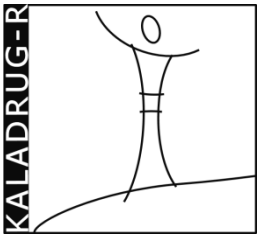
## KALADRUG-R Clinical SOP#A

**BPKIHS *L. donovani* isolation from bone marrow aspirates**

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Author: ITMA

### A. Material required

1. Sterile 2 ml tubes to put 1 ml bone marrow (BM). If less BM, then add Locke without antibiotics (see point E. preparation of Locke) till 1 ml. If BM or spleen is also needed for PCR take 1.5 ml or dilute till 1.5 ml
2. Plastic culture tubes
3. Sterile rabbit blood (see point B. cardiac puncture of a rabbit)

### B. Cardiac puncture of a rabbit

1. A syringe with 19G needle is filled with heparin (0.1 ml = 25 IU heparin/ml blood)
2. Rabbit is placed on a rabbit-stand with its ventral surface facing upwards. Both ears are hold by an assistant.
3. Forelimbs, hind limbs and neck are tied to the stand. Care should be taken that it should be able to breathe.
4. Apex of heart is localised with the surface marking (i.e.mid point between upper and lower border of sternum 0.5cm left laterally, just below the left nipple) and alcohol and hibitane mixture is applied as antiseptic.
5. After feeling heartbeat, the needle is inserted in the intercostal regions until the jerking motion is felt to the syringe.
6. Syringe is fixed with the left hand and required amount of blood is withdrawn with the right hand slowly.
7. Mixing of blood with the anticoagulant is done by gently moving the syringe up and down immediately after its withdrawing.
8. Rabbit is untied and kept back in the cage. Date of bleeding and the amount of blood drawn are noted in a tag attached to the cage. Usually 20 ml of blood is withdrawn from a rabbit weighing 3-5kg every 2 weeks

## C. Preparation of Tobie + Locke

| Ingredients TOBIE'S BLOOD-AGAR (Tobie E.J., 1949) | |
|---|---|
| bidistilled water | 1000 ml |
| Bacto-Tryptose (Difco) | 15.0 g |
| NaCl | 4.0 g |
| $Na_3PO_4.12H_2O$ | 5.0 g |
| KCl | 0.4 g |
| Bacto-Agar (Difco) | 15.0 g |
| pH 7.6, adjusted with HCl or NaOH 1N | |

1. Dissolve by boiling onto heating plate with magnetic stirring
2. Dispense per 80 ml in 250 ml screw-cap bottles
3. Autoclave at 121°C for 20 min and store in refrigerator
4. For use, melt in boiling water or microwave then cool down to 56°C for 30 min in a water bath
5. Add 20 ml rabbit blood obtained by aseptic heart puncture onto heparin (0.1 ml = 25 IU heparin/ml blood)
6. Mix gel and blood, dispense in tubes (±1 ml in 10x100 mm plastic test tubes, flat bottom), slant
7. Incubate 1 tube at 37°C for 24 hrs for sterility control, prepared medium can be kept for maximum 3 weeks.
8. Add between 1 to 3 ml Locke (depending on the growth of the parasites) / tube just before use

| LOCKE (OVERLAY TOBIE) | |
|---|---|
| bidistilled water | 1000 ml |
| NaCl | 8.0 g |
| KCl | 0.20 g |
| $KH_2PO_4$ | 0.30 g |
| $MgSO_4.7H_2O$ | 0.10 g |
| $NaHCO_3$ | 1.00 g |
| Glucose | 2.50 g |
| Penicillin | 200,000 IU |
| Streptomycin | 200,000 µg |
| pH adjusted to 7.4 with HCl or NaOH 1N | |
| Sterilize through a 0.22 µm membrane filter | |

Note: In case Locke is needed to dilute the bone marrow or spleen sample, don't add antibiotics

## D. Inoculation of the medium and maintenance of the culture

1. Two Tobies tubes, each with 1.5 mL Locke overlay, are inoculated with 0.5ml BM just below the surface, and incubated at room temperature or in a stove at 26°C. One tube without antibiotics and one with antibiotics. (see table use antibiotics)

**Use antibiotics in culture:**

| | |
|---|---|
| **A.** Maximum non-toxic concentration of αβ/ml culture medium: | |
| Penicillin | 5000 IU/ml |
| Gentamycin | 200 µg/ml |
| **B.** Commercial vials (to be kept at 4°C) | |
| **Penicillin** (Benzylpenicillin natrium) 6g | 10.000.000 IU/vial |
| Add 10 ml of sterile $H_2O$ to make your stock solution, (1.000.000 UI/ml) store at -20°C<br>Add 7 µl to a Tobies + 1.5 mL Locke before inoculation | |
| **Gentamycin** (gentamycin sulphate) | 50 mg/ml |
| Ready to use, store at -20°C<br>Add 2 µl to Tobies + 1.5 mL Locke before inoculation | |
| **! Streptomycin** is not used for isolation as it is inhibiting partially transformation from mammalian to procyclic stage | |

2. Culture is checked each week during at least 4 weeks, when positive transfer a small amount with a pipette and inoculate to fresh biphasic medium such as Tobie + Locke.
3. Cultures are sub-cultured to fresh medium each 3 or 4 days, when there is sufficiently grow, parasites are cryo preserved (see laboratory SOP#5).
4. Keep meticulous record of all parasite isolation attempts and culture growth in the lab culture book.