Protocol: Standardization of the Miniculture Technique for Parasitological Diagnosis of *Leishmania* spp.

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Abstract

Culture media are employed for the isolation of numerous biological agents causing diseases in humans and animals. The traditional culture method (TCM) is regarded as the gold standard in the diagnosis of Visceral and Tegumentary Leishmaniasis (VL / TL), as it is known that *Leishmania* spp. have a good growth on culture media used in TCM. The miniculture method consists in the use of tubes with a smaller volume, promoting a microaerophilic environment with high CO_2 levels, which enhances the amastigote-to-promastigote differentiation. One of the disadvantages of this method is the possibility of contamination by bacteria and fungi, originating from the biological samples or the environment. It is important to ensure sterility during sample collection and processing. Therefore, the sample handling must be carried out with sterile material in a biological safety cabinet. This methodology requires trained personnel as well as sites adapted to biosafety standards for handling microorganisms class II. The advantages of miniculture include better sensitivity compared to traditional methods and a shorter time to achieve positivity. Additionally, miniculture is easy to implement, using the same equipment and materials as traditional culture but in smaller volumes, which can lead to cost reduction. Furthermore, like traditional culture, these tubes allow access to the isolate for post-culture analyses, such as species characterization.

Keywords: Visceral Leishmaniasis, Canine Visceral Leishmaniasis, Tegumentary Leishmaniasis, Miniculture, Parasitological diagnosis.

Step 1 - Preparation of Tubes

The tubes used for this procedure must contain a biphasic culture media composed of a solid phase of Novy and McNeal agar modified by Nicolle (NNN) and a liquid phase of Schneider's Insect Medium (S9895 – Sigma) supplemented with 10% fetal bovine serum (FBS) and antibiotics penicillin and streptomycin, according to the previously described protocol (https://dx.doi.org/10.17504/protocols.io.22tggen). The miniculture method, initially described by *Boggild et al., 2008* for the diagnosis of cutaneous leishmaniasis in Peru, involved distinct approaches compared to our protocol. Unlike the original method, which used a single-phase medium and only aspirates, our adaptation employs a biphasic medium, skin fragments and aspirates, and evaluates two types of tubes for the miniculture method.

In the miniculture technique, two tubes with different volumes can be used:

- **Miniculture 1:** Vacuum blood collection tube (13 x 75 mm), made of clear PET plastic, without additives, sterile, with total capacity of 4 mL. In this study the technique was standardized with the **VACUPLAST®** tube (Code: GD040SADT)
- **Miniculture 2:** Polypropylene microtube with an external screw cap and sealing ring, sterile and autoclavable, with total capacity of 2 mL. In this study the technique was standardized with the **Nalgene®** cryogenic vials (Code: 5012-0020)

- In the biological safety cabinet, the tubes should be opened and positioned to facilitate the distribution of the solid phase in a bevel shape.
- After this, the tubes should be exposed to ultraviolet light (UV) for at least 30 minutes.
- Following the aseptic step, the tubes should be prepared as described below:
    - **Miniculture 1:**
        - NNN = 1 mL
        - Schneider = 1.5 mL

- **Miniculture 2:**
  - NNN = 0.5 mL
  - Schneider = 1.0 mL

Step 2 - Collection of Clinical Samples

- Veterinarians perform biopsies of the skin and spleen and aspirates of the bone marrow and lymph nodes in dogs with two positive serological tests for canine visceral leishmaniasis, following euthanasia.
- Lymph nodes and bone marrow aspirates should be seeded on the same day and maintained for a maximum of 6 hours under refrigeration at 4-8°C.
- In the biological safety cabinet, 0.2 mL of each aspirate should be seeded in duplicate in the culture tubes.
- If it is not possible to collect the necessary volume, the collected aspirate volumes should be equally distributed in the culture tubes.
- Tissue fragments are kept in microcentrifuge tubes containing sterile saline solution with penicillin, streptomycin and 5' fluorocytosine, as previously described in protocol (https://dx.doi.org/10.17504/protocols.io.22tggen).
- In the biological safety cabinet, these fragments are washed in another tube containing the same saline solution, gently agitated.
- Subsequently, the tissue fragment is transferred to a third tube containing the same solution and stored at 4-8°C overnight.
- After this period, in the biological safety cabinet, the fragments should be divided into two similar sizes on a sterile Petri dish and seeded separately into the culture tubes containing biphasic culture medium.
- The tubes should be labeled with the encoded patient data and incubated in a Biochemical Oxygen Demand incubator (BOD) at 26-28°C.
- For diagnosis, a small aliquot of the liquid phase from the culture tubes is collected using a sterile pipette.
- This aliquot is placed on a microscope slide, and the examination is performed under an optical microscope with magnification lenses of 100x and 400x.

