CHAPTER IV
MATERIAL AND METHODS
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This study was carried out in the Department of Medicine and Microbiology at Christian Medical College and Hospital, Ludhiana from June 1996 to December 1997.

Study Group:

The study group consisted of 150 patients who came to Christian Medical College and Hospital, Ludhiana from June 1996 to December 1997. The study group consisted only microbiologically or histologically proven patients.

All patients were subjected to a detailed clinical history, physical examination and relevant investigations in accordance with the protocol, including Antigen A60 specific serum IgM and IgG.

The diagnostic criteria for tuberculosis were as follows:

a) Clinical history and presentation with past history of tuberculosis or history of contact.

b) Clinical examination and findings.

c) Supportive investigations

i) Raised erythrocyte sedimentation rate

ii) Chest x-ray PA view for radiographic evidence of tuberculous lesions
iii) Positive PPD

iv) Body fluid examination includes cytology, sugar, protein LDH, ADA
wherever required.

d) **Confirmative investigations:**

   i) Sputum AFB smear and culture for 3 consecutive days

   ii) Biopsy of tissue wherever required.

   Antigen A60 specific serum IgM and IgG in all patients.

**Control group**

The control group included 100 healthy patients with no apparent clinical suspicion of tuberculosis will be taken as the controls. The control group will include 75 healthy blood donors and 25 doctors and staff of Microbiology Department who are frequently exposed to patients with tuberculosis or frequently handle tuberculous body fluids and tissue for analysis.

**Sample collection:**

In both the study group and control group 3-4 ml of venous blood was drawn which was transported to Microbiology Department in a sterile test tubes. In the study group blood sample was taken before PPD was given.
Clinical study:

All the 150 patients who were suspected to have tuberculosis who were came to T.B.Clinic or admitted in the medical or surgical wards. A detailed history with emphasis on past medical history and family history of tuberculosis was obtained. A detailed clinical examination was done on first visit who came to OPD or on admission and same was assessed periodically as they were followed up. The attached protocol was followed for each patient and accordingly every patients was subjected to investigations including serum A60 specific IgM and IgG.

Laboratory studies:

1) All patients included in this study were subjected to serum antigen A60 specific IgM and IgG detection.

2) Other tests
   a) Erythrocyte sedimentation rate
   b) Chest x-ray PA view
   c) Mantoux test
   d) Sputum for Ziehl Neelson stain and culture in LJ medium
   e) Body fluid for Ziehl Neelsen stain and cultures LJ medium whereever required.
   f) Tissue biopsy where ever required.
Serum IgM and IgG estimation:

**Principle:** Detection of serum IgM and IgG antibodies to *micobacterium* A60 antigen was done by commercially available enzyme immunoassay kits. The wells are coated with A60 antigen.

Patients' serum is added into these wells and the plates are incubated. Their incubation allows the formation of antigen antibody complexes. The unbound antibodies in the sera are eliminated by washing. The wells are thereafter incubated with peroxidase labelled antihuman IgG or IgM antibodies that bind to the antibodies complexes present in the wells. The unbound antibodies are eliminated by washing. The peroxidase substrate tetramethyl-benzidine (TMB), containing hydrogen peroxidase is thereafter added to the wells. A colour develops in the well due to hydrolysis of substrate by the conjugated enzyme. The intensity of the colour is proportional to the quantity of specific antibodies present in the serum sample.

A reference curve is constructed by plotting the optical densities of the reference control sera. The concentration of the antibodies in unknown serum was analysed at the same time as the references in then determined from the reference curve and transformed into relative serounits, which allows the user to take into account the inevitable daily variations which appear during the determinations.
Reagents:
1) 1 bottle (100ml) of sample diluent
2) 1 vial (10ml) of peroxidase labelled anti human IgG or IgM in protein stabilized buffer.
3) 1 bottle (1.6ml) of 10 fold concentrated TBMC (tetramethyl benzidine)
4) 1 bottle (16ml) of TMB diluent. Citric acid buffer containing 0.02% of H2O2
5) 1 bottle (16ml) sulphuric acid (H2SO4)
6) 1 vial (0.15ml) of weak positive serum (around 300 U/ml)
7) 1 vial (0.15ml) of strong positive serum (around 800 U/ml)
8) 1 vial (1ml) of negative reference serum
9) 1 vial (1ml) of reference serum containing 1 units/ml
10) 1 vial (1ml) of reference serum containing 2 units/ml
11) 1 vial (1ml) of reference serum containing 4 units/ml
12) 1 vial of reference serum containing 8 units/ml
13) 1 vial (1ml) of reference serum containing 16 U/ml
14) 1 vial (50ml) of concentrated (20X) washing buffer solution
15) 12 strips of 8 wells coated with antigen A60 from mycobacterium bovis (BCG)
Material required:

1) Pipettes to deliver 10 ml
2) Pipettes to deliver 100 and 1000ml
3) A photometer reading at 450 nm
4) Tubes for serum dilutions
5) Distilled or demineralized water
6) A 37°C incubater

Sample collection:

All the blood samples were received in sterile well covered test tubes. Hemolysed samples were discarded. Blood was allowed to clot at room temperature. Serum was separated by centrifugation. All sera were stored at -20°C till the test was put up.

