KALADRUG-R: Clinical SOP#G

Indian *L. donovani* isolation from spleen aspirates BHU method

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A. Medium

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 liter 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.

Preparation of NNN-Blood agar (10 mL) and NNN tubes:

- 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 new NNN tubes.
- Do all this work under laminar flow.
- Put 800 µL of blood agar mixture in each NNN tubes using steri pipette and leave NNN tube in slant position.
- Close the cap of NNN tubes.

- Leave it in laminar flow for 5 minutes, in this time it will be solidified.
- Now store at 4oC (it can be used for maximum 15 days).

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, now 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. Isolation of parasites in NNN-tubes

1. Splenic aspirate is taken by expert physician using sterile syringe in 1 mL complete M199 media.

2. Put 100 µL of diluted splenic material in NNN tube along with 500 µL complete M199 media (biphasic media).

3. Keep NNN tube in 26° C BOD incubator for parasite stage conversion and isolation.

4. Check NNN tube for parasite positivity after 7 days (take one drop of NNN liquid on glass slide and confirm presence of parasite by inverted microscope).

5. If parasites are present on the slide and there density per field is ~ 100 then subpassage to flask with monophasic medium = primary culture (see point C).

5. If the number of parasites during microscopy on slide is ~10 then leave the NNN tube for 3-4 additional days in the incubator. Check parasite density again after 3-4 days, if ~100 parasites per field then subpassage to flask with monophasic medium = primary culture (see point C).

6. If parasites are not present after 7-14 days, consider the NNN tube negative for *Leishmania.*

C. First passage of isolated parasites = PRIMARY CULTURE

1. Take 2 mL complete M199 media in $25cm^2$ culture flask and carefully transfer 1-2 drops of the culture in the NNN tube to the flask, the freshly isolated parasites will now adapt to monophasic media.

2. Keep the M199 flask in BOD incubator at 26°C.

3. Check the flask after 4-5 days, the parasite culture should be in good condition (~ 1 X 10^7 par/mL. For few isolates, it may take up to 10 days to reach that density.

4. When a good density of 1×10^7 per/mL is reached, subpassage the culture and prepare for cryopreservation as described in point D.

D. Second passage of parasite isolate

1. Transfer 100 µL of parasite culture from primary culture flask to a new flask with 5 mL complete M199 medium (make as many flasks as needed for desired cryopreservation).

2. Keep the flask in BOD incubator at 28°C and after 5-6 days the parasite culture will be in good condition (i.e-.1×10^7/ mL).

3. The resulting culture can already be used for cryopreservation (follow Kaladrug-R I-SOP#5) or for DNA isolation (follow Kaladrug-R laboratory SOP#1b).

Reference:
Belkaid, Y., S. Mendez, R. Lira, N. Kadambi, G. Milon, and D. Sacks. 2000. A natural model of Leishmania major infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity. *J Immunol* 165:969-977.