Chapter-3

Material and Methods

Study Area:

Saharanpur is situated at longitude 77°09’16” East and latitude 30°61’67” North the different parts of Saharanpur Lohani Sarai, Dholikhal, Nakhasa Bazar, Shada Nagar, Ram Jeevan Nagar, and its adjoining areas (dudhali, Kailashpur, Pilakhni, Paper mill road, Tapri, Hareti) were selected for this study. These areas have a population of approximately two lacs. The blood and sputum samples of suspected tuberculosis patients were collected almost daily (except holiday) from different centre’s and clinics such as Urmila Nursing Home, ITC Medical Centre, Mittal Diagnostic centre and personally from the villages. These samples were examined at Pathology centre, Saharanpur and in the laboratory of Shree Bankey Bihari Group of Institutions, Meerut for different Parameters.

The persons reactive to Mantoux test were considered to be positive (Induration diameter ≥ 10mm) and negative (Induration diameter < 10 mm) on the basis of induration at the site of injection on the arm. The mantoux negative cases were considered as normal. The mantoux positive cases were further examined for sputum AFB test to categories them into AFB positive (infectious) and AFB negative (non-infectious) groups. These were subjected to three consecutive sputum examinations for the AFB before declaring them AFB negative or AFB positive i.e. bacillary cases. Normal cases were the mantoux negative. The acute and chronic cases were grouped under the term symptomatic. i.e having symptoms particular to pulmonary tuberculosis.

A. EPIDEMIOLOGY OF PULMONARY TUBERCULOSIS (PTB):

A total number of 1430 suspected patients of pulmonary tuberculosis were examined for tuberculosis infection and were categorized into different groups as follows-

1. Seasonal (Temperature, humidity and rainfall).

2. Infected/Reinfected
3. Age (0-10, 11-20, 21-30, 31-40, 41-50, 51-60, 61-70 and >70 years).

4. Sex (Male and Female)

5. Socio economic Classes/Social status (Lower, Middle and Upper)

6. Habit (Vegetarians and Non-vegetarians, Smokers and Non-smokers)

7. Habitat (Rural and Urban)

8. Blood group (ABO)

The ecological parameters viz. temperatures, humidity and rainfall were observed and recorded almost daily. Temperature, humidity and rainfall were measured using ordinary Thermometer, Arenoid hygrometer and Rain gauge respectively.

(a) Diagnosis :

(i) Mantoux Test/Tuberculin Skin Test :

The Mantoux test (Mx. test) is the preferred and standard skin test for detecting TB.

Principle :

Infection of \textit{M. tuberculosis} results in hypersensitivity to tuberculoproteins in the body intradermally injected purified tuberculin PPD (Purified Protein Derivative) produces erythema and induration of the skin around the point of injection. The diameter of induration is directly proportional to the degree of sensitisation.

Test Procedure :

Mantoux text was performed by injecting SPAN's Tuberculin PPD 5 TU (Tuberculin units)/0.1 ml. with tuberculin syringe intradermally.

1. The preferred site the test was the flexor or dorsal surface of the forearm about 4 inches below the elbow joint.

2. The skin at the chosen site was cleaned with spirit and allowed to dry.

3. The stopper of the PPD vial was cleaned with spirit and 0.1ml of tuberculin PPD solution was drawn into the sterile tuberculin syringe fitted with a short 26-gauge needle.
4. Then PPD was injected by inserting the tip of the needle into the most superficial layers of the skin with the needle bevel pointing upwards. As the solution was injected, a pale white bleb, 6-10 mm in diameter rose at the needlepoint. This quickly absorbed and hence no dressing was required.

The diameter of induration (not erythema) was considered and measured transversely to the long axis of the forearm and recorded in mm after 48-72 hours of injection. Erythema of less than 10mm was disregarded. If the diameter of erythema was greater than 10mm or more was considered Mx. +ve. This indicated hypersensitivity to tuberculoprotein and post or present infection with *M. tuberculosis*. The induction of less than 10mm was considered Mx.-ve. This indicated lack of hypersensitivity to tuberculosis and tuberculous infection was almost unlikely.

Reactivity to tuberculin may be depressed or suppressed for as long as 4 weeks by viral infections, live-virus vaccines (eg. Measles, Smallpox, Polio, Rubella, Mumps etc.) or by the administration of corticosteroids. Malnutrition may also have a similar effect. About 20% of patients with active incidence of false-negative results. For example, false-negative rates up to 50% have been reported in patients with advanced HIV infection. Alternately, false-positive results may occur in patients infected by other nontuberculous mycobacteria (e.g. *M avium* complex.) malnutrition, severe bacterial infections including TB itself viral infections, cancer and immunosuppressive drugs. Therefore, a negative skin test rules out TB and a positive skin test alone does not establish the diagnosis. Therefore sputum microscopy for detecting AFB is needed for more accuracy in diagnosis of PTB infection in patients.

**(ii) Sputum Acid and Alcohol Fast Bacilli (AFB) Test :**

**Sputum Collection :**

The suspects were asked to produce sputum by forced coughing at the time of specimen collection (early morning preferred as secretions are built up in the air ways overnight). The collection was made in a paper cup Beetle, tobacco and blood was avoided in the specimen collection.
Preparation of Smear :

A clear glass slide was taken for each specimen. With the help of sterile platinum loop, most sticky part of sputum was taken over the glass slide evenly for a diameter of about 2cms.

Drying of Smear:

All the smears were air dried.

