Chapter 2

Material & Method
2. MATERIAL AND METHODS

2.1 Haemoglobinopathies and survey:

The survey was conducted in 15 separate centers spread over all three panchayats of the region. The help of the health service personnel and local leaders were sought for the mobilization of the tribals. Care was taken to mobilize all the subjects to attend the camps irrespective of whether they were healthy or sick. In the colonies and hamlets this was not a problem since all available members were examined. Similarly in the schools and tribal hostels all the children belonging to the tribal communities were examined. Even in one instance where the primary health centre was taken as the survey site, the selection bias was minimized by examining all the healthy bystanders also.

Selection of places for survey was not random in that the convenience of travel and contacts of the local organizers were taken into account. The selection of the individuals to be tested in each centre was however as unbiased as possible. Care was also taken to have adequate samples from all the three tribal communities.

Generally no difficulty was experienced in subject compliance. One reason for the general acceptance we feel, is because blood was collected by the finger prick method only and not by the conventional venepuncture. The list of centers where survey was conducted and the number of people tested in each center is given below.

Name, age, sex and community were noted for each person along with the clinical history of illnesses if any. History of recurrent jaundice, bone and joint pain, abdominal pain, leg ulcers were specifically asked for. Physical
examination was conducted particularly looking for growth retardation, anemia and hepatosplenomegaly.

**Table 2.1: Survey centres**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Centre Number</th>
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<tbody>
<tr>
<td>1.</td>
<td>Cheerakadavu Irula Ooru 36</td>
</tr>
<tr>
<td>2.</td>
<td>Bhoothivazhi Irula Ooru 70</td>
</tr>
<tr>
<td>3.</td>
<td>Anavayi Kurumba Ooru 48</td>
</tr>
<tr>
<td>4.</td>
<td>Mukkali Muduga Ooru 17</td>
</tr>
<tr>
<td>5.</td>
<td>Varakampady Irula Ooru 20</td>
</tr>
<tr>
<td>6.</td>
<td>Pudur Tribal Boys Hostel 91</td>
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<tr>
<td>7.</td>
<td>Pudur Government High School 36</td>
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<td>8.</td>
<td>Pudur Primary Health Centre 121</td>
</tr>
<tr>
<td>9.</td>
<td>Agali Tribal Girls Hostel 91</td>
</tr>
<tr>
<td>10.</td>
<td>Sholayur Government High School 198</td>
</tr>
</tbody>
</table>

**Preparation of Blood collecting anticoagulated containers:**

Containers for blood collection were prepared before collecting the blood. These containers or vials were thoroughly cleaned with no trace of detergents. As the amount of anticoagulant added to the container is meant for a specific amount of blood specimen, hence the container was marked previously for the amount of blood sample to be collected (i.e. 5 ml level). Then 10% solution of the anticoagulant i.e. EDTA was prepared as follows.

EDTA (Disodium Dipottasium Salt) : 10 gm
Distilled water : 100 ml
10 gm of the salt was dissolved in 80 ml of water in a 100 ml volumetric flask and then the volume of the solution was made up to 100 ml. Then about 0.1 ml of EDTA solution (approx. 10 mg EDTA) was poured in 5 ml marked bottles. (the EDTA concentration will be 2 mg/ml of whole blood.) The anticoagulant within the bottle was dried by keeping the latter overnight in an incubator (37° C). These prepared containers were later capped and stored at room temperature.

**Equipment and preparation:**

Gloves, tourniquet, alcohol pads, needles, syringes or evacuated tube holders, blood collection tubes, needle disposed containers were keeping ready for blood collection. Hands must be washed before specimen collection and a clean pair of gloves put on.

Before proceeding to collect the blood from the patient a tourniquet was applied to the arm of the patient so as to slow the blood flow and make the veins more prominent, which helps to select the puncture site for blood drawing. A decontaminant i.e. 70 % isopropanol pad was applied on the skin before the puncture was made. Let the site air dry to prevent unnecessary discomfort (stinging).

