MATERIAL AND METHODS
MATERIAL AND METHODS

The study was conducted on twenty five cases of measles attending the M.L.B. Medical College Hospital, from August 1988 to June 1989.

Fifteen cases of measles with complications constituted Group A and 10 cases of measles without complications formed group B. Ten healthy children of comparable age served as controls and were taken as group C.

Group A - 15 cases of measles with complications.
Group B - 10 cases of measles without complications.
Group C - 10 healthy children of age and sex comparable to above groups.

Selection of cases:

Cases were selected on the basis of clinical grounds. Healthy children who did not have any history of measles or measles vaccination were selected from Well Baby Clinic of the Department of Paediatrics, M.L.B. Medical College, Jhansi.

The cases of measles were selected strictly on clinical grounds. These cases had history of fever which was continuous type and moderate to high grade, dry hacking cough running of nose, redness of eyes and excessive lacrimation. Presence of Koplik spots during fever was diagnostic of measles.
The typical morbilli form rashes first appeared behind the ears, near the hair-line on the forehead, face and neck. History of measles in siblings & neighbourhood and absence of immunization against measles corroborated our clinical diagnosis. Thorough physical examination was undertaken in all cases. Special emphasis was given on clinical data regarding age, sex, nutritional status and complications encountered.

**Nutritional status of the cases was graded according to classification given by Indian Academy of Paediatrics (I.A.P., 1972).**

<table>
<thead>
<tr>
<th>Grade of malnutrition</th>
<th>Weight expressed in percentage of the reference of Harvard Standard (50th percentile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade I</td>
<td>71 - 90%</td>
</tr>
<tr>
<td>Grade II</td>
<td>61 - 70%</td>
</tr>
<tr>
<td>Grade III</td>
<td>51 - 60%</td>
</tr>
<tr>
<td>Grade IV</td>
<td>≤ 50%</td>
</tr>
</tbody>
</table>

**Relevant investigations including TLC, DLC, ESR, Urine examination, Stool examination, X-ray chest - PA view, CSF examination, Fundus examination were also carried out and recorded on proforma.**
Methods:

Blood samples were collected from all cases within a week from the onset of rash for serological confirmation of measles antibody levels and for determination of immunoglobulins namely IgG, IgM & IgA.

Determination of measles antibody level was done by Haemagglutination Inhibition technique (Enders, 1969). Immunoglobulin levels were measured by single radial immunodiffusion technique of Macini et al (1965).

Collection and storage of blood sample:

For serological confirmation by HAI test and determination of immunoglobulins, about 3 ml of blood was withdrawn from peripheral vein by a sterile syringe under all aseptic precautions and transferred to sterile vial. Blood was allowed to clot at room temperature and then kept overnight in an ordinary refrigerator ( +4°C ). Serum was separated and was kept in a deep freeze ( -20°C ) till tested.

Haemagglutination Inhibition (HAI) Test:

Haemagglutination inhibition test (HAI), the simplest and most commonly done test was used to determine the antibodies against measles in patient's serum as described by Enders (1969).

1. Principle

A wide variety of animal viruses have been shown to possess the capacity of absorbing to red blood cells and in
many cases causing their agglutination. Virus haemagglutination is readily inhibited by specific antibody. Haemagglutination inhibition test furnishes a sensitive method for detecting viral antibodies.

II. Equipment and Reagents:

1. Measles haemagglutination (HA) antigen (available from Central Research Institute, Kasauli).

2. Rhesus monkey erythrocytes.

3. Physiological saline, pH 7.2.

4. Alsever's solution (contents of Alsever solution - Glucose - 24.6 gm, Trisodium citrate dehydrate - 9.6 gm, NaCl - 50.04 gm, Distilled water - 1200 ml. pH was adjusted to 6.1 using citric acid. The solution was sterilized by low pressure autoclave and stored in refrigerator).

5. Known positive and negative measles antisera (available from Central Research Institute, Kasauli).

6. Graduated centrifuge tube (15 ml capacity).


10. Mirror for reading the plates.

III. Procedure:

(a) Collection of Monkey's blood - Rhesus monkey blood was collected under sterile conditions in Alsever's solution in the ratio of 1:4 and was stored at 4°C. These cells could be used up to 10 days.

(b) Washing of Erythrocytes - Required quantity of preserved blood was taken in a graduated centrifuge tube and was centrifuged for 10 minutes at 2000 rpm. RBCs were then washed thrice with normal saline. Physiological saline was added to make 10% suspension of RBCs.

(c) Treatment of sera -

(i) Serum sample was inactivated at 56°C for 30 minutes,

(ii) The inactivated serum was kept overnight in a refrigerator with 50 ml of 50% Rhesus monkey erythrocytes to adsorb inactivate non-specific inhibitors.

(d) Titration of Antigen -

(i) With a wax pencil a V microtitre plate was marked as antigen dilutions - 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256 and cc. (cell control).

