MATERIAL & METHODS
The present study was carried out in the Department of Paediatrics, M.I.E. Medical College, Jhansi, in collaboration with the Department of Pathology, M.I.E. Medical College, Jhansi, over a period of 11 months from May 1981 to March 1982. Pre-school children (1-5 years age), attending the Well Baby Clinic and those admitted in the Paediatric ward, were selected for this study.

Cases were grouped as:

A  Healthy normal controls.

B  Children suffering from protein-calorie malnutrition (PCM).

SELECTION OF CONTROLS:

Twelve normal healthy pre-school children were taken as control. Criteria for selection of control cases were:

1. Weight more than 80% of the 50th percentile of Harvard standard for age.

2. Exclusion of all possible factors known to affect the complement status viz. infections, liver and renal disorders, immunodeficiency diseases and corticosteroids.

SELECTION OF CHILDREN SUFFERING FROM PCM:

Thirty two children having PCM were taken for the present study. Criteria for selection of PCM cases were:

1. Weight less than 80% of the 50th percentile of Harvard standard for age.
2. Children having primary liver disorders, renal disorders and immunodeficiency diseases were excluded from the study.

3. No case was receiving corticosteroids.

Children suffering from PCM were treated with broad spectrum antibiotics, intravenous fluids, supplemental vitamins and minerals as per the requirements. All patients were put on nutritional rehabilitation schedule to raise the daily intake of food to more than 100 calories/kg along with 3-4 gm proteins/kg of the expected body weight.

An attempt was made to follow the cases at 3 weeks, 4-7 weeks and 10-12 weeks interval.

Besides name, age, sex, address and socio-economic status following facts were recorded in each case:

**DIETARY HISTORY:**

Dietary history was recorded with special emphasis on the following points:

a) The age upto which breast milk was given.

b) Age at which artificial milk was started.

Type of artificial milk and the quantum of dilution were also recorded.

c) Age at which semi-solids and solids were started, was also noted.
d) Present diet in terms of quantity and quality of food material used in feeding the child was recorded. Total calorie and protein intake per day were thus recorded in every case to ascertain the cause of PCM.

**IMMUNIZATION STATUS:**

History of immunization was taken from the parents or family members. For small pox and BCG 
vaccination confirmation was made by careful inspection of scar marks. For polio and DPT vaccination, however, 
verbal statements were relied upon, confirmation was done by records, if available.

**ANTENATAL, NATAL AND POSTNATAL HISTORY:**

To rule out any secondary factor which could give rise to malnutrition, relevant antenatal, natal and 
post natal history was recorded. Special emphasis was also given to birth weight and gestational age of the 
child.

**MILESTONES:**

Mile stones were recorded under 4 headings: motor, manipulative, social and speech. The age, at 
which the child attained these was ascertained, by 
objective and subjective assessment.

**PRESENT, PAST AND FAMILY ILLNESSES:**

Present ailment relating to various systems were recorded.
Efforts were made to find out the occurrence of any acute or chronic illness in the past, that might have affected the nutritional status of the child. Past illness was mainly recorded in two categories:

Category 1 - History of acute illness viz., fever, vomiting, diarrhoea and convulsion, lasting more than 4 days during the previous two weeks; category ii - History of cough, fever, vomiting, convulsions and diarrhoea, lasting more than 2 weeks any time during the previous 6 months. Besides these, definite history of primary complex, pertussis, measles or worm infestation was also recorded.

An enquiry was made about the history of any familial illness such as tuberculosis and diabetes.

**Physical Examination:**

A thorough clinical examination was made including those related to psychomotor changes, pallor, oedema, skin changes, hair changes, amount of subcutaneous tissue and muscle mass. Eyes were examined for the presence of xerosis and Bitot's spots. Skin was examined for any evidence of xerosis, hypopigmentation, hyperkeratosis and any dermatosis. Lips, gums and tongue were examined for the presence of angular lesions, cheilosis, gum swelling and glossitis.

Skeletal system was examined for the presence of any deformity and signs of vitamin D deficiency such
as craniotabes, cranial bossing, persistent open anterior fontanelle, costo-chondral bossing and epiphyseal widening. Thyroid gland was examined to find out any abnormality.

Thorough systemic examination was made to detect any abnormality in cardiovascular, digestive, respiratory and central nervous systems.