The blood was collected by venipuncture of the median cephalic vein of the forearm by the needle and the blood was expelled gently into the container. The container was capped and swirled gently to mix the anticoagulant with the blood. Blood (2 ml) was collected in another plain container (i.e. without anticoagulant) so as to obtain the serum for biochemical analysis.
The blood collection containers were labeled with the patients' lost and first name, sex, age and date of collection.

**Blood Collection and Hemolysate Preparation:**

Blood was collected by a deep finger prick using a disposable lancet. The finger was lightly squeezed so as to elicit four to five large drops of blood, which is collected directly into a 4 ml test tube containing normal saline. One drop of blood was used to prepare a peripheral smear.

Preparation of hemolysate was done immediately after the camp. Red cells were washed thrice in normal saline by centrifuging at 3000 rpm for three minutes and pipetting the supernatant. After the last pipetting, 5 drops of hemolysate reagent were added to the cell button and shaken to obtain the hemolysate. In those cases where less amount of blood was obtained and consequently the cell button was small; 28 fewer drops of hemolysate reagent were used. The hemolysates were run in cellulose acetate strips at pH 8.4. Hemoglobin electrophoresis was done within 24 hours.

**Mass screening method for thalassaemia:**

Information about laboratory methods and counseling was obtained from interviews with hematologists, laboratory technicians and nurse specialists plus various reports from Amravati Thalassemic Society. The most effective and feasible approach for a vast country like ours is preventive genetics and major efforts need to be directed for applying simple and unexpensive screening test. NESTROFT was suitable test for screening the suspected cases of b-thalassaemia trait.
2.2 Blood analysis:

NESTROFT: (Naked Eye Single Tube Redcell Osmotic Fragility Test):

NESTROFT was easy to perform, inexpensive and does not require any sophisticated equipments. Reagents used were stock solution of 10% buffered saline (pH 7.4) comprising of NaCl 90 g, Na2HPO4 13.65 g and NaHPO4, 2 H2O 2.4 g dissolved in distilled water. Final volume was adjusted to one liter.

Procedure of the test:

1) Two ml of buffered saline was taken in one tube (10 cms X 1 cm diameter) and 2 ml distilled water was taken in another test tube.

2) A drop of anticoagulated blood is added to both tubes and were left undisturbed for half an hour at room temperature.

3) After half an hour both tubes were shaken and then held against a white paper on which a thin black line was drawn.

4) The line is clearly visible through the contents of the tube containing water. If the line is similarly visible through the contents of the tube with the buffered saline, the test is considered negative.

5) If the line is not clearly visible, the test is considered positive.

6) The number of true positive (TP), true negative (TN), false positive (FP) and false negative (FN) were determined.

By using following formulae we can easily find out the results of Nestroft

1) Sensitivity = TP x 100 / TP + FN

2) Specificity = TN x 100 / TN + FP

3) Predictive value of a positive test = TP x 100 / TP + FP

4) Predictive value of a negative test = TP x 100 / TN + FN
Estimation of Foetal haemoglobin:

Principle:
The estimation of HbF by alkali denaturation is based on the resistance of HbF to denaturation at alkaline pH. Except foetal haemoglobin all other haemoglobin are easily denatured by an alkali.

Reagents:
1) Drabkins solution or cyanide solution.
2) Saturated ammonium sulphate solution.
3) Sodium hydroxide solution.

Procedure:
A red cell lysate of 5 ml of the specimen (anticoagulated blood) was prepared. Then it was reacted with Drabkins solution and the resultant cyanmethaemoglobin solution was used for determining foetal haemoglobin: total haemoglobin.

Preparation of foetal hemoglobin solution:
To the resultant cyanmethaemoglobin solution, 1.2 N NaOH was added after 2 minutes saturated ammonium sulphate solution was added and allowed to stand for 5 minutes. Filtered through a double layer of Whatmann filter no. 42. The absorbance of the filtrate was read at 415 nm in a photoelectric colorimeter using drabkins solution as blank.