Fixation of smear:

Air dried smears were allowed to pass over the flame gently (avoiding over heating) for fixation.

Staining:

Smears were covered with hot carbol fuchsin for 8-10 minutes. After washing the smear with decolourisation was by 3% sulphuric acid (H₂SO₄) till the original colour of stain was washed off (acid alcohol-3ml H₂SO₄ in 97ml ethanol). Counter staining was done by methylene blue for 1-2 minutes. Then this was washed and air dried.

Observation:

Stained smears were examined under oil immersion lens and the presence of small rods, not decolouriesed by acid alcohol red in colour, indicated the specimen to be AFB positive.

(b) Blood Groups (ABO):

Principle:

The test is based on the principle of agglutination. Normal human red blood cells possessing antigens clump in presence off antibody directed towards the antigens.

Reagents required:

1. Blood Grouping Serum Anti – 'A'
2. Blood Grouping Serum Anti – 'B'
3. Blood Grouping Serum Anti – 'D'
**Procedure:**

1. 10% suspension in saline of the cells was prepared.

2. A clean glass slides were marked on the left side as Anti-A and on the right side as Anti-'B'

3. A drop of 'Anti-A' and 'Anti-B' blood grouping serum were put on their respective marked areas on the slide.

4. Next to each Antisera drop, 1 drop of 13% saline suspension of unknown red cells was added taking care that the contents on the left and right sides not get mixed up.

5. With one half of an applicator stick, the red cell-suspension was mixed with Anti-A serum and with the other half the one with Anti-B serum.

6. The slide was rocked back and forth for about one minute and observed for agglutination.

Agglutination of red blood cells with a given antiserum was considered to be positive, which indicated the presence of the corresponding antigen on the red blood cells, on the other hand absence of the agglutination indicated negative test.

**(B) Pathology of PTB:**

In other to study the pathogenicity (if any) of PTB infection the patients were classified to determine various haematological and biochemical parameters as follows:

**Group I (Normal):** Healthy patients i.e. not having PTB infection (Mantoux negative patients.)

**Group II (Acute):** The patients which were symptomatic for tuberculosis, having persistant cough for more than three weeks of duration with expectoration, haemoptysis, fever, breathlessness and chest pain. These were the first time infected tuberculosis patients with no previous history of the disease.

**Group III (Chronic):** Long term tuberculosis cases i.e. > 5 months of confirmed diagnosis ; incompletely treated patients reinfected cases with a previous history of
active disease failure cases i.e. smear-positive at 5 months or more after starting treatment and patients who were initially remained smear-positive after completing a retreatment regimen.

For each group, the following hematological and biochemical parameters were studied-

**Hematological Parameters:**

1. Haemoglobin (Hb)
2. Total Erythrocyte Count (TEC)
3. Erythrocyte Sedimentation Rate (ESR)
4. Packed Cell Volume (PCV)
5. Mean Corpuscular Volume (MCV)
6. Mean Corpuscular Haemoglobin (MCH)
7. Mean Corpuscular Haemoglobin Concentration (MCHC)
8. Total Leucocyte Count (TLC)

**Biochemical Parameters:**

1. Serum Glutamic Oxaloacetic Transaminase (SGOT)
2. Serum Glutamic Pyruvic Transaminase (SGPT)
3. Serum Bilirubin (SB)
4. Serum Alakline Phosphatase (ALP)
5. Serum Total Proteins (STP)

The study was carried for two years i.e. from October 2009 to September 2010 (first year) and October 2010 to September 2011 (Second year). The mantoux test, sputum and blood samples of suspected patients were examined following standard techniques (Oser, 1965; Varely, 1966; Dey, 1986 and Sood 1994). The data was statistically analysed using standard statistical methods (Mather, 1973).
(a) Determination of Haematological Parameters

(i) Haemoglobin (Hb):

Principle:
This is based on conversion of haemoglobin to acid haematin, which is brown in color.

Reagents required:
1. Distilled water
2. N/10 HCL

Procedure:
Sahli's acid haematin method was followed to determine the haemoglobin concentration of the blood as follows:

1. Haemoglobin tube was filled till 20 mark with N/10 HCL.
2. Blood was sucked up to the specific mark (20cc) in the haemoglobin pipette and waited for 5-6 minutes.
3. Added N/10 HCL until a match was obtained with the brown glass standard.

ii. Total Erythrocyte Count (TEC):

Principle:
Hayem's fluid (Isotonic solution) is used as diluting fluid so that RBCs are not haemolysed.

Reagents required:
1. Sodium Citrate
2. Hayem's Fluid

Procedure:
1. The blood was taken directly into the standard red cell diluting pipette and was diluted in the ration of 1:200 using the Hayem's diluting fluid.
2. The diluted blood was shaken gently for 1-2 minutes for thorough mixing.
3. A drop of diluted blood was put on to improved Neubauer’s haemocytometer.

4. It was allowed to settle for 3-5 minutes before counting.

5. Red blood cells were counted under 40 objective in 80 smallest squares of the 5 central squares.

**Calculations:**

\[
\text{No. of RBC/cu. mm} = \frac{\text{No. of RBC counted} \times \text{Dilution factor}}{\text{Area counted} \times \text{Depth Factor}}
\]

Where,

Dilution is 1 in 200, Depth is 1/10 mm. Area counted was 80/400 = 1/5 sq.m

\[
\text{No. of RBC/cu. mm} = \frac{\text{No. of RBC counted} \times 200 \times 10}{1/5} = \frac{\text{Number of RBC counted} \times 10,000}{1/5}
\]

**iii. Erythrocyte Sedimentation Rate (ESR):**

**Principle:**

This is the rate at which erythrocyte sediment on their own weight when anticoagulated blood is held in a vertical column. It is expressed as the fall of RBCs in mm at the end of first hour.

