ANNEXURE - I
MEDIA AND REAGENTS

I. ZIEHL-NEELSEN METHOD

Ziehl-Neelsen carbol fuchsin stain -

Basic fuchsin 5 gm
Phenol 25 gm
Alcohol (95 % of absolute) 50 ml
Distilled water 500 ml

- The fuchsin was dissolved in phenol by placing them in a liter of flask over a boiling water bath for about 5 minutes,
- The contents were shaked from time to time.
- Alcohol was added to complete solution and mixed thoroughly.

De-colorizing agent 25% sulphuric acid
Water 750 ml
Concentrated sulphuric acid 250 ml

- The acid was poured slowly down the side of the flask into the water about 50 ml at a time.

Counter stain: Methylene blue
Methylene blue chloride - 100 µgm
Distilled water - 100 ml
• Methylene blue were mixed in distilled water and stored in a amber colored bottle after labeling with name of the reagent and dates of preparation and date of expiration.
• It was stored at room temperature for 6-12 months.

II. PREPARATION FOR LOWENSTEIN-JENSEN MEDIA

Ingredients for 1600ml

- Asparagine 3.60gm
- Monopotassium Phospate 2.50gm
- Magnesium Citrate 0.60gm
- Magnesium Sulphate 0.24gm
- Potato flour 30.00gm
- Malachite green 0.40gm
- Eggs (fresh, whole) 1000.00ml
- Glycerol 12.00ml
- Distilled water 600ml

Then all above mixture were autoclaved and cooled it at room temperature.

Now 1000ml homogenized eggs were added with this mixture.

HOMOGENIZATION OF WHOLE EGG

Fresh hen’s egg not more than 7 days old are cleaned by scrubbing thoroughly with a hand brush in warm water and a plain alkaline soap. The eggs were soaked for 30 minutes in the soap solution. Eggs were rinsed thoroughly in running water and
soaked in absolute alcohol for 30 minutes. The eggs were cracked with a sterile probe in to a sterile bottles containing glass beads and homogenized by hand shaking.

III. PREPARATION OF PETROFF’S METHOD

(Decontamination method)

Reagent Preparation

A. Sodium hydroxide – Trisodium citrate

- 40g of NaOH was dissolved in 1000ml of distilled water (DW) in a separate clean and dry flask

- 29g of trisodium citrate (di-hydrate) or 26g of trisodium citrate (anhydrous) was dissolved in 1000ml of Distilled Water in a separate clean and dry flask.

- Both were autoclaved at 121°C for 15 minutes.

- Equal volume of NaOH and trisodium citrate were mixed aseptically just prior to use. The final volume was prepared according to the need.
B. NALC-NaOH reagent

- 0.5% of NALC was added to the above sodium hydroxide-trisodium citrate mixture. This always should be prepared fresh just prior to use.

<table>
<thead>
<tr>
<th>Total volume required for Decontamination (ml)</th>
<th>Sterile NaOH - Na citrate (ml)</th>
<th>NALC(g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>50</td>
<td>0.25</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>0.5</td>
</tr>
<tr>
<td>250</td>
<td>250</td>
<td>1.25</td>
</tr>
<tr>
<td>500</td>
<td>500</td>
<td>2.50</td>
</tr>
<tr>
<td>1000</td>
<td>1000</td>
<td>5.0</td>
</tr>
</tbody>
</table>

C. Phosphate buffer 0.067 M, pH 6.8

9.47g of disodium hydrogen phosphate (Na$_2$HPO$_4$, anhydrous) was dissolved in 1000ml distilled water in a clean flask.

- 9.07g of potassium dihydrogen phosphate (KH$_2$PO$_4$) was dissolved in 1000ml Distilled water in a clean flask.

- Equal volumes of both were combined and mixed thoroughly.

- The pH was checked with a calibrated pH meter.
This was autoclaved for 15 minutes at 121°C.