**ANTHROPOMETRIC MEASUREMENTS:**

**Weight**

Weight was recorded nearest to 0.1 kg by using adult type weighing machines. For children who could not stand, Infant weighing scale was used, capable of measuring weight to the nearest 0.05 kg. Same machines were used for subsequent follow up, to minimise the error.

**Length/Height**

Reclined length was measured by an Infantometer and standing height was taken by a locally fabricated Stadiometer. These measurements were recorded nearest to 0.1 cm.

**Mid-Arm Circumference**

Circumference of left upper arm at the point midway between the tip of the acromion process of scapula and olecranon process of ulna, was measured, while arm was hanging freely, to the nearest 0.1 cm. A flexible steel tape was used to record this.

**Laboratory investigations viz. haemoglobin, leucocyte count (total and differential), total serum**
proteins, serum albumin, urine and stool examinations were carried out routinely in each and every case. Radiological and other relevant investigations were performed if necessary.

Blood was collected by venepuncture. Samples of sera were harvested and stored frozen at -20° until ready for assay, but never for more than 4 months.

1 - DETERMINATION OF TOTAL HAEMOLYTIC COMPLEMENT (CH₅₀)

LEVEL 1:

Total haemolytic complement was determined by the technique of Mayer (1961).

Principle:

Measurement of total serum haemolytic complement is a useful screening test for the integrity of complement system. The test is based on the ability of sheep red cells, properly sensitized by rabbit antibody to sheep erythrocytes, to lyse in the presence of all 9 classical pathway components. Haemoglobin released by such lysis can be measured spectrophotometrically with great precision and related to the percentage of cells lysed.

Amount of complement required to lyse 50% SRBC constitutes one unit of CH₅₀. Complement titre is defined as the number of CH₅₀ units contained in 1 ml of serum.
Material:

1. Asever's Solution

- Glucose 24.6 gm, trisodium citrate (dihydrate)
- 9.6 gm and sodium chloride 5.04 gm were dissolved in
- 1200 ml distilled water. The pH of solution was adjusted
to 6.1 with 10% citric acid. It was then sterilized by
- low pressure autoclaving and stored in refrigerator.
- This solution was prepared fresh every 4 weeks. One
- volume or more was used for each volume of whole blood.

2. Stock Veronal Buffered Saline (Stock VBS)

- A concentrated (5 times) solution was prepared
- by dissolving sodium chloride 83.0 gm, sodium 5.5 diethyl
- barbiturate 10.19 gm in 1.5 litres of distilled water.
- The pH of solution was adjusted to 7.35 ± 0.05 with
- 1 N HCl and volume was made upto 3.0 litres. This
- solution was stored for 1 month at 4°C.

3. Isotonic Gelatin Veronal Buffered Saline (GVBS)

- One part of stock VBS was mixed with 4 parts
- of distilled water. Sufficient dry gelatin was added to
- give final gelatin concentration of 0.1%. Gelatin was
- dissolved by gently heating and mixing the solution.

- One ml each of 0.3 M CaCl₂ and 2 M MgCl₂ were
- added to each 1 litre of GVBS. This solution was
- prepared fresh every week.

4. Antisheep Haemolysin (procured commercially).

This was used as a source of complement (procured from a healthy donor).


These were procured from jugular vein of a healthy sheep with the help of a dry sterilised syringe.

7. Test Serum.

(This was collected from the patient under investigation.)

**Procedure:**

1. Preparation of Sheep Red Blood Cells (SRBC) Suspension.

SRBC were collected in Alsever's solution and used 3-5 days after collection and within 12 days of collection. SRBC were stored at 4°C.

On the day of the test sheep erythrocytes were washed thrice in 0.9% NaCl solution. One volume of the packed cells was suspended in 10 volumes of buffer to give a slightly greater than 5% suspension. One ml of this suspension was lysed with exactly 14 ml of distilled water and the optical density (O.D.) was measured at 541 nm with distilled water as blank.

A lysate with O.D. of 0.7 was considered to contain 5% or \(1 \times 10^9\) Cells/ml. From the O.D. of the sample tested and volume of the suspension (V1), final volume (Vf) to which the suspension was
adjusted to make a standardized suspension, was calculated according to the relationship:

\[ V_f = \frac{V_1 \cdot g \cdot D}{0.7} \]

2. Titration of Haemolysis

This was first performed so that the complement titration was independent of the concentration of haemolysis. 5.0 ml volumes of 5% SRBC were treated with equal volumes of 1:50, 1:100, 1:200, 1:400 and 1:800 diluted haemolysis in GVBS for 15 minutes at 37°C. This suspension of sensitized SRBC was now called EA.