**Reagents required:**

1. Sodium citrate solution (3.8%) – Anticoagulant.

**Procedure:**

Westergreen’s pipette (open at ends) is about 30 cms long with the bore diameter of about 2.5mm. The lower 20 cm are marked from 0 (top) to 200 (bottom.)

1. 0.5 ml of sodium citrate (3.8%) was added to 2 ml of blood.

2. This blood was sucked in Westergreen pipette and the pipette was clamped vertically in the westergreen rack.

3. The upper level (mm) of the red cells was noted exactly after one hour.
iv. Packed Cell Volume (PCV):

**Principle:**

Haematocrit is a volume of red cells expressed as a percentage of the volume of whole blood in the sample.

**Reagents required:**

1. Dried EDTA salt.

**Procedure.:**

Packed cell volume was estimated by using wintrobe's tube method.

1. The blood was taken in Wintrobe's tube till 100 mark on the top with pasture pipette. It was ensured that there was no air bubble in the blood column.

2. This tube was centrifuged for 15 min. at 3500 rpm until packing was complete.

3. After centrifugation, the blood got separated into 3 layers:

   (a) column of red blood cells at the bottom,

   (b) A narrow middle layer buffy coat of white blood cells and platelets,

   (c) The top most fluid column of plasma.

The percentages of the height of the column of blood occupied by packed red cells constitute the haematocrit.

V. MEAN CORPUSCULAR HAEMOGLOBIN (MCH):

MCH was measured of the average haemoglobin content of a red blood cell expressed in picrograms (pg) or (μg).

\[
MCH(\mu g) = \frac{Hb \times 10}{RBCCount \text{ (millions/mm}^3)}
\]

vi. Mean corpuscular volume (MCV):

MCV was measured form the average volume of red blood cells per litre expressed in cubic micrometers (μ^3) of femtoliters (fl).
\[ MCV(\mu^3) = \frac{\text{Haematocrit} \times 10}{\text{RBC counting (millions/mm}^3)\] 

vii. Mean Corpuscular Haemoglobin Concentration (MCHC):

MCHC was measured from the average haemoglobin concentration per unit volume of red blood cells expressed in g/dl or percent.

\[ MCV(\%) = \frac{\text{Hb} \times 10}{\text{PCV}} \]

viii. Total Leucocyte Count (TLC):

A white cell count (TLC) estimates the total number of white cells in a cubic millimeter of blood. Turk’s fluid is used as WBC diluting fluid, which contains a weak acid to lyse the red blood cells and a stain for staining the nucleus of WBCs.

**Reagents required:**

1. Sodium citrate
2. Turk’s fluid

**Procedure:**

1. Using a WBC pipette of a haematocytometer well mixed venous blood or capillary blood was drawn and filed till the 0.5 mark. The tip of the tuber was cleaned.
2. WBC diluting fluid was drawn till the 11 mark.
3. The fluid and blood were mixed gently avoiding bubbling.
4. The fluid blood mixture was shaken and transferred using a fine bore pasteur pipette on the counting chamber (called charging the chamber) taking care that the mixture does not overflow.
5. The cells were allowed to settle to the bottom of the chamber for mins.
6. Using 10 or low power objective WBCs were counted in the four larger corner squares.
Calculation:
\[
\text{Number of WBCs/cu.mm} = \frac{\text{WBCs counted} \times \text{blood dilution} \times \text{chamber dept}}{\text{Area of Chamber counted}}
\]
\[
= \frac{\text{WBCs counted} \times 20 \times 10 \text{ dept factor}}{4}
\]
\[
= \text{WBCs counted} \times 50
\]

**IX. DIFFERENTIAL LEUCOCYTE COUNT (DLC):**

Reagents required:
1. Leishman's stain
2. Distilled water
3. Buffered water

**Procedure:**
1. A thin blood film was made by spreading a drop of blood evenly across a clean grease free glass slide using a smooth edged spreader and air dried.
2. Few drops (about 8) of Leishman's stain were poured on the slide and waited for 2 mins.
3. About double the amount (16 drops) of buffered water was added and mixed by rocking the slide (not by blowing) and waited for 7-10 mins.
4. The stain was flooded off with distilled water in 2-3 seconds and air-dried.
5. The slide was observed under the microscope. For DLC an area was chosen where the morphology of the cells was clearly visible and ensured that there is no tailing of the WBCs. DLC was done by moving the slide in order to include central and peripheral areas of the smear. At least 100 cells were counted and the percentage of the cells seen was given using laboratory cells counter which give a ring after every 100 cells counted.
Observation:

Five types of leucocytes were distinguished on basis of their size and shape, size and shape of their nucleus, and their cytoplasmic inclusions:

Eosinophils:

These comprise 1% to 4% of total WBC count in blood, i.e. 70 to 300 per cu. mm. of blood. Their nucleus is distinctly bilobed with the lobes connected only by a thin strand. Their granules are larger, contain important digestive enzymes and stain by acid dyes like eosin.

Basophiles:

These comprise only about 0.5% to 2% of the leucocytes (35 to 150 per cu. mm. of blood) Two or three lobes of their twisted, S-shaped nucleus are less distinct. Their granules are larger and fewer. These stain with basis dyes like methylene blue.

