CHAPTER 2

Material and Methods
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A range of estimates for thalassaemia trait and disease were derived for the different ethnic groups living in the different parts of the world, which reflected uncertainty over true population value in certain countries and the heterogeneity within an between countries of origin comprising the same ethnic group.

2.1 Screening and Diagnostic tests.

The study was undertaken to determine the prevalence of β-thalassaemia major and β- thalassaemia trait in Amravati region. The study included 1100 patients of specific group (unmarried individuals above 14 years, married couples planning for children and anemic patients). These patients hailed from various part of the Amravati district.

2.1.1 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 Dipotassium 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 prepare containers were later capped and stored at room temperature.

2.1.2 Equipment and preparation

Gloves, tourniquet, alcohol pads, needles, syringes or evacuated tube holders, blood collection tubes, needle dispensed containers were keeping ready for blood collection. Hands must be washed before specimen collection and a clean pair of glove 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 patient's last and first name, sex, age and date of collection.

2.1.3 Mass screening method for thalasaemia.

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 β-thalassaemia trait.

2.1.3a 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

2.1.3b Hemoglobin electrophoresis.
All those samples which were detected to be positive in the NESTROFT were then subjected to a confirmatory test i.e. hemoglobin electrophoresis. Cellulose acetate electrophoresis was used to eluted and quantitated the band of variant haemoglobin i.e. quantitation of HbA2 level. Cellulose acetate paper is micro porous and this prevents spreading of bands and hence excellent separation is achieved at low voltage.

Preparation of haemolysate

1) Centrifuge 3 ml of anticoagulated blood at 3000 rpm for 15 min.
2) Plasma and WBC were removed and wash the red cells 3 times in 3 volumes of 9 gm/l NaCl (saline) at 3000 rpm for 5 min.
3) After third wash, traces of plasma proteins and as much as WBC were removed.
4) Adding 2 volumes of distilled water lysed RBCs.
5) Shake well for 5 min in a stoppered centrifuge tube.
6) Centrifuge at 3000 rpm for 30 min, pipette the clear supernatant lysate into a test tube.
7) Upper layer was removed and centrifuged again obtaining perfectly clear haemolysate.

Cellulose Acetate Electrophoresis

Equipment

1) Cellulose Acetate strips – 4 X 2 cm
2) Horizontal electrophoretic tank
3) Power pack
4) Small forceps
5) Applicator coverslip
6) Blotting paper.

Reagents

i) Buffer

Tris EDTA borate buffer (pH 8.6)
Tris powder = 10.2 gm
EDTA = 0.6 gm
Boric acid = 3.2 gm
Distilled water = 1000 ml

ii) Stain :- Ponceau S solution : Dissolve 5 gm Ponceau S powder per liter of 5 % Trichloroacetic acid.

iii) Destaining solution :- 5 % Acetic acid

iv) Clearing solution :- Methanol : Acetic acid (88:12)

Procedure

1) compartments of electrophoretic tank were filled with TEB buffer. Soak and position.

2) Cellulose acetate membranes was soaked in TEB buffer for 5 minutes. It is important to immerse the membranes slowly so as to avoid trapping air bubbles.

3) Strip was positioned on the bridge of the electrophoresis chamber with porous surface facing up.

4) Four or two samples were loaded on the strip such that the samples will be approximately 15 mm from strips cathodic edge.

5) Strip was stain with Ponceau S solution.
6) Clear in 20 % acetic acid in methanol for 6-8 min.
7) Strips were then oven dried at 65° C for 4-6 min.

2.2 Hematopathology

Hematopathology is somewhat unique in its approach to the patient and the disease in that a) many diseases are understood at the molecular level, b) the patient’s tissue is easily obtainable in large quantities and easily kept viable for studies, c) the function of the blood is relatively simple when compared to that of other organ systems.

In the present study different hematological techniques were used for the screening of the thalassemic patients.

2.1.1 Peripheral blood smear examination.

Specimen : - A fresh venous sample collected in EDTA was used to prepare PBS.

