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ศูนย์วิทยาการ  
 จุฬาลงกรณ์มหาวิทยาลัย

## Appendix

### Reagent for acid-fast staining

#### 1. Ziehl - Neelsen stain

##### a. Carbol - fuchsin

Basic fuchsin 3.0 g

Ethanol, 95% 10.0 ml

Phenol solution 5% in carbolic acid 90.0 ml

Dissolve the basic fuchsin in the ethanol, mix the basic fuchsin solution with phenol solution. Let stand for several days and filter before use.

##### b. 3% Acid alcohol

HCl (concentrated) 3 ml

Ethanol, 95% 97 ml

##### c. Methylene blue counterstain

Methylene blue chloride 0.3 g

Distilled water 100.0 ml

#### 2. Kinyoun stain

The formula is similar to Ziehl-Neelsen except concentration of basic fuchsin which is ten fold of basic fuchsin in Ziehl-Neelsen stain.

### Staining procedures

#### 1. Ziehl - Neelsen staining procedure

a. Cover the heat-fixed film with strong carbol fuchsin.

b. Heat the stain-covered slide with a flame to steaming but do not allow to dry, and kept it steaming for 5 minutes, replenished the stain if necessary.

c. Wash with water, and allow to drain.

- d. Decolorize with 3% acid-alcohol until no more stain appears.
- e. Wash with water, and allow to drain
- f. Counterstain with methylene blue for 1 to 2 mins.
- g. Wash with water, and air dry.
- h. Examine with 100% oil immersion objective over the entire smear.

Mycobacteria are stained red and the background light blue.

## 2. Kinyoun staining procedure (cold method)

The procedure is similar to the former method but there is no heat in step b. So, strong carbol fushsin is used for 5 minutes and then is followed as step c to h.

### Culture media

#### 1. Lowenstein - Jensen media

|   |        |    |
|---|--------|----|
| KH <sub>2</sub> PO <sub>4</sub> anhydrous | 2.40   | g  |
| MgSO <sub>4</sub> . 7H <sub>2</sub> O     | 0.24   | g  |
| MgCO <sub>3</sub>                         | 0.60   | g  |
| Asparagine                                | 3.60   | g  |
| Potato flour                              | 30.00  | g  |
| Glycerol                                  | 12.00  | ml |
| Distilled water                           | 600.00 | ml |
| Homogenized whole eggs                    | 1,000  | ml |
| Malachite green 2%                        | 200.00 | ml |

a. Dissolve the salts and asparagine in the water.

b. Admix the glycerol and potato flour, autoclave at 121° C for 15 mins., and cool to room temperature.

c. Scrub eggs in 5% soap solution. Allow to stand for 30 mins. in soap solution : then rinse thoroughlt in cold running water.

- d. Immurse in 70% ethyl alcohol for 15 mins.
- e. Break eggs into a sterile flask. Homogenize by hand shaking and filter through four layers of gauz.
- f. Add homogenized eggs to the potato salt mixture.
- g. Prepare the malachite green and admix thoroughly.
- h. Dispense 5 ml screw-capped tubes.
- i. Slant and inspissate at 85° C for 50 mins.
- j. Incubate for 48 hr at 37° C to check sterility and store at 4° to 20° C.

## 2. Ogawa and Kudo's P.D. Media

|                                 | Ogawa | Kudo's P.D. |    |
|---------------------------------|-------|-------------|----|
| KH <sub>2</sub> PO <sub>4</sub> | 3     | 2           | g  |
| Mg citrate                      | -     | 0.1         | g  |
| Sodium glutamate                | 1     | 0.5         | g  |
| Soluble starch (Dextrin)        | -     | 3           | g  |
| Homogenized egg                 | 200   | 200         | ml |
| 2% Malachite green              | 5     | 4           | ml |
| Pyruvic acid                    | -     | 0.2         | g  |
| Distilled water                 | 100   | 100         | ml |

### Procedure for Ogawa media

- a. Dissolve KH<sub>2</sub>PO<sub>4</sub>, sodium glutamate in distilled water by heating.
- b. Add glycerol, 2% malachite green and homogenized egg and mix together.
- c. Add these mixture to screw-capped tube container (5 ml/tube) and then incubate at 90° C, 90 min in the slope position (45°)
- d. Incubate for 48 hr at 37° C to check sterility and store store at 4°-10° C.

Procedure for Kudohs' P.D. media

a. Dissolve  $\text{KH}_2\text{PO}_4$ , sodium glutamate, Mg citrate and dextrin in distilled water and then sterile by autoclave at  $121^\circ\text{C}$ , 30 min, then add homogenized egg and then homogenate these mixture.

b. Add glycerol, 2% malachite green and pyruvic acid, mix together.

c. Contain these mixture with screw-capped tube container (5 ml/tube) and then incubate at  $90^\circ\text{C}$ , 60 min in the slope position ( $45^\circ\text{C}$ ).

d. Incubate for 48 hr at  $37^\circ\text{C}$  to check sterility and store at  $4 - 10^\circ\text{C}$

3. Middlebrook 7H10 media

Solution 1 : keep at room temperature

|                          |     |    |
|--------------------------|-----|----|
| $\text{KH}_2\text{PO}_4$ | 15  | g  |
| $\text{K}_2\text{HPO}_4$ | 15  | g  |
| Distilled water          | 250 | ml |

Solution 2 : keep at  $4 - 10^\circ\text{C}$

|   |     |    |
|---|-----|----|
| Ammonium sulfate                                  | 5   | g  |
| Monosodium glutamate                              | 5   | g  |
| Sodium citrate. $2\text{H}_2\text{O}$ , U.S.P.    | 4   | g  |
| Ferric ammonium citrate                           | 0.4 | g  |
| Magnesium sulfate. $7\text{H}_2\text{O}$ , A.C.S. | 0.5 | g  |
| Biotin, in 2 ml of 10% NaOH                       | 5   | mg |
| Distilled water aq                                | 250 | ml |