Neutrophils (Hetrophils):

These are the most abundant (60% to 70% = 4000 to 5000 per cu. mm. of blood) and most active type of WBCs. Their nucleus has 2 and 5 distinct lobes. Their granules are smaller, but most abundant, take all stains and contain hydrolytic enzymes like the lysosomes. Certain neutrophils in female mammals possess a small spherical lobe attached to their nucleus by a stalk. This lobe is called drumstick.

Lymphocytes:

These are small and roughly spherical (6 μ to 16 μ in diameter) corpuscles., comprising about 20% to 40% of the leucocytes (about 1500 to 2500 per cu. mm. of blood). These are comparatively less motile, possess a large, subshperical, central nucleus and produce antiboides.

Monocytes:

These comprise only about 25 to 10% of the leucocytes (i.e. 200 to 700 per cu. mm. of blood) but are the largest cells of the blood (12 μ to 20 μ in diameter) These
have a large uniform or horse-shoe-shaped nucleus and are actively motile and phagocytic.

(b) Determination of Biochemical parameters:

i. Serum Glutamic Oxaloacetic Transaminase (SGOT):

Principle:

Transamination is the process by which an amino group of an amino acid is transferred to an alpha keto acid with the formation of keto acid corresponding to the original amino acid. The oxalocetic acid formed is spontaneously converted into pyruvic acid. The pyruvic acid formed in the reaction reacted with dinitrophenyl hydrazine to give pyruvate dinitrophenyl hydrazone. The yellow colour is modified to an intense brown colour by the addition of sodium hydroxide.

Reagents Required:

1. Buffer substrate hyophilized (Reconstituted each vial with 5 ml of distilled water).
2. Solution B: DNPH colour developer.
3. Solution C. Conc. sodium hydroxide solution (4N) prepared working solution (0.4N) required for estimation by pipeting exactly 1 ml of solution C with 9 ml distilled water.
4. Solution D. Calibration standard (2 m. mol/l)

Procedure:

1. Pipetted 1 ml of working buffer solution into a clean test and placed it at 37° for 5 mins.
2. 0.2 ml of serum was added and mixed it by shaking the tube and then kept it at 37° C for 60 mins.
3. After incubation added 1 ml of solution B kept it at room temperature for 20 mins.
4. Added 10 ml of working solution C (0.4N) and allowed it to stand for 10 mins.
5. The ODs of all tubes was measured at 520 nm against distilled water blank adjusted to zero using Spectrophotometer (D.N. Mitra Lab system analyser.)
Calculations:

The SGOT (IU UNITS) were calculated using calibrating curve.

Calibration Curve:

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Working Buffer Solution (ml)</th>
<th>Solution 'D' (Standard)</th>
<th>Distilled water (ml)</th>
<th>SGOT (IU units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>0.0</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
<td>0.1</td>
<td>0.2</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>0.8</td>
<td>0.2</td>
<td>0.2</td>
<td>51</td>
</tr>
<tr>
<td>4</td>
<td>0.7</td>
<td>0.3</td>
<td>0.2</td>
<td>114</td>
</tr>
<tr>
<td>5</td>
<td>0.6</td>
<td>0.4</td>
<td>0.2</td>
<td>190</td>
</tr>
</tbody>
</table>

1. 1ml. of solution 'B' (DNPH) was pipetted into each tube and allowed it to stand at room temperature for 20 mins.

2. Then added 10ml of working solution (0.4N-NaoH-Solution) to all the tubes. Kept these tubes at room temperature for 10 mins.

3. ODs of all tubes was measured at 520 nm using Spectrophotometer (DN Mitra lab system analyser).

4. Plotted the curve of ODs against SGOT unites as above.

ii. Serum Glutamic Pyruvic Transaminase (SGPT):

Principle:

Transamination is the process by which an amino group of an amino acid is transferred to an alpha keto acid with the formation of keto acid corresponding to the original amino acid. The pyruvic acid formed in the reaction is reacted with dinitrophenyl hydrazone. The yellow colour is modified to an instant brown colour by the addition of sodium hydroxide.
**Reagents Required:**

1. Buffer substrate hyophilized (Reconstituted each vial with 5ml of distilled water)
2. Solution B : DNPH colour developer.
3. Solution C: Concentrated sodium hydroxide solution (4N). Prepared working solution (0.4N) required for the estimation by pipetting exactly 1 ml of solution C with 9 ml distilled water.
4. Solution D : Calibration standard (2m. mol/l)

**Procedure:**

1. Pipetted 1 ml of working buffer solution in clean test tube and placed it at $37^0$C for 5 mins.
2. Added 0.2 ml of serum and kept it at $37^0$C for 30 mins.
3. After incubation added 1 ml solution 'B' (DNPH) and kept it at room temperature for 20 mins.
4. Added 10ml of working solution 'C' (0.4N) and allowed it to stand for 10 mins.
5. The ODs of all tubes was measured at 520 nm against distilled water as blank adjusted to zero, using spectrophotometer (D.N. Mitra lab system analyser).
6. Plotted the curve of ODs against SGOT units.