Preparation of total haemoglobin solution:
To the lysate instead of NaOH solution distilled water was added followed by ammonium sulphate. The solution was filtered diluted ten fold with...
drabkins solution. The absorbance of the diluted filtrate was read at 415 nm.

**Calculation**: \[
\text{Absorbance of HbF solution} \times 100
\]
\[
\% \text{HbF} = \frac{\text{Absorbance of Hb (total) solution} \times 20}{\text{Absorbance of HbF solution} \times 100}
\]

**Complete Blood Count**:

This method simply involves counting the number of blood cells per unit volume of whole blood. Manual methods using the hated hemocytometer have been universally replaced by automated counting.

In the present study automated system were used. A venous sample was collected from all the patients and controls and the following parameters are used.

1) Red blood cell count (RBC)
2) Hemoglobin (HGB)
3) Hematocrit or Packed cell volume (PCV)
4) Mean corpuscular volume (MCV)
5) Mean corpuscular hemoglobin (MCH)
6) Mean corpuscular hemoglobin concentration (MCHC)
   1) White blood cell count (WBC)
   2) Platelet count

**Hemoglobin Electrophoresis**:

The method followed by us is briefly outlined.16

**Equipment**:

1. Electrophoresis chamber and Power pack
2. Cellulose acetate membrane
3. Applicator
4. Wicks and blotting paper
5. Glass plate
6. Staining set-up

Reagents:
1. Hemolysate reagent (Helena Biosciences)
2. Tris-EDTA-Boric acid buffer pH 8.4 (Helena Biosciences)
3. Working buffer: Stock buffer powder dissolved in 1000 ml. distilled Water
4. Staining solution Ponceau red 200 gm Trichloroacetic acid 7.5 gm Sulfo salicylic acid 7.5 gm Distilled water Upto 100 ml
5. Destaining solution Glacial acetic acid 5 ml Distilled water 100 ml
6. Clearing solution Glacial acetic acid 30 ml Methanol 70 ml
7. Clear aid (Helena Biosciences) 4 ml

Procedure:
1. Electrophoresis chamber is filled with working buffer. Whatman No. 3 filter paper is cut to suitable size and kept in each chamber serving as wick.
2. Cellulose acetate membrane is cut as per requirement. The membrane is dipped in buffer for 5 minutes and blotted between two pieces of Whatman filter paper quickly and evenly.
3. Hemolysate (1 to 1.5 microlitres) is applied with applicator on the mark of origin.
4. It is then placed on wicks, directing the mark of origin towards the cathodal end.
5. Glass plate is placed over it.
6. A current of 150 to 200 volts is applied for 30 minutes.
7. Power supply is switched off. The membrane is removed and stained with Ponceau red for five minutes. Excess dye is drained off and the membrane destained with 5% acetic acid till the background is white.
7. The strip is then put in clearing solution for 7-10 minutes and dried in incubator. The dried and transparent strips are then numbered and filed. Hemolysates from known cases of sickle cell anemia served as controls for hemoglobin S. Cord blood was used as control for hemoglobin F. Normal blood had the HbA.

**Peripheral smear examination:**

The peripheral smear was prepared, stained with Leishman's stain and screened in all the subjects particularly for hypochromic microcytic anemia. In all cases of sickle cell anemia the following points were specifically looked for: presence or absence of irreversibly sickled cells, target cells, anisopoikilocytosis, polychromasia and nucleated red cells. Irreversibly sickled cells and target cells were assigned a grading of 3+ when they were present in almost all fields, 2+ when seen frequently and 1+ when only occasional cells were present.

**Methodology:**

A Registered Nurse Specialist at Children's Medical Services will work directly with the infant screening coordinator from the referral centers, following up on referrals of newborns in Hospital. This nurse will provide initial contact and counseling, assure that confirmatory testing is done and treatment initiated, as well as coordinate care for children with thalassemia and
sickle cell disease. Letters will be sent by the partnership to families whose children are identified with thalassemia and sickle cell trait, providing them with information about who to contact at the Sickle Cell Foundation for counseling. There are approximately 426 newborns identified annually in this region with one of the traits. Follow-up contact will be made to encourage families to obtain counseling. The partnership will also provide information to all pediatricians and family practitioners in the area about resources available for children and families who are identified sickle cell disease or trait.