6.5 ml volumes of 1:50, 1:100, 1:200 and 1:400 diluted normal human serum (NHS) were also prepared and taken in tubes A to D in 5 sets.

In tubes E and F, 6.5 ml each of GVBS and distilled water were taken, respectively. Then 1 ml of EA 1:50 was poured in 1st set of tubes from A to F. The same procedure was repeated for EA 1:100, 1:200, 1:400 and 1:800 for rest of 4 sets of tubes.

<table>
<thead>
<tr>
<th></th>
<th>NHS (1:50)</th>
<th>NHS (1:100)</th>
<th>NHS (1:200)</th>
<th>NHS (1:400)</th>
<th>GVBS</th>
<th>Distilled water</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>6.5 ml</td>
<td>6.5 ml</td>
<td>6.5 ml</td>
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<tr>
<td>A</td>
<td>1 ml</td>
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<td>B</td>
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<td>C</td>
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<td>D</td>
<td>1 ml</td>
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<tr>
<td>E</td>
<td>1 ml</td>
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<td></td>
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<tr>
<td>F</td>
<td>1 ml</td>
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</table>

| EA (1:50)|1 ml  |
|--------------------------|
|                            |
| EA (1:100)|1 ml  |
|--------------------------|
|                            |
| EA (1:200)|1 ml  |
|--------------------------|
|                            |
| EA (1:400)|1 ml  |
|--------------------------|
|                            |
| EA (1:800)|1 ml  |
|--------------------------|
After mixing the contents of tubes in each set, these were incubated at 37°C for 60 minutes. The tubes were then centrifuged and optical density (O.D.) measured at 541 nm. Percentage of haemolysis was calculated by the formula:

\[
\text{Haemolysis (\%)} = \frac{\text{O.D. Rep A to } \beta - \text{O.D. Rep } F}{\text{O.D. Rep F}} \times 100
\]

Dilution factors regarding antiserum (used in EA suspension) and normal human serum were read and used for further titration. These dilutions were found to be 1:100 and 1:50 respectively in the present series.

3. Titration of Complement

5% suspension of SRBC was prepared. Equal volume of 1:100 diluted haemolysis was added to SRBC suspension. This mixture was then incubated at 37°C for 15 minutes and was stored in a refrigerator till use (1 to 2 hours usually).

Test serum was diluted to 1:50 in GVBS and titration was set up as follows:

<table>
<thead>
<tr>
<th>TUBES</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>GVBS</td>
<td>4.0</td>
<td>3.0</td>
<td>1.5</td>
<td>0.5</td>
<td>6.5</td>
<td>-</td>
</tr>
<tr>
<td>Distilled water</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6.5</td>
</tr>
<tr>
<td>EA</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Test serum (1:50)</td>
<td>3.5</td>
<td>3.5</td>
<td>5.0</td>
<td>6.0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
After mixing the contents of tubes in each column, the test material was incubated at 37°C for 60 minutes. Then tubes were centrifuged and optical density of the supernatant in each tube was read at 541 nm with distilled water containing tube as a blank.

4. Calculations to Determine the Number of Units of Total Haemolytic Complement per ml of Serum (CH₅₀)

Haemolysis (Y) was calculated for each tube as follows:

\[ Y = \frac{O.D. \text{ tube 1 to 5}}{O.D. \text{ tube 6}} \]

A graph was plotted. The log of the amount of test serum added (log X) was plotted on the abscissa; log \( \frac{Y}{1-Y} \) was plotted on the ordinate. The antilog of X where straight line crossed 0 (zero) on the ordinate gave the volume of test serum needed for 50% lysis.

CH₅₀U/ml of undiluted serum was calculated as follows:

\[ \text{CH₅₀U/ml} = \frac{\text{dilution of serum}}{\text{volume required for 50% lysis}} \]

II. Determination of Alternative Pathway Activity (AP₅₀):

Alternative pathway activity was assessed by the technique described by Platta-Hills and Ichimaka (1974).

Principle:

Unsensitized rabbit erythrocytes (RRBC) activate the alternative pathway of complement in
normal human serum. Hence the lysis of RBC is used to assess the functional activity of the alternative pathway components.