**Calculation :**

The SGPT (IU UNITS) were calculated using calibration curve as follows.
Calibration Curve:

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Working Buffer Solution (ml)</th>
<th>Solution 'D' (Standard)</th>
<th>Distilled water (ml)</th>
<th>SGOT (IU units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>0.0</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
<td>0.1</td>
<td>0.2</td>
<td>28</td>
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<tr>
<td>3</td>
<td>0.8</td>
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<td>0.2</td>
<td>57</td>
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<td>4</td>
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<td>0.2</td>
<td>97</td>
</tr>
<tr>
<td>5</td>
<td>0.6</td>
<td>0.4</td>
<td>0.2</td>
<td>157</td>
</tr>
</tbody>
</table>

1. 1 ml of solution 'B' (DNPH) was pipetted into each test tube and allowed it to stand at room temperature for 20 mins.

2. After incubation added 10 ml of working solution (0.4N-NaOH solution) to all the test tube. Kept these for 10 mins at room temperature.

3. ODs of all tubes was measured at 520 nm using spectrophotometer (D.N. Mitra lab system analyser).

4. Plotted the curve of ODs against SGPT units.

iii. Serum bilirubin (SB):

**Principle:**

Direct (Unconjugated): Bilirubin couples with diazotised sulfanalic acid forming azobilirubin, a red purple coloured product in acidic medium.

Indirect (Unconjugated): Bilirubin is diazotised only in presence of its dissolving solvent, methanol. Thus, the red-purple coloured azobilirubin and thus represents total concentration. The difference of total and direct bilirubin gave indirect (Unconjugated) bilirubin.
The intensity of red purple colour developed was measured colorimetrically and it was directly proportional of the appropriate fraction of bilirubin. The reaction can be represented as:

Bilirubin + Diazotized Sulfanalic Acid \rightarrow Azobilirubin (red-purple colour)

**Reagents Required:**

1. Diazo reagent A
2. Diazo reagent B
3. Diazo blank
4. Bilirubin reagent D
5. Bilirubin standard (artificial) (10 mg/dl).

**Procedure:**

1. Preparation of Diazo working reagent (just before use) 3.3ml of Diazo reagent A was mixed with 0.1 ml of Diazo reagent B.
2. Four test tubes were labeled as total – T1, Total Blank – T2 and Direct – D1, Direct blank – D2.
3. 0.2ml of Serum/Plasma was pipetted in all 4 tubes i.e. T1, T2, D1 and D2.
4. Then 1.8ml distilled water was pipetted in tubes D1 and tube D2; and 2.5 ml of Bilirubin reagent D in tubes T1 and T2 respectively.
5. 0.5ml of Bilirubin Diazo blank was added into tubes T2 and D2 each and 0.5 ml of Diazo working reagent in tube T1 and tube T2.
6. Then 2.5ml distilled water was pipetted in tubes D1 and tube D2 and 2.5 ml of Bilirubin reagent D in tubes T1 and T2 respectively.
7. These were then mixed well and ODs of D1 and D2 were read after one min. against distilled water adjusted to zero at 540 nm.
8. The tubes T2 and T1 were kept in dark at room temperature for 30 mins. then their ODs measured against distilled water adjusted to Zero at 540 nm.
Measurement of standard:

The OD of Bilirubin artificial standard was read against distilled water at 540 nm.

Calculation:

**Bilirubin concentration (mg/dl):**

Total (A) = \[
\frac{OD_{T1} - OD_{T2}}{OD \text{ of Standard}} \times 10
\]

Direct (B) = \[
\frac{OD_{D1} - OD_{D2}}{OD \text{ of Standard}} \times 10
\]

Indirect = (A) – (B)

iv. Serum Alkaline Phosphatase (ALP):

**Principle:**

Serum is incubated with phenyl phosphathe buffered at pH 10.0 for 15 mins. at 37\(^\circ\)C. The hydrolysis product Phenol is condensed with 4- aminoantipyrine (4-AAP) and then oxidised with alkaline ferricyanide to given red coloured complex which is measured colorimetrically at 520nm. The reaction can be represented as-

\[
\text{PhenylPhosphathe} \times \text{Alkaline Phosphate pH 10.0} \times \text{Phenol + Pi}
\]

\[
\text{Phenol + 4AAPe} \times \text{Potassium Ferricyanide OH}^- \times \text{Orange, red Coloured complex}
\]

**Reagents Required :**

1. Reagent A - Buffered substrate, pH- 10.0
2. Reagent B - Sodium hydroxide
3. Reagent C - Sodium bicarbonate
4. Reagent D - 4- Aminoantipyrine
5. Phenol Standard - Stock 1 mg/dl
**Procedure**

a. Preparation of working buffer solution. 1 vial of buffered substrate was reconstituted with 9ml. distilled water and mixed well.

b. Preparation of working phenol standard (0.01 mg/ml) : 0.1 ml. of stock standard was diluted to 10 ml with distilled water.

1. Four test were labeled as B-Blank, S-Standard, C-Control, T-Test (Serum).
2. 2.0 ml of the reagent A was pipetted into tubes C and tube T each 2.0 ml distilled water was pipetted in tube B and 1.1ml distilled water in tube S.
3. These were incubated at 37°C for 3 mins.
4. 1.0 ml working standard solution was incorporated into tube S and 0.05 ml serum was added to tube T.
5. These were again incubated at 37°C for 15 mins.
6. After incubation 0.4 ml of reagent B was pipetted into all the 4 tubes
7. Then 0.05 ml of serum was pipetted into tube C.
8. 0.5 ml of reagent C, reagent D and reagent, each, was added into all the 4 tubes respectively.
9. These were mixed well and kept for 20 mins. at room temperature.
10. The ODs of all the tubes were measured at 520 nm against distilled water adjusted to zero using Spectrophotometer (D.N. Mitra lab system analyser).
Calculation:

Alkaline phosphatase (IU/L) = \( \frac{RT - RC}{RS - RB} \times 20 \)

Where,

- \( RT \) = OD of the test (Serum)
- \( RS \) = OD of the test (Standard)
- \( RC \) = OD of the test (Control)
- \( RB \) = OD of the test (Blank)

v. Serum Total Proteins (STP):

Principle:

Proteins and peptides containing at least 2 adjacent peptide bonds react with cupric ions in alkaline solution forming violet coloured complex having absorption at 540nm. The concentration of low mol. wt. peptides is too less to interface.