2.3 Biochemical Analysis:

2.3.1 Estimation of serum alkaline phosphatase – P-nitrophenol method.

Phosphatases are enzymes which catalyse the splitting of a phosphate from mono-phosphoric esters.

**Principle of the method:**

Paranitrophenyl phosphate, which is colourless, is hydrolysed by alkaline phosphatase at pH 10.5 and 37°C to free paranitrophenol, which is coloured yellow. The addition of NaOH stops the enzyme activity and the final colour shows maximum absorbance at 410 nm.

**Reagents:**

1) 2-amino 2-methyl 1 propanol (AMP) buffer pH 10.5
2) Magnesium chloride (1.5 m mol/L)
3) Sodium hydroxide 0.25 m
4) Substrate: Obtained by dissolving 83.5 mg disodium paranitrophynyl phosphate in 1 ml magnesium chloride solution.
5) Stock paranitrophynol (PNP) 10.8 m mol/L
6) Working PNP 54 m mol/l and serum was used.

**Procedure:**

The protocol of the procedure was described below.

Preparation of standards (S1-S6)

Spectrophotometer was set at 410 nm

Enzyme measurement in test

**Calculation:**

Activity of ALP was calculated by the formula = Test absorbance / std.

Absorbance X 40

2.3.2 Estimation of serum urea – Diacetyl Monoxime method.

**Principle of the method:**

Urea reacts directly with diacetyl monoxime under strong acidic conditions to give a yellow condensation product. The reaction is intensified by the presence of ferric ions and thiosemicarbazide. The intense red colour formed is measured at 540 nm / yellow green filter.

**Reagents:**

Stock acid reagent – obtained by dissolving 1.0 gm of ferric chloride hexahydrate in 30 ml of distilled water and also add 20 ml orthophosphoric acid and mixed.

**Mixed acid reagent:** Obtained by adding 100 ml of conc. H₂SO₄ to 400 ml distilled water. Again 0.3 ml or stock acid reagent was added mix and store.

**Stock colour reagent A** – 2 gm diacetyl monoxime dissolved in distilled water and volume make upto 100 ml.
Stock colour reagent B - 0.5 gm thiosemicarbazide dissolved in distilled water and volume make upto 100 ml.

Mixed colour reagent - 35 ml stock colour reagent A + 35 stock colour reagent B and volume make upto 500 ml with distilled water

Stock urea standard - 1.0 gm analytical grade urea + 100 ml benzoic acid.

Working standard 50 mg/dl - 5 ml stock urea standard + 100 ml benzoic acid.

Procedure: The protocol of the procedure was described below.

Dilution of standards (S1 - S3), and test

Colour Development

The colour reagent is prepared fresh at the time of analysis by mixing distilled water, mixed acid reagent and mixed colour reagent in the ratio 1:1:1.

Calculation:

Patients samples was analyzed and the results calculated by using formula.

Absorbance of test

Urea in test sample = \[
\frac{\text{Absorbance of standards}}{150 \text{ mg/dl}}
\] X 150 mg/dl

Absorbance of standards

2.3.3 Estimation of serum creatinine - Jaffe’s method:

Principle of the method:

Creatinine present in serum directly reacts with alkaline picrate resulting in the formation of a red colour, the intensity of which is measured at 505 nm / green filter.

Reagents:

Reagent A - obtaining by 400 ml distilled water + 4.4 NaOH mixed + 9.5 gm
trisodium phosphate + 9.5 gm sodium tetraborate, volume make upto 500 ml.

**Reagent B** – obtained by 20 gm sodium lauryl sulfate + 500 ml distilled water

**Reagent C** - 4.6 gm anhydrous picric acid + 500 ml distilled water

**Working reagent** - Prepared by freshly mix equal volumes of the above three reagents. Stock creatinine standard 100 mg/dl

100 mg pure creatinine + 0.1 M HCl and volume make upto 100 ml.