(ii) With 25 ul dropper one drop of physiological saline was added in all the wells.

(iii) Twenty five ul of measles antigen was then put in
first well and then doubling dilutions were made with the help of microdiluters in subsequent wells.

iv) With 25 ul droper one drop more of saline was added into each well.

v) 50 ul volume of 0.5% normal Rhesus monkey RBC suspension was added to each well with the help of microdropper.

vi) Cells and antigen dilution were mixed by shaking or by tapping the plate.

vii) The plate was covered and incubated at 37°C for 1 - 2 hours.

viii) Titration were read and then recorded in proforma.

Complete agglutination consisted of a layer of uniformly agglutinated cells covering the bottom of the well. The results could be read by tilting the plate and looking for the free flow of cells indicating absence of agglutination. The reciprocal of the highest dilution of antigen with complete agglutination was the end point and contained that number of haemagglutination (HA) units.

(E) Procedure for Haemagglutination Inhibition (HI):

1. Titrated antigen and treated test and known positive and negative sera were used.

2. The microtitre plate was marked to accommodate the number of sera to be tested for each antigen.
3. With the help of 25 ul dropper pipette, 25 ul of physiological saline was placed in all the wells except first and 7th well.

4. 25 ul of undiluted sera was added to first, second and 7th well.

5. Doubling dilutions of sera was made.

6. The last two wells had control serum dilution of 1 : 16 and 1 : 32.

7. 25 ul of measles antigen having 4 HA units was then added from first to sixth well of each row.

8. 25 ul of physiological saline was added in seventh and eighth well.

9. The plate was kept at room temperature for one hour.

10. 50 ul of 0.5% normal monkey erythrocytes was added to all the wells.

11. The contents were mixed on a shaker and incubated at 37°C for 1½ to 2 hours.

12. With each test the positive serum control, cell control and antigen control were put up to check the efficacy of the result.

13. The cell pattern was read and recorded -

   (a) RBC control was checked. These should settle down as a button.

   (b) the titration of antigens, which should contain 4 HA unit, was checked.
(c) the serum control was checked, there should not be any haemagglutination.

(d) positive control serum should give known HI titres.

(e) the test sera was checked. The end point was designated by the titre of the antibody.

**Determination of Immunoglobulins:**

Single radial immuno-diffusion technique of Manzini *et al* (1965) was used for determination of immunoglobulins (IgG, IgM and IgA). Tripartigen immuno-diffusion plates supplied by Immunodiagnostic Laboratory were used for this test.

I. **Principle:**

When an unknown amount of antigen is allowed to diffuse radially from a well in a uniformly thin layer of antibody containing agar for a sufficient time to allow all antigen to combine, the final area reached by the precipitate is directly proportional to the amount of antigen employed and is inversely proportional to the concentration of antibody. By standardizing the technical conditions of the experiment, it is possible to use this principle for the immuno-chemical determination of antigen.

II. **Materials Required:**

1. Tri-partigen* * immuno-diffusion plates IgG, IgA and IgM types.
2. *Accuracy control* - WHO recognised lyophilised stable human serum, for the immunoglobulins IgG, IgM and IgA.

3. *Partigen*\(^{(R)}\) dispenser (5 ul) or suitable 5 ul pipette.

4. Measuring devise with 1/10 mm division e.g. partigen ruler or measuring projector for immuno-analysis.

5. Isotonic saline.

6. Test tubes.

7. Suitable pipettes.


9. Table with reference values.

Tri-partigen immuno-diffusion plates contain a prepared agar gel in which M-chain specific antiserum to the respective immunoglobulin is incorporated. The antiserum is produced by immunization of sheep and goats.

III. *Method*:

The following steps were followed:

1. The plates were kept open and left to stand for about 5 minutes at room temperature to allow any condensation water that may have accumulated in the wells to evaporate.

2. IgA and IgM were determined using undiluted serum while for IgG determination, the serum to be tested and the control serum used were diluted 1:10 with isotonic saline.
3. Well-1 to 3 were filled with 5 ul of control serum.

4. Well-4 to 12 were filled with 5 ul of the respective sera under test.

5. The plates were closed tightly and left at room temperature.

6. Evaluations were made after a minimum diffusion time of 50 hours for IgG and IgA and 80 hours for IgM determination.

7. At the end of the given diffusion time, the diameter $D$ of the precipitation rings were measured accurately to 0.1 mm using a suitable measuring device (partigen ruler).

IV. Evaluation:

1. The immunoglobulin concentration related to the measured diameter were read directly from the table of reference values, when determining IgG, the values found were multiplied by the dilution factor.

2. When the protein concentrations of the serum samples diverge considerably from the normal value, i.e. the resulting precipitation ring diameters fall outside the assay range of the plate, the examination were repeated using higher or lower dilutions of serum samples.

3. The results were relied only when the value found for the control serum applied to well-1 lies within the confidence range taken from the table of values enclosed with each pack of the control serum.

The data were finally analysed by using standard statistical methods.