Reagents required:

1. Mitra total protein reagent.

Procedure:

1. 5 ml. Protein reagent was pipetted into three test tubes labeled as B-blank, S-Standard, and T-Test (Serum).
2. 100 ul distilled water was added into B 100 ul protein standard into S and 100 ul serum into T.
3. These were mixed by lateral shaking to ensure thorough mixing.
4. All tubes were incubated at 37\(^{0}\)C for exactly 10 mins. or at room temperature for 15 min.
5. The od’s of all tubes were measured at 540 nm using spectrophotometer (D.N. Mitra lab system analyser) against blank adjusted to zero.
Calculation :

Total Protein (g/dl) = \frac{RT}{RS} \times 6

Where

\begin{align*}
RT &= \text{OD of the test} \\
RS &= \text{OD of the Standard.}
\end{align*}

(c) IN VITRO EFFICACY OF DRUGS AGAINST MYCOBACTERIUM SP:

The antimicrobial activities of various Allopathic, Ayurvedic and Homeopathic drugs commonly used for treatment of tuberculosis were tested against *Mycobacterium* *sp.*

(a) Characteristics of Mycobacteria:

Mycobacteria require special methods for study because many of these grow very slowly and require special media and many are rapidly growing species. Rapid growers can be identified reasonably easily, but the slow growers show several complexes of very similar species, which are hard to distinguish. Slow growers require over 7 days at optimum temperature on nutritionally rich media to yield easily visible single colonies from very dilute inoculam while the rapid growers show visible colonies in 7 or less days. Some species are intermediate in their growth rates. *M. leprae* is not cultivable and several other species are cultivated only with great difficulty. Mycobacteria are relatively resistant to chemical disinfectants.

'Mycobacteria' group contains a single genus 'Mycobacterium' These are slender rod shaped characteristically acid fast aerobic slow growing and free living or pathogens of vertebrates. The property of acid fastness, due to waxy material in the cell walls, is particularly important for recognizing mycobacteria. These bacteria are commonly described as acid-alcohol-fast, implying that after staining, they resist decolorization with acidified alcohol as well as with strong mineral acids.

**Genus Mycobacterium**: Straight or Slightly curved rods, 0.2 -0.7 × 1.0 -10 um sometimes branching, filamentous or mycelium like growth may occur but is readily fragmented into rods or cocci and due to branching filamentous forms resembling
fungal mycelium, hence the name 'Mycobacteria, meaning fungus like bacteria. Acid alcohol fast at some stage of growth. Not readily stained by gram's method, usually weakly gram positive. No aerial hyphae are grossly visible. Non-sporing without conidia or capsules. Aerobic and chemo-organotrophic. Growth is slow or very slow, visible colonies appear in 2-60 days at optimum temperature. Colonies are often pink, orange or yellow, especially when exposed to light, pigment is not diffusing and surfaces commonly dull or rough. Some species are fastidious, requiring special supplements (e.g. M. Para tuberculosis or are none-cultivable M. Leprae). Catalase positive, arylsulfatase positive and lysosomes resistant. Widely distributed in soil and water, some species are obligate parasites and pathogens of vertebrates.

Mycobacterium genus includes obligate parasites, opportunistic pathogens and saprophytes of the pathogenic species belonging to the M. tuberculosis. Complex the frequent and important agent of human disease. M. bovis, M. africana, M. leprae etc. also infect human.

M. tuberculosis is a straight or slightly curved rod-shaped, 1-4 μm × 0.2-0 μm. occurring singly, in pairs or in small clumps. The size depends on condition of growth and long, filamentous, club shaped and branching forms may sometimes be seen. They stain readily and are neutral to gram's staining. They are stained by carbol fuchsin by the Ziehl-Neelson method or by fluorescent dyes (auramine O, rhodamine). They resist decolourisation by 20% H₂SO₄ and absolute alcohol for 10 mins (acid fast bacilli/AFB). Acid fastness is due to the organism’s high content of mycolic acids long chain cross linked fatty acids and other cell wall lipids. Mycolic acid is an unsaponifiable wax present around the cell Staining may be uniform or granular. Beaded or barred forms are frequently seen in M. tuberculosis.

In the Mycobacterial cell wall, lipids (eg. mycolic acids) are linked to underlying arabinogalactun and peptidoglycan. This structure is responsible for very low permeability of the cell wall and thus for the ineffectiveness of most antibiotics against the organism. In addition, lipids such as acylated trehaloses, or cord factors any play a role in the virulence of M. tuberculosis by inducing cytokine mediated events. Another molecule in the mycobacterium cell wall, lipoarabinomannan, is involved in pathogen-host interaction and facilitates the survival of M. tuberculosis.
within macrophages. The several proteins characteristic of *M. tuberculosis* include those in purified protein derivative (PPD) tuberculin, a mixture of non-species specific molecules in an extract from a culture filtrate.