**Working creatinine standard** - Obtained by diluting 2, 4, 6 and 8 ml of stock creatinine standard each to 100 ml with 0.1 M HCl.

**Procedure** :- The protocol of the procedure was described below.

Leave at room temperature (25-35°C) for 30 minutes. Spectrophotometer set to zero with blank at 505 nm/green filter and measure the absorbance of the other tubes. After measuring the absorbance, solutions pour back into the respective tubes. Then add 0.2 ml of 30% acetic acid to the test and QC tubes, mixing well and leave at room temperature (25-35°C) for 5 minutes. Again by setting the spectrophotometer to zero with blank at 505 nm/green filter and measure the absorbance of test and QC.

**Calculation** :

Serum creatinine was obtained by using formula

Serum creatinine = Test absorbance / Standard absorbance X 6 mg/dl

2.3.4 Estimation of Bilirubin :

**Principle**:

A pink colour is produced when bilirubin in methyl alcohol is coupled with diazotized sulphanilic acid. Methyl alcohol releases bound bilirubin and catalyses the reaction of freed bilirubin with diazo reagent.
Reagents:

Absolute methyl alcohol:

**Diazo Blank solution**: 15 ml. of conc. HCL in 985 ml. of distilled water.

**Diazo reagent**: 3 ml. of Diazo reagent B is added to 100 ml. of Diazo reagent A before use.

**Dilute standard bilirubin solution**: 1 ml. of standard stock solution (1 ml = 0.4 mg. bilirubin).

**Corrected reading of unknown**: = Reading of unknown minus reading of blank.

**Calculation**:

Total bilirubin in mg. per 100 ml. of serum. = Corrected reading of unknown X 5 / Reading of standard

Direct reacting bilirubin can be determined in the same way as above, using distilled water in place of methyl alcohol.

Indirect reacting bilirubin: = Total bilirubin minus direct reacting bilirubin.

2.3.5 Estimation of sodium and potassium – Flame photometry:

**Principle of the method**:

when a solution of an inorganic salt such as sodium chloride is sprayed into the flame, the elements in the compound are partly converted into the atomic state. Due to the heat energy of the flame a very small proportion of these atoms is excited and the electrons move to a higher energy level. The proportions of the atoms that are excited depends upon the concentration of the particular element and on the temperature of the flame. In the excited state the
electrons are unstable and they rapidly revert back to their former lower energy level. As they change from the excited state or higher energy level back to the lower energy level, they emit the light in the form of a fixed wavelength, to produce a spectrum. Under carefully controlled conditions the amount of light emitted is directly proportional to the number of atoms that are excited, which in turn is proportional to the concentration of the structure in the sample.

Reagents:

Stock sodium 1000 m mol/L

Prepared by dissolving 29.25 gm dried NaCl in 400 ml of distilled water and then make up volume up to 500 ml.

Stock potassium 100 m mol/L

Prepared by dissolving 0.746 gm dried KCl in 80 ml of distilled water and then make up volume upto 100 ml.

Combined standard for sodium and potassium

Prepared by diluting 14 ml of stock sodium and 5 ml of stock potassium together to 100 ml with distilled water.

Sample dilution Obtained by diluting serum sample 1:100 with distilled water by mixing 0.1 sample with 9.9 ml distilled water.

Procedure:

The blank was set by atomizing water over flame, followed by the standards of sodium and potassium (combined working solutions of highest concentration). Dial readings were adjusted and then the test sample (diluted serum) was taken for the determination of sodium and potassium.
Calculations:

After aspirating the standard solution, the digital reading for Na\(^+\) was adjusted to 140 and that of K\(^+\) to 50. This was done in order to represent Na\(^+\) and K\(^+\) values in undiluted serum. Since the test sample was diluted initially 1:100 and then aspirated, the initial standard values for Na\(^+\) and K\(^+\) multiplied by 100.