

APPENDIX 4

Culture of *Leishmania* from aspirate or biopsy samples

Wherever possible use a blood agar based culture medium, as for initial isolation of *Leishmania*, they are the most reliable. In VL, isolations are usually made from aspirates of bone marrow, spleen or lymph gland. Aspirated material is taken aseptically, and into an anticoagulant in the case of bone marrow and spleen, and inoculated into one of the culture media described below. Do not inoculate large volumes of marrow or splenic aspirates as these contain substances inhibitory to the growth of *Leishmania* promastigotes. Using aseptic precautions inoculate two or at most three drops of splenic or marrow aspirate into each culture tube. Inoculate several tubes, and incubate at 25°C or below. Examine under a microscope a small drop of culture medium taken aseptically from each tube every 48 hrs for the growth of promastigotes. The majority of cultures destined to become positive will do so within 7 - 10 days. Those still negative at 10 days should be blind passaged into fresh culture medium and then examined as before. Discard any culture still negative after 20 days.

Culture media recipes

Proline Balanced Salts Solution (PBSS)

PBSS although not a complete culture medium in itself is a component of most of the media described below, and a stock of this should always be available. Its composition is as follows:

KCL	0.4 gm
NA ₂ HPO ₄ . 12H ₂ O	0.06 gm
KH ₂ PO ₄	0.06 gm
CaCl ₂ . 2H ₂ O	0.185 gm
MgSO ₄ . 7H ₂ O	0.1 gm
MgCl ₂ . 6H ₂ O	0.1 gm
NaCl	8.0 gm
L-proline	1.0 gm
Phenol red	0.001 gm
Distilled water	1000 ml

Dissolve the ingredients one at a time in approx 70 ml of distilled water. Adjust the pH to 7.2 with a few crystals of Tris (Tris[hydroxymethyl]-aminomethane), make up the volume to 1000 ml, dispense into convenient screw-capped bottles, and sterilise by autoclaving at 121°C for 15 min. Store preferably at 4°C, although it will keep for several months at room temperature.

"Sloppy" Evans Medium

One of the most successful culture media for isolating *Leishmania* from patients with VL is "Sloppy Evans" medium.

PBSS (see above)	85 ml
Bacteriological peptone	0.1 gm
Beef extract	0.03 gm
*Defibrinated rabbit blood	15 ml
Agar (plain non-nutrient)	0.3 gm

To prepare, mix the ingredients (*omit the defibrinated rabbit blood*) in a screw-topped bottle. Sterilise by autoclaving at 121°C for 15 min, cool to about 50°C, add the blood then a solution of gentamycin to give a final concentration of 50 µg/ml of medium (i.e. 5 mg gentamycin in 100 ml of complete medium as above). Mix well and dispense while still molten into suitable sterile tubes or bottles (3 ml into a bijou bottle of 7 ml capacity is ideal). Inoculate the material aspirated from the patient deeply into the sloppy agar.

Biphasic blood agar media

(a) NNN medium

Solid phase: heat 1.4 gm agar (plain non-nutrient), 0.6 gm NaCl and 90 ml of distilled water together in a flask; keep the contents well mixed until the agar melts. Transfer the molten agar to a screw-topped bottle and sterilize by autoclaving at 121°C for 15 min, cool to about 50°C, add 10 ml of defibrinated rabbit blood to which 5 mg gentamycin has been added, then dispense whilst molten into sterile culture tubes or bottles. Place the tubes or bottles in a sloped position until the agar has set, then transfer to a refrigerator.

Liquid phase: classically this consists of the water that condenses at the bottom of the slopes, but in practice most workers add additional liquid phase such as PBSS or even sterile distilled water. If additional liquid phase is added do not add more than 5 drops to a slope made in a 7 ml bijou bottle. Inoculate the material from the patient into the liquid portion of the medium.

* Rabbit blood collected aseptically and agitated with sterile glass beads to remove fibrin.

(b) USMARU medium₁ (DIFCO blood agar medium)

Solid phase: 4 gm 'Bacto' blood agar base and 100 ml distilled water. Preparation as for NNN medium, including the addition of defibrinated rabbit blood and gentamycin. The liquid phase is also the same as for NNN.

Notes

Use of bloods other than rabbit blood in biphasic media

If you cannot obtain rabbit blood it is worthwhile trying other bloods that may be available. Sheep, horse or human bloods have all been used with some degree of success. Use them either defibrinated or with an anticoagulant, but always heat inactivate (56°C for 30 min) before use and if possible increase the agar-agar concentration in the medium to 2%.

Storage. Store prepared tubes at 4°C, these media are best used within one week of adding the blood, and ideally should be discarded after three weeks storage at 4°C. The medium without the blood may be stored for several months at ambient temperature.