Electron-micrographs of thin sections show that the thick cell wall is composed of 03 layers enclosing a plasma membrane, which also has got 03 layers. Spheroplast formation occurs when grown in the presence of lysozyme L-type growth can also be induced.

*M. tuberculosis* was discovered in 1882 by R. Koch. It is aerobic, non-motile and grows slowly, the generations time in vitro being 14-15 hours. Colonies appear only in about 2 weeks and sometimes may be delayed up to 6-8 weeks.

Optimum temperature is 37\textdegree{}C and growth does not occur below 25\textdegree{}C or above 40\textdegree{}C Optimum pH 6.4 -7.0. Growth is stimulated by 5-10\% CO. It is eugonic, i.e. grows luxuriantly in culture. The addition of glycerol (0.5\%) improves the growth of human strains. Sodium pyruvate also improves the growth.

Tubercle bacilli do not have exacting growth requirements but are highly susceptible to even traces of toxic substances like fatty acids in culture media. The toxicity is neutralized by serum, albumin or charcoal, Koch originally grew the bacillus on heat coagulated bovine serum. The organisms grow on selective media and several media both solid and liquid have been described for its cultivation. The solid media contain egg (Lowenstein Jensen. Petragnini or Dorset), blood (Tarshis medium), serum (Loeffler's serum slope) or potato (Pawlowsky's). Among the several liquid media described, Dubosm YK Medium, middle brook's Proskauer and Beck's. Sula's and Sauon's media are more common. Liquid media are not generally employed for routine cultivation but are used for sensitivity tests, chemical tests and preparation of antigens and vaccines.

On solid media dry, rough-irregular colonies with a wrinkled surface appear. They are creamy white initially, becoming yellowish or buff colored later. They are tenacious and not easily emulsified. In liquid media without dispersing agents the growth begins at the bottom creeps up the sides and forms a prominent surface pellicle that may extend along the side above the medium. Diffuse growth is obtained in Dubos
medium containing a detergent Tween-80 (sorbitan monooleate). Virulent strains tend to form long serpentine cords in liquid media, while avirulent strains grow in a more dispersed fashion. Tubercle bacilli may be grown in chick embryos and in tissue culture.

**Resistance:**

Mycobacteria are more resistant to external effects as compared to other non-spore forming bacteria as result of their high lipid content (25-40%). They are not especially heat resistant, being killed at 60°C in 15-20 mins. Cultures may be killed by exposure to direct sunlight for 2 hr. but bacilli in sputum may remain alive for 20-30 hrs. Bacilli may remain viable in droplet nuclei for 8-10 days. Cultures remain viable for 6-8 months at room temperature and may be stored for 2 years in the deep freeze cabinet at -20°C. The organisms survive in the flowing water for over a year, in soil and manure up to 6 months, on the pages of books over a period of 3 months in dried sputum for 2 months, in distilled water for several weeks and in gastric juice for 6 hours. They are easily rendered harmless at temperature ranging from 100 to 120°C.

Mycobacteria are relatively resistant to chemical disinfectants, surviving exposure to 5% phenol, 15% H₂SO₄, 3% HNO₃, 5% oxalic acid and 4% NaOH. It is destroyed by tincture of iodine in 5 mins and by 80% ethanol I has been recommended as a disinfectant for skin, rubber gloves and clinical thermometers. It sterilises pieces of cloth in 10 mins.

**Biochemical reactions/tests:**

Several biochemical tests have been described for the identification of mycobacterial species, the more important of them are Niacin test, Aryl Sulphatase test, Neutral red test, Catalase- Peroxidase test, Amidase test and Nitrate reduction test.

**Isolation and culture of Mycobacterium sp :**

The tubercle bacillus does not grow on ordinary media. Primary cultures are usually made on some form of egg medium. The international Union against Tuberculosis (IUT) has prescribed Lowenstein- Jensen (LJ) Medium for cultivation of mycobacteria. This egg medium is probably the most widely used. After the primary
culture has been established it is possible to make subcultures on media without egg, such as Dubos, Proskaure and Beck, Kirschner, Youman's media and YK media etc. Sputum may contain microorganisms other than mycobacteria. This is tested by inoculating the material in EMB and NA plates and incubated for 18 to 24 hours. If no growth is observed on the plates than it is considered that the material does not contain any bacteria other than mycobacteria. On the other hand, if the material is found contaminated it is than decontaminated with a reagent which is less lethal to mycobacteria only.

In present study, the bacteria were isolated at Tyagi Pathology Center Saharanpur from the AFB positive sputum of active disease patient, using standard method for isolation of culture (Sood, 1994 and Bhattacharya, 1993). Lowenstein – Jensen Media tubes were used for culturing the organism. Following procedure was adopted:

**Petroff's Method:**

1. The sputum specimens were collected in clean, sterile containers and treated with 4% NaOH to destroy organisms other than mycobacteria.
2. To the sputum in the container was added an equal quantity of 4% NaOH.
3. The container was closed and inverted twice or thrice and placed in the incubator for 30 minutes, inverting it every 10 mins for shaking.
4. The mixture was than centrifuged at 3000 r.p.m. for 30 mins.
5. The supernatant was discarded and a few drops of neutral red indicator were added to the deposit.
6. It was than neutralised with 8% HCL.
7. This neutralised deposit was inoculated on the L.J. Medium screw caped tubes, aseptically. The medium favours the growth human tubercle bacilli and suppresses the growth of other organisms.

