KALADRUG-R: Clinical SOP#H

Indian *L. donovani* isolation from blood samples BHU method

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Author: BHU (Vijay Kumar Prajapati, Saskia Decuypere)

A. Media

I. NNN Medium:

NNN medium in 1L of triple distilled water:

- 50 g Bacto beef (Difco,USA)
- 20 g Neopeptone
- 20 Bactoagar
- 5 g NaCl
- Mix well with magnetic stirrer and adjust pH to 7.4.
- Filter under laminar flow using whatman filter-1 (cat no- 1001090).
- Autoclave the 1 litre medium.
- After autoclave leave it to cool.
- Make 10 new aliquots of 7 ml NNN medium in 15 ml Borosil glass culture tube (Cat No-14991007).
- Keep all the glass tube at 4°C and whenever need to prepare NNN tube then take out 1 aliquot.

II. Preparation of NNN-Blood agar (10 mL) in microtiter plates:

- Take a 7 ml NNN medium aliquot and bring it in liquid condition at 43°C water bath (higher temperature will lyse red blood cells).
- Mix 7 ml of NNN medium with 3 ml of rabbit blood (aspirated from the ear of the rabbit) with the help of sterile pastette.
- Take 96-well micro titer plates (TPP®, Trasadingen, Switzerland) and put them in a vertical slanted position in the laminar flow.
- Continue all the next steps in the laminar flow

- Add 50 µL blood agar (BA) to each well of the 96-well microtitre plate and leave the plate in vertical slanted position so that the blood agar only covers 1 slanted half of the well. This allows examination of parasites growth using an inverted microscope.
- Leave the micro titer plate in laminar flow for 10 minutes in vertical position.
- When the plate is dry, wrap it carefully to guarantee sterility
- Store the wrapped plates at 4 °C for maximum 15 days.

III. Incomplete RPMI-1640 medium for 1000 ml:

- RPMI-1640 powder (Hyclone, Cat No- SH30011.02) 10.4 gm
- Sodium hydrogen carbonate GR (Merck) 2 gm
- Hepes (Himedia, cat no.- RM-380-100g) 2.8 gm
- Penicillin and streptomycin (10 ml) (Gibco, cat No-15140, which contains 10,000 units/mL Penicillin and 10,000 µg/mL streptomycin).
- Gentamycin 1250 µL
- Triple distill water 1000 mL

Make RPMI complete by adding 15% heat inactivated fetal bovine serum and filter it using 0.22 µm filter paper. = **complete RPMI medium = work solution**

IV. Complete M199 for 1000 ml:

- 1X M199 Hank's salt (Gibco, Cat No- 12350)
- Heat inactivated 20% Fetal calf serum
- 1M Hepes (Gibco, cat no.- 15630, 40 ml)
- 10mM adenine in 50 mM hepes (10 ml)- (dilute 500 µL 1M HEPES in 10 mL triple distill water, add 17.0 mg adenine in above)
- 2ml 0.25% hemin (sigma) in 50% triethanolamine (2.5 mg/ml) (2.5 mg Hemin in 1mL 50% TE)
- 0.1% biotin (sigma) in 95% ethanol (1 ml) (10 mg biotin in 95% ethanol)
- Penicillin and streptomycin (10 ml) (Gibco, cat No-15140, which contains 10,000 units/mL Penicillin and 10,000 µg/mL streptomycin).
- L-glutamine (10ml) (100 X Glutamax-I Gibco, cat No- 35050)
- 10X M199 Hank's salt (28.5 ml) (Gibco, Cat No- 11825)

B. Blood preparation

1. 5 mL heparinised blood drawn from patient in 15 mL falcon tube.
2. Divide blood into two parts, 3 ml for buffy coat and 2 ml for PBMC isolation.

C. PBMC isolation of blood

1. Make 1:1 dilution of whole blood with 1x PBS.

2. Take 3 ml Ficoll in a new 15 ml centrifuge tube. (mix Ficoll stock well before taking aliquot)

2. Now with the help of pasture pipette, pipette the blood as a layer on top of the Ficoll by pipetting gently against the side of the tube.

3. Spin at 400g, 30 min, 18°C

4. After centrifugation you observe 3 layers: top is clear supernatant, middle is opaque fluid containing PBMC, and bottom is RBC. Carefully harvest the PBMC layer and transfer to 15 ml tubes.

5. Fill up the tube with PBS, mix well and pellet the cells by centrifugation at 1500 rpm for 10 min at 10°C). Keep the cells at 4°C from this step onwards.

6. Add 500 µl cold ACK lysis buffer (Cat No- 10-548E, Lonza, USA) to lyse red blood cells if necessary, vortex while adding the ACK buffer.

