the viability of the cryopreserved parasites according to the methodology developed and described in this study shows that this is a suitable method that can be used for various *Leishmania* isolates, as the parasites can be cultivated and studied without being observed changes in structure and movement. The proposed optimized protocol uses glycerol as a cryoprotectant, which avoids the toxic effects of dimethyl sulfoxide and is less used in *Leishmania*'s proposed cryopreservation techniques. This method is reliable and reproductive for cryopreservation of an adapted culture of parasites and can be used when the cultivation of isolated parasites is not possible.

CONCLUSIONS

Cryopreservation is a specific long-term storage method that saves time and reagents and allows laboratory-maintained parasitic species to remain viable. Determining the optimal conditions for freezing and thawing cryopreserved parasites is an important item, which depends on the type and equipment available in each laboratory.

The developed and described method of cryopreservation of *Leishmania donovani* at -80°C with cryoprotectant glycerol is an easy and reliable method that allows continuous protection of these parasites and their subsequent cultivation in need of study or other purposes.

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REFERENCES

1. Herwaldt B. Leishmaniasis. *Lancet*, 354(9185):1191–1199, 1999.
2. Harizanov R. Visceral leishmaniasis, In: *Clinical Parasitology and Tropical Medicine* (Ed. P. Petrov and R. Kurdova). East-West, pp. 206-214, 2016.
3. CDC. Leishmaniasis: Biology. [Available from: <https://www.cdc.gov/parasites/leishmaniasis/biology.html>].
4. Torres-Guerrero E., Quintanilla-Cedillo M., Ruiz-Esmenjaud J., Arenas R. Leishmaniasis: a review, *F1000Research*, 6:750, 2017.
5. Montavlo A., Fraga J., Montano I., Monzote L., Auwera G., Marín M., Muskus C. Molecular identification of *Leishmania* spp. clinical isolates from Colombia based on hsp70 gene. *Biomedica*, 36:37-44, 2016.
6. Mann S., Frasca K., Scherrer S., Henao-Martinez A., Newman S., Ramanan P., Suarez J. A review of Leishmaniasis: current knowledge and future directions. *Curr Trop Med Rep*, 8:121-132, 2021.
7. Evans D. In: In vitro methods for parasite cultivation: *Leishmania*. Taylor AR, Baker JR, editors. New York: Academic, pp. 52–75, 1987.
8. Grekov I., Svobodová M., Nohýnková E., Lipoldová M. Preparation of highly infective *Leishmania promastigotes* by cultivation on SNB-9 biphasic medium. *J of Microbial Methods*, 87:273-277, 2011.
9. Ahmed N., Cultivation of Parasites. *Trop Parasitol*, 4(2):80-89, 2014.
10. Kousha S., Mohebbali M. Cryopreservation methods for different species of *Leishmania*. *Researcher bulletin of medical sciences (Pejouhanden)*. 2 (18):157-164, 2000.
11. Baltz T., Baltz D., Giroud C., Crockett J. Cultivation in a semi-defined medium of animal infective forms of *Trypanosoma brucei*, *T. equiperdum*, *T. evansi*, *T. rhodesiense* and *T. gambiense*. *The EMBO Journal*, 4 (5):1273-1277, 1985.
12. World Health Organization. Information on the epidemiology and control of the Leishmaniases by country or territory. World

- Health Organization (WHO), Geneva, Switzerland. *Leishmania* 91:30, 1991.
13. Boelaert M., Sundar S. 47-Leishmaniasis. In: Farrar J., Hotez P., Junghanss T., Kang G., Lalloo D., White N., editors. *Manson's Tropical Infectious Diseases* (Twenty-third Edition). London: W. B. Saunders, pp. 631-651, E4, 2014.
 14. Harizanov R., Rainova I., Tzvetkova N., Kaftandjiev I., Bikov I., Mikov O. Geographical distribution and epidemiological characteristics of visceral leishmaniasis in Bulgaria, 1988 to 2012. Surveillance and outbreak reports. *Euro Surveill*, 18(29):pii=20531, 2013.
 15. Palomino J. Peptone-yeast autolysate-fetal bovine serum 10, a simple, inexpensive liquid medium for cultivation of *Leishmania* spp. *J Clin Microbiol*, 15(5):949-950, 1982.
 16. Castelli G., Galante A., Lo Verde V., Migliazzo A., Reale S., Lupo T., Piazza M., Vitale F., Bruno F. Evaluation of two modified culture media for *Leishmania infantum* cultivation versus different culture media. *J Parasitol*, 100(2):228-230, 2014.
 17. Reale S., Maxia L., Vitale F., Glorioso N., Caracappa S., Vesco G. Detection of *Leishmania infantum* in dogs by PCR with lymph node aspirates and blood. *J of Clin Microbiol*, 37:2931-2935, 1999.
 18. Schnur L., Jacobson R. In *The Leishmaniases in Biology and Medicine*, Vol. 1: Biology and Epidemiology, eds Peters W., and Killick-Kendrick R., London Academic Press, pp. 500-541 1987.
 19. Ihalamulla R., Liyanage C., Karunaweera N. A simple device to maintain in vitro cultures of *Leishmania* in tropical countries. *Transaction of the Royal Society of Trop Med Hyg*, 98:315-317, 2004.
 20. Callow L., Farrant J. Cryopreservation of the promastigote form of *Leishmania tropica* var *major* at different cooling rates. *Int J for Parasitology*, 3:77-88, 1973.
 21. Bhattacharya J., Chandra G., Hati A. A simple method for cryopreservation of *Leishmania donovani* promastigotes. *Indian J Med Res*, 93:245-246, 1991.
 22. Pereira T., Grogl M. Standardization of a cryopreservation technique to facilitate the characterization of *Leishmania* parasites. *Mem Inst Oswaldo Cruz*, 92 (Suppl. I), 079, 1997.
 23. Çavuş İ, Ocak F, Kaya T, Özbilgin A. Cryopreservation of *Leishmania* Species in Manisa Province. *Türkiye Parazit Derg*, 41(3):152-155, 2017.