The slants were incubated at 37°C for 2-4 weeks. The screw caped tubes were loosened once in a week at the time of inspection for good growth.
(c) **Preparation of culture medium:**  
YK Medium given as Growth Medium No. 52 in MTCC catalogue of IMTECH Chandigarh was used for the experimental work.

**Composition of YK medium:**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asparagin</td>
<td>5.0g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>5.0g</td>
</tr>
<tr>
<td>Na-citrate</td>
<td>2.5g</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>0.5g</td>
</tr>
<tr>
<td>Fe-ammonium citrate</td>
<td>50.0g</td>
</tr>
<tr>
<td>Tween – 80</td>
<td>0.8ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.0litre</td>
</tr>
</tbody>
</table>

Adjust pH. to 7.2-7.4  
Add glycerol 32.0 g before autoclaving.

All the above ingredients were weighed accurately and dissolved in distilled water. pH of the medium was adjusted to 7.2-7.4. 32.4g glycerol/litre was added, before sterilization/autoclaving. Then the media was dispensed in required quantities in the clean culture tubes, screw-capped tubes and conical flasks and proper plugging was done. These were then sterilized in autoclave at 15 lbs pressure for 20 mins. at 121.5°C.

(d) **Test drugs:**

In present study the following drugs (Photoplate I) were used for studying their antimicrobial efficacy against mycobacterium sp.:

I. **Allopathic (Powder form):**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin</td>
<td>Sharabhai</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Lupin</td>
</tr>
</tbody>
</table>
Pyrazinamide  
Novartis 
Isoniazid  
Pfizer.

II. Ayurvedic (Powder form):

<table>
<thead>
<tr>
<th>Drug</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swarn Vasant Malti Ras</td>
<td>Baidyanath</td>
</tr>
<tr>
<td>Mahalakshmi Vilas Ras</td>
<td>Baidyanath</td>
</tr>
<tr>
<td>Rajmrigank Ras</td>
<td>Baidyanath</td>
</tr>
<tr>
<td>Shringyadi Churana</td>
<td>Gurukul Kangri Pharmacy</td>
</tr>
</tbody>
</table>

III. Homeopathic (Liquid form):

<table>
<thead>
<tr>
<th>Drug</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arsenic iodatum 30</td>
<td>Medisyunth</td>
</tr>
<tr>
<td>Stannum iodat 30</td>
<td>Medisyunth</td>
</tr>
<tr>
<td>Kalium iodat 30</td>
<td>Medisyunth</td>
</tr>
<tr>
<td>Silicea 30</td>
<td>Medisyunth</td>
</tr>
</tbody>
</table>

(e) Preparation of test drugs concentrations:

The Allopathic and Ayurvedic drugs were either in tablet/capsule or in powder form. Therefore 1gm. of each drug was dissolved in 9ml sterilized distilled water in culture tube, separately. This was treated as the mother/full strength solution of the drug. These were further serially diluted in culture tubes, each containing 9 ml sterile distilled water to get concentrations of 1 : 100, 1 :1000, 1 : 10000 and 1 : 100000 ratios for each drug, separately. As homeopathic drugs were in liquid form, 1 ml (1g=1ml) of each drug was dissolved in 9 ml sterile distilled water, separately in tubes and serially diluted as above. These drug concentrations were used to test their anti-microbial activity against Mycobacterium sp.
(f) **Preparation of inoculum and standardization of the culture:**

The Mycobacterium sp. was isolated from the sputum on LJ Medium slants and maintained on it. This served as the mother culture for the experiment (Photo plate II). Under aseptic conditions colonies from the LJ media slant were picked up using a sterile platinum inoculation loop and transferred into the screw capped culture tubes containing 5 ml sterile YK medium. These were incubated at $37^0C$ in BOD incubator for 7 days. After 7 days, 1 ml bacterial suspension from these tubes was transferred into different tubes containing 9 ml. YK Medium (sterilized) each aseptically and incubated further at $37^0C$ for 2 weeks. After 2 weeks these subcultures were again standardized for the second time (Photo plate III an IV) the standardization was done to bring bacteria under log phase of growth. The second time standardized subcultures served as the inoculum for the experiment.

(g) **Procedure for Antimycobacterial Activity :**

(i) **Preparation of Control :**

The culture tubes each containing 9 ml. of sterilized YK medium and 1 ml. sterilized distilled water was inoculated with *mycobacterium sp.* (two loops each), aseptically. No drug was added in these tubes. These were used as controls.

(ii) **Preparation of Blanks.**

The culture tubes each containing 9 ml. sterilized YK medium and 1 ml. of each of the drug concentrations were prepared aseptically to use as blanks.

(iii) **Preparation of Test Solutions:**

The culture tubes containing sterilized YK medium (9 ml. each) were inoculated with *mycobacterium sp.* (two loops each), aseptically using a sterilized platinum loop. Under aseptic conditions 1 ml. each of the drug concentrations were added to individual culture tubes and labelled accordingly. These were used as test solutions.
(iv) **Incubation:**

All the tubes (Blanks, controls and test solutions) were incubated at 37°C for 4 weeks in BOD incubator and observed at weekly intervals. Shaking of the tubes were ensured from time to time.

(v) **Reading absorbance:**

The optical density (absorbance) of the test solutions were read at 520 nm in the Spectrophotometer using the corresponding blank solutions set to zero absorbance. The absorbance of the control solutions observed at weekly intervals was used for comparing the antimicrobial efficacies of the drugs against the bacteria. The experiments were conducted in three replicates. Twice i.e. in six replicates and the mean values of ODs were calculated.