Preparation of the reagents

a) Washing solution
   Add 19 volumes of distilled water to one volume of washing buffer solution (concentrated 20 times)

b) Diluted TMB diluent
   Add 9 volumes of TMB diluent to one volume of TMB concentrated 10 times.
Preparation of enzymatic substrate solution

<table>
<thead>
<tr>
<th>Number of Wells</th>
<th>TMB concentrated</th>
<th>TMB diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>0.10 ml</td>
<td>0.90 ml</td>
</tr>
<tr>
<td>16</td>
<td>0.20 ml</td>
<td>1.80 ml</td>
</tr>
<tr>
<td>32</td>
<td>0.35 ml</td>
<td>3.15 ml</td>
</tr>
<tr>
<td>48</td>
<td>0.50 ml</td>
<td>4.50 ml</td>
</tr>
<tr>
<td>64</td>
<td>0.70 ml</td>
<td>6.30 ml</td>
</tr>
<tr>
<td>80</td>
<td>0.90 ml</td>
<td>8.10 ml</td>
</tr>
<tr>
<td>96</td>
<td>1.00 ml</td>
<td>9.00 ml</td>
</tr>
</tbody>
</table>

Test procedure

1. Bring the A60 sensitised wells at room temperature.
2. Dilute the sera to be tested 1 : 100 in the serum dilution medium.
3. The weak (300 U) and strong (800 U) positive sera must be diluted just like the other analysed samples.
4. The reference sera are already diluted and should be used as such.
5. Introduce 100ml of diluted serum into a microtitration well. Use two wells per sample and identify each well proceed in the same way for the reference sera.

6. Incubate 1 hour at 37 °C

7. Dilute the concentrate washing buffer solution during this incubation period.

8. Wash the wells under a light streams of washing buffer solution fill and empty the wells 5 times. At the end of the washing process, the wells must be entirely dry.

9. Distribute 100 ml of anti Hum IgG-POD or Anti-Hum IgM POD conjugate in each well. Incubate 30 minutes at 37 C.

10. Dilute the TMB just before the end of the 30 minutes.

11. Wash the wells under a light stream of diluted washing buffer solution: fill and empty 5 times. At the end of the washing process, the wells must be entirely dry.

12. Distribute rapidly 199ml of the substrate solution in each well of the microtitration plate. Incubate at 37 C during exactly 15 minutes.

13. Add H2SO4 100 ul to each well to stop the reaction

14. Measure the results at 450 nm in Elisa reader

15. The blank of the photometer is adjusted on air unused wells.
Determination of results (IgG)

1) Calculate the mean optical density (OD 450 nm) for each sample and reference serum.

2) On semi-log graph paper, trace the reference curve by bringing the optical density of each reference serum except for the negative reference sera, on the vertical axis in relation to the number of corresponding units, put on the horizontal axis. This curve should be essentially a straight line between 2 and 16 units.

3) Using the mean absorbance value for each sample, determine the corresponding concentration of antibodies expressed in units/ml as follows. From the reference curve, find on the 4 axis the absorbance value and extend a horizontal line to the reference curve. At the intersection point, extend a vertical line to the x axis and read the corresponding concentration in specific IgG for the unknown. Multiply by the applied dilution factor (normally 100) to obtain the ELISA units.

Determination of result (IgM)

1) Calculate the mean absorbance (A450 nm) for each sample and reference serum.

2) To reduce daily variations and for comparison of data obtained at different days, divide the absorbance of the analysed samples by the
absorbance of the positive reference obtained during the same analysis.

A 450 sample
A 450 pos. ref.

The normalized value of the positive reference is then one. All samples whose normalised absorbance value is below 0.8 are considered negative indicating levels of specific antibodies inferior to those expected to confirm an infection. All samples whose normalized value comprised between 0.8 and 1.0 are considered dubious and all samples whose normalized value is located above 1.0 are considered positive for IgM antibodies.