- Microscopic observations should be performed at the following intervals after sample seeding:
    - 1st reading: 3 to 5 days
    - 2nd reading: 7 days
    - 3rd reading: 14 days
    - 4th reading: 21 days
    - 5th reading: 28 days
- The culture is considered positive when one or more *Leishmania* promastigotes is detected in any of the readings performed.

Step 3 - Maintenance of Parasites Isolated

- After parasite isolation in culture, perform the first passage between 1 and 7 days, according to the intensity of parasite growth.
- Inoculate the entire volume of samples to be expanded into a traditional culture tube containing biphasic NNN medium and Schneider's Insect Medium (S9895 – Sigma) supplemented with 10% FBS and antibiotics penicillin and streptomycin.
- Maintain the tubes in a BOD incubator at a temperature of 26-28°C and monitor parasite growth by observation of an aliquot under the optic microscope.
- After 5-7 days from the initial subculture, based on parasite growth, transfer the entire volume of traditional culture tubes to cell culture flasks (75 cm^2) containing 2 mL of NNN and 3-5 mL of Schneider's Insect Medium (S9895 – Sigma) supplemented with 10% FBS and antibiotics penicillin and streptomycin with an additional supplement of sterile human urine (5%), which was obtained from a healthy male child. The urine should be sterilized on the same day of collection by a vacuum filtration system with 0.22 µm pore size membrane.
- Monitor parasite growth using an inverted light microscope.
- Add culture medium to the flasks at intervals of 2-3 days according to the parasite growth until it reaches the final volume of 10 mL.

- Then, the total culture volume can be centrifuged and the parasite pellet obtained can be cryopreserved in liquid nitrogen for an indefinite period.

References:

BOGGILD, A. K. et al. Optimization of microculture and evaluation of miniculture for the isolation of Leishmania parasites from cutaneous lesions in Peru. **The American Journal of Tropical Medicine and Hygiene**, v. 79, n. 6, p. 847–852, dez. 2008.

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Reagents:

- Antifungal 5' fluorocytosine (stock solution with 10,000 µg/mL)
- Bacteriological agar
- Calcium chloride
- Defibrinated and sterile rabbit blood
- Distilled water
- Hydrochloric acid (HCl)
- Penicillin (stock solution with 63,000 Units / mL)
- Schneider´s Insect Medium – S9895- SIGMA
- Sodium bicarbonate ($NaHCO_3$)
- Sodium chloride (NaCl)
- Sodium hydroxide (NaOH)
- Sterile fetal bovine serum (FBS).
- Sterile human urine
- Sterile saline
- Streptomycin (stock solution with 50,000 µg /mL)

Materials:

- Beaker
- Bistoury
- Cell culture flasks
- Culture tubes
- Filter Paper
- Filter with a membrane porosity of 0.22 microns
- Microcentrifuge tubes
- Microscope slides and coverslips
- Pasteur Pipette
- Sterile Petri dish
- Sterile serological pipette

Equipments:

- Autoclave
- Biological safety cabinet
- Centrifuge
- Freezer
- Incubator (BOD)
- Inverted microscope
- Laminar flow cabinet
- Liquid nitrogen tank
- Magnetic stirrer
- Magnets
- Optical microscope
- pH meter
- Precision analytical balance
- Refrigerator
- Vacuum pump

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