Material:

1. Alsever’s Solution.
2. Stock Veronal Buffered Saline (Stock VBS)
3. Isotonic Gelatin Veronal Buffered Saline (GVBS)

For alternative pathway activity, only 1 ml of 3 M MgCl₂ was added to each 1 litre GVBS. This was prepared fresh every week.
4. Stock EDTA

Disodium ethylene diamine tetra acetate
37.3 gm was dissolved in 800 ml distilled water. The pH was adjusted to 7.65 ± 0.05 with freshly prepared 2 M NaOH and volume was made to 1 litre. This was stored for 3 weeks at 4°C.
5. GVBS - EDTA

Nine parts of isotonic GVBS (without CaCl₂ and MgCl₂) was mixed with 1 part of stock EDTA. This was prepared fresh every week.
6. Rabbit Red Blood Cells. (These were procured by giving a cut on the ear margin of a healthy rabbit and blood collected under sterile conditions).
7. Test Serum (This was collected from the patient).

Procedure:

RBC, collected in Alsever’s solution, were used from day 0 – 15 of collection. These were kept in the refrigerator after collection.
On the day of the test a 2.5% suspension of RBC in buffer was prepared in the same way as SRBC in the determination of CH50.

Test serum was diluted to 1:45 in GVBS and titration was set up as follows:

<table>
<thead>
<tr>
<th>Tubs</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>GVBS</td>
<td>ml</td>
<td>0.40</td>
<td>0.30</td>
<td>0.15</td>
<td>0.05</td>
<td>0.65</td>
</tr>
<tr>
<td>Distilled water</td>
<td>ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SRBC</td>
<td>ml</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Test serum (1:45)</td>
<td>ml</td>
<td>0.35</td>
<td>0.35</td>
<td>0.30</td>
<td>0.60</td>
<td>-</td>
</tr>
</tbody>
</table>

After mixing the tubes in each column, test material was incubated at 37°C for 30 minutes. Reaction was stopped by adding 3 ml of GVBS - EDTA. Tubes were then centrifuged and optical density (O.D.) of the supernatant was measured at 413 nm.

Calculation was done exactly in the same way as for CH50 and expressed as AP50 U/ml.

III- Determination of C3 Concentration:

Serum C3 levels were estimated by single radial immunodiffusion technique as described by Mancini et al (1965), with suitable modifications according to Pahey and McKeelvey (1965).
**Principle:**

Antigen diffuses radially from the point of application into an antibody containing gel and a circular precipitate (ring) is formed at the zone of equivalence. Keeping antibody concentration and gel thickness constant, the area covered by precipitation ring is proportional to the concentration of antigen.

**Material:**

1. Stock Barbitone Buffer (0.12 M, pH 8.6)

   Sodium barbitone 20.6 gm and barbitone 4.0 gm were dissolved in distilled water to a final volume of 1 litre with pH 8.6. One gm sodium azide was added per litre as a preservative.

2. Working Barbitone Buffer (0.06 M)

   Stock was diluted to 1 : 2.

3. Monospecific Antiserum Against C3 (anti-C3)

   (This was procured commercially).

4. Standard Normal Human Serum with Known Amount of C3

   (This was procured commercially).

5. Unknown Test Serum

   (This was obtained from the patient).

6. Glass slides of size 7.5 x 5.0 cm, gel punch, moist chamber, immunomicroscope.

**Procedure:**

Two percent Agar gel was prepared in working barbitone buffer. 2.5 ml volume of melted agar in a test tube was kept in a water bath maintained at 45°C.
2.5 ml of anti-C3 diluted 1:9 in buffer was added to the tube containing 2.5 ml agar and mixed thoroughly and poured over a clean glass slide kept on a horizontal table. Air bubbles, whenever present, were removed with a hot wire loop. Gel was allowed to set for 10-15 minutes.

Fifteen evenly spaced wells, of 3 mm size each, were cut, with the help of a punch using a predesigned template.

Measured volume (0 ml each) of various dilutions of standard serum and appropriately diluted test serum, were run into the wells. The plate was then incubated in a moist chamber at room temperature and diffusion was allowed for 20 hours. Finally the diameter (d) of precipitin rings was measured by immunomicroscope and $d^2$ (diameter square) was obtained in mm (Fig.: 4).

A standard curve was drawn by plotting $d^2$ vs known concentrations of standard serum. Concentrations of unknowns (with known diameter) were obtained from the curve. Final results were obtained after multiplying the readings with the dilution factor and expressed in mg/dl.

...
Fig. 4: Showing Single Radial Immunodiffusion.