Solution 3 : keep at  $4 - 10^\circ\text{C}$

|  |     |    |
|--|-----|----|
| $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , A.C.S. | 50  | mg |
| $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , A.C.S. | 100 | mg |
| $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , A.C.S. | 100 | mg |
| Pyridoxine HCl                                     | 100 | mg |
| Distilled water aq                                 | 100 | ml |

Solution 4 : keep at room temperature

Glycerol reagent

Solution 5 : keep at 4-10° C, in light-resistant container

Malachite green 0.01% solution

Procedure

|                    |        |
|--------------------|--------|
| a. Distilled water | 975 ml |
| Solution 1         | 25 ml  |
| Solution 2         | 25 ml  |
| Solution 3         | 1 ml   |
| Solution 4         | 5 ml   |

Mix these solution together

b. Adjust the pH of these solution to 6.6 by using

6 N HCl

c. Add solution 5, 2.5 ml and agar 15 g

d. Autoclave at 121° C, 15 min

e. After cooling, add OADC enrichment (commercial product)

100 ml, Homogenate and then contain the media with screw-capped tube  
(5 ml/tube) or plate (20 ml/plate)

4. Middlebrook 7H11 media

|                                   |         |
|-----------------------------------|---------|
| Middlebrook 7H10 media            | 1 litre |
| Pracreatic digest of casein U.S.P | 1.0 g   |

5. Selective Middlebrook 7H11 media

|                        |             |
|------------------------|-------------|
| Middlebrook 7H11 media | 1 litre     |
| Carbenicillin          | 0.05 mg     |
| Amphotericin-B         | 0.01 mg     |
| Polymyxin-B            | 20,000 unit |
| Trimethoprim           | 0.02 mg     |

6. Sula liquid media

|                           |          |    |
|---------------------------|----------|----|
| $\text{Na}_2\text{HPO}_4$ | 2.5      | g  |
| $\text{KH}_2\text{PO}_4$  | 1.5      | g  |
| Sodium citrate            | 0.5      | g  |
| Asparagine                | 2.0      | g  |
| Alanine                   | 0.15     | g  |
| Glycerin                  | 25.00    | ml |
| Ferri ammonium citrate    | 0.05     | g  |
| malachite green (0.2%)    | 1.00     | ml |
| Distilled water aq        | 1,000.00 | ml |

7. Middlebrook 7H9 media

|                           |       |    |
|---------------------------|-------|----|
| $\text{KH}_2\text{PO}_4$  | 1.0   | g  |
| $\text{Na}_2\text{HPO}_4$ | 2.5   | g  |
| L-Glutamic                | 0.5   | g  |
| Sodium citrate            | 0.1   | g  |
| Ammonium Sulphate         | 0.5   | g  |
| Pyridoxine                | 0.001 | g  |
| Ferric ammonium citrate   | 0.04  | g  |
| $\text{MgSO}_4$           | 0.05  | g  |
| $\text{ZnSO}_4$           | 0.001 | g  |
| $\text{CuSO}_4$           | 0.001 | g  |
| Biotin                    | 0.5   | g  |
| $\text{CaCl}_2$           | 0.5   | g  |
| Distilled water           | 900   | ml |
| ADC enrichment            | 100   | ml |

8. Fluid media

|                           |     |   |
|---------------------------|-----|---|
| $\text{KH}_2\text{PO}_4$  | 1.5 | g |
| $\text{Na}_2\text{HPO}_4$ | 2.5 | g |
| Sodium citrate            | 1.5 | g |
| $\text{MgSO}_4$           | 0.5 | g |

|                        |      |    |
|------------------------|------|----|
| Casitone               | 5.0  | g  |
| Feric ammonium citrate | 0.05 | g  |
| Glycerine              | 25   | ml |
| Malachite green        | 1    | ml |
| Distilled water sq     | 1000 | ml |

**9. Kirchner media**

|  |     |    |
|--|-----|----|
| $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ | 19  | g  |
| $\text{KH}_2\text{PO}_4$                             | 2   | g  |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$            | 0.6 | g  |
| Sodium citrate                                       | 2.5 | g  |
| L-asparagine   | 5   | g  |
| Glycerine  | 20  | ml |
| 0.4% phenol red                                      | 3   | ml |
| Casein hydrolysate                                   | 0.5 | g  |
| Distilled water sq                                   | 900 | ml |
| Calf serum   | 100 | ml |

**10. Selective Kirchner media**

|                |         |       |
|----------------|---------|-------|
| Kirchner media | 1       | litre |
| Carbenicillin  | 100     | mg    |
| Amphotericin-B | 10      | mg    |
| Polymyxin-B    | 200,000 | unit  |
| Trimethoprim   | 10      | mg    |

**11. Transport media**

|   |         |      |
|---|---------|------|
| $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ | 200     | g    |
| Ammonium sulphate                                   | 5       | g    |
| $\text{MgSO}_4$                                     | 500     | mg   |
| Ferric ammonium citrate                             | 250     | mg   |
| Penicillin  | 100,000 | unit |
| Distilled water to                                  | 1000    | ml   |

Liquid media procedure

All ingredients were dissolved in distilled water and then sterile by autoclave at 121°C, 15 min, after cooling. Middlebrook 7H9 were added with ADC enrichment in 10% of media volume, Kirchner media were added with calf serum in 10% of media volume and were made selective by the addition of antibiotics, Transport media were also added with penicillin after cooling.

All media were dispensed in Erlenmeyer-flask in amount of 40-50% of container volume, stored at 4°-10° C.

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