Equipment and Reagents: - New slides, cover slip, DPs, Romanovski stain (Leishman stain, methanol (as a fixative).

Procedure :- 1) Small drop of blood (with or without anticoagulant ) was place on a new slide.
2) Push forward the spreader with a quick, smooth and single movement so as to make 2-3 cm long smears with convex edge.
3) Smear was dried quickly and stained the slides by using Leishman stain and methanol was used as a fixative.
4) Permanent slide was prepared by covering with cover slip.

2.2.2 Estimation of Haemoglobin (Acid haematin / Sahli method)

Specimen : - A fresh venous sample collected in EDTA was used to determine
haemoglobin concentration.

Equipment and Reagent :- N/10 HCl, Sahli's haemoglobinometer

Technique :- a) N/10 HCl was taken upto the mark of 20 percent in haemoglobinometer tube.

b) 200 mm of whole blood collected in haemoglobinometer pipette was transfer and mixed with N/10 HCl.

c) Fresh N/10 HCl was sucked in the pipette and dropped into the tube 3 times.

d) The tube was shaken thoroughly.

e) The colour of the diluted blood in the tube was compared with the reference tube.

f) The haemoglobin concentration was read directly from the mark reached.

2.2.3 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 and 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:

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

**2.2.4 Complete Blood Count**

This method simple involves counting the number of blood cell 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)
7) White blood cell count (WBC)
8) Platelet count.
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 monophosphoric esters.

Principle of the method

Paranitrophenyl phosphate, which is colourless, is hydrolysed by alkaline phosphatase at pH 10.5 and 37°C to from 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 paranitrophenyl phosphate in 1 ml magnesium chloride solution.
5) Stock paranitrophenol (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)

<table>
<thead>
<tr>
<th></th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
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<td>3.0</td>
<td>4.0</td>
<td>5.0</td>
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<td>3.0</td>
<td>2.0</td>
<td>1.0</td>
<td>**</td>
</tr>
<tr>
<td>Activity U/L</td>
<td>40</td>
<td>80</td>
<td>160</td>
<td>240</td>
<td>320</td>
<td>400</td>
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Mix Well

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<th></th>
<th>Blank</th>
<th>Test</th>
<th>QC</th>
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</thead>
<tbody>
<tr>
<td>AMP buffer (ml)</td>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
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<tr>
<td>Mix and Incubate at 37°C for 5 minutes</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Test sample/QC (ml)</td>
<td>--</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Substrate (ml)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Mix and Incubate at 37°C for 15 minutes</td>
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</tr>
</tbody>
</table>

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 g 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 up to 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 = \[\text{Absorbance}\] \times 150 \text{ 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 tetaborate, volume make up to 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 mixing equal volumes of the above three reagents. Stock creatinine standard 100 mg/dl

100 mg pure creatinine + 0.1 M HCl and volume make up to 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.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>Test</th>
<th>QC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ml)</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
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<tr>
<td>Distilled water</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(ml)</td>
<td>0.2</td>
<td></td>
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<tr>
<td>Standard</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>(ml)</td>
<td></td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
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<tr>
<td>Test sample/QC</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Mix Well
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 0.2ml 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

\[
\text{Serum creatinine} = \frac{\text{Test absorbance}}{\text{Standard absorbance}} \times 6 \text{ mg/dl}
\]

### 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:

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

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

Dilute standard bilirubin solution : 1ml 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.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Colorimeter tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>Standard</td>
</tr>
<tr>
<td>Methyl Alcohol</td>
<td>5 ml.</td>
</tr>
<tr>
<td>Diazo Blank Solution</td>
<td>1 ml.</td>
</tr>
<tr>
<td>Diluted Bilirubin Standard</td>
<td>X</td>
</tr>
<tr>
<td>Diazo Reagent</td>
<td>X</td>
</tr>
<tr>
<td>Serum (diluted 1 in 10)</td>
<td>4 ml.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>X</td>
</tr>
</tbody>
</table>

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.