3.5ml 0.2% phenol red indicator was added to 1000ml of the above prepared reagent. The pH should be just neutral (pH6.8). If the colour of the indicator was yellow (acidic) or dark pink (alkaline), the pH was adjusted by using sterile acidic buffer or alkaline buffer accordingly, to bring the pH to neutral.

IV. SENSITIVITY DRUGS

ISONIAZID (H)

Stock solution preparation:
20mg of isoniazid was weighed in 40 ml of sterile distilled water (500 g/ml).

Working solution:
The working solution was prepared on the day of drug media preparation (Sterilise by filtering through a 0.22 membrane filter).

2 ml of stock solution (500 g/ml) + 48 ml of sterile distilled water (=50 ml of 20 g/ml)
ETHAMBUTOL (E)

**Working solution preparation:**

20mg of ethambutol was weighed out and dissolved in 100 ml of sterile distilled water to get 200 μg/ml of stock solution. It was sterilised by filtering through a 0.22 membrane filter.

The working solution was prepared on the day of drug media preparation (Sterilise by filtering through a 0.22 membrane filter). Stock solution of E was not stored.

DIHYDRO STREPTOMYCIN SULPHATE (S)

**Working solution:**

20mg of dihydro streptomycin sulphate was added in 50 ml of sterile distilled water to obtain 400 μg/ml of stock solution. It was sterilised by filtering through a 0.22 membrane filter.

RIFAMPICIN (R)

**Working solution:**

40mg of rifampicin was weighed and dissolved in 5 ml of absolute methanol, followed by addition of 5 ml of 99% ethanol to get 4000 μg/ml of stock solution. This solution was not stored.

2 ml of stock solution (500 μg/ml) + 48 ml of sterile distilled water (=50 ml of 20 μg/ml)
V. MEDIA FOR IDENTIFICATION TESTS

PNB-

0.5 gm PNB was weighed and dissolved in the minimum amount of dimethylformamide (~ 15ml). It was added to 1litre of LJ, distribute & was insipissated once for 50 minutes at 85°C. These were stored in cold room.

NIACIN TEST

REAGENTS

1) O-tolidine-1.5%

O-tolidine – 1.5g
Ethanol – 100ml

It is mixed in an amber bottle and was stored in the dark in the refrigerator, it was prepared fresh weekly.

2) Cyanogen bromide solution, approx. 10%

A saturated aqueous solution of cyanogen bromide is approx 10%. It was stored at 4°C in the refrigerator.
CATALASE TEST at 68°C/pH 7.0

Reagents

1) 0.067M phosphate buffer solution, pH 7.0

\[
\begin{align*}
\text{Na}_2\text{HPO}_4 &\quad - 9.47\text{g} \\
\text{Distilled water} &\quad - 1 \text{ litre}
\end{align*}
\]
Disodium phosphate was dissolved in water to provide 0.067 M solution
Solution 1

\[
\begin{align*}
\text{KH}_2\text{PO}_4 &\quad - 9.07\text{g} \\
\text{Distilled water} &\quad - 1 \text{ litre}
\end{align*}
\]
It was dissolved in water to give 0.067 M KH$_2$PO$_4$ solution
Solution 2

61.1 ml of solution 1 was mixed with 38.9 ml of solution 2. The pH was adjusted to 7.

2) Hydrogen peroxide, 30%. It was stored in the refrigerator

3) Tween-80, 10%

\[
\begin{align*}
\text{Tween -80} &\quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots10\text{ml} \\
\text{Distilled water} &\quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots90\text{ml}
\end{align*}
\]
Tween-80 was mixed with distilled water and autoclave at 121°C for 10 minutes. It was allowed to cool & stored in the refrigerator

4) Complete catalase reagent (Tween-peroxide mixture):
Immediately before use, equal parts of 10% Tween-80 and 30% hydrogen peroxide were mixed. 0.5ml reagent were used for each strain to be tested.