7. Incubate 5 min at room temperature (below 25°C).

8. Wash once more with PBS, pellet cells and resuspend in 5 ml cRPMI.

9. Load the 10 µL suspension cells on Hemocytometer. Count the lymphocytes in Trypan blue (Cat No- 17-924-E, Lonza, USA). Count only viable cells, viable cells are glowing yellow color and dead cell are shrinking black.

D. Buffy coat isolation from blood

1. Buffy coat cells (includes WBC) are isolated from 3 ml of blood by centrifugation at 1300 g for 15 min (=separated thin layer between plasma and red blood cells).

2. Remaining red blood cells in Buffy coat layer were reduced by lysis using 1.5 mL hypotonic (0.2%) NaCl solution for 20 seconds, to achieve an isotonic solution an equal volume of hypertonic (1.6%) NaCl solution was added.

3. The cell pellet was dissolved in 100-150 µl complete M199 media.

E. Parasite isolation from Culture in NNN micro-titer plates

1. Take a BA plate out of storage and add 100 µL of complete M199 medium to each well except first vertical row of the plate (see fig plate setup).

2. Inoculate 150 µl of Buffy coat or PBMC cell suspensions in the first vertical row

3. Make a serial 3-fold dilution from the first to the 12^{th} well (see fig. plate setup)

3. Seal the culture plate with parafilm and keep in incubator at 27°C.

4. Examine cultures on alternate days under the inverted microscope.

5. After detection of positive culture, transfer 100 µL of promastigotes culture flask to culture flask containing complete M199 medium and follow protocol cSOP#G for further culturing.

Reference:

Maurya RS, Mehrotra S, Prajapati VK et al; J. Clin. Microbiol.doi:10.1128/JCM.01733-09. Evaluation of Blood agar micro-titer plates for culturing *Leishmania* parasites to titrate the parasite burden in spleen and peripheral blood of VL patients

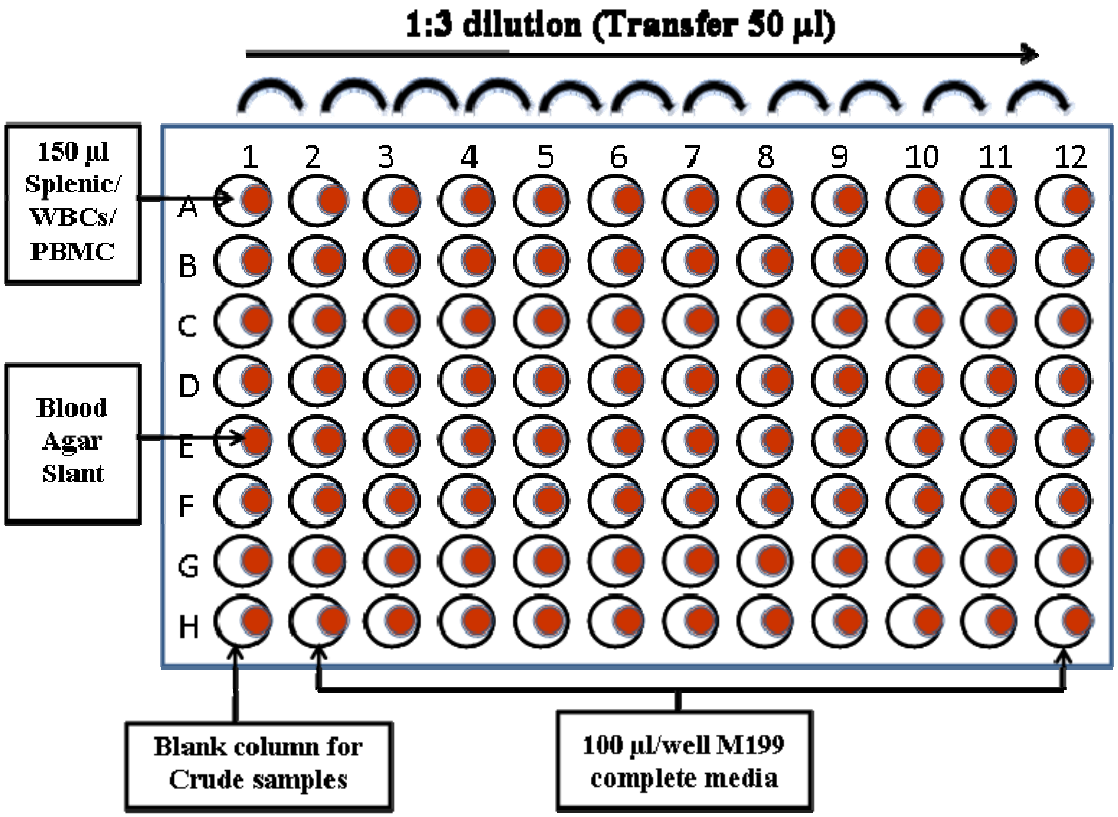

Plate setup for parasite isolation using PBMC or WBC on NNN blood agar micro-titer plates