MATERIALS AND METHODS
Selection of cases

1. One hundred consecutive anemia patients attending the Dr. J. C. Patel Department of Hematology K. E. M. Hospital, Bombay were taken for study. Patients with anemia due to bleeding disorder or leukemia were excluded.

2. One hundred and thirty four family members of 34 diagnosed beta thalassemia cases (homozygous, heterozygous or double heterozygous) attending the Hematology Department were studied.

3. Twenty apparently healthy individuals were taken up for study as a control group.

4. Individuals of Lohana community residing in a particular locality in Bombay were approached and explained the purpose of population screening of their community to detect the beta thalassemia trait. 201 individuals volunteered for the purpose.
In the first 3 groups, the clinical examination was done as per specified proforma (See Annexure-1). From the individual cases 4 ml of blood was collected in EDTA bulb and 10 ml blood in plain tube were collected. Peripheral smear was prepared from blood before adding to anticoagulant. The smear was air dried, fixed for 5 minutes in methanol and air dried for staining in Giemsa Stain. The smear were stained and morphology of RBCs studied as described by Dacie & Lewis (1975)\textsuperscript{128}.

From the EDTA blood Hb\%, PCV, RBC count was done by electronic cell counter. RBC indices like MCV, MCH, MCHC were calculated. After the indices, various discriminant factors were calculated as per formulae given below. Reticulocyte preparation was done using Supravital Stain (Brilliant Cry|yl Blue) from EDTA blood and percentage of reticulocyte was determined as described by Dacie & Lewis (1975)\textsuperscript{128}.

Osmotic fragility was done as per Parpart et al (1947)\textsuperscript{123} at 0.4 concentration of buffered saline. Single tube osmotic fragility test was done as described by Katamis et al (1981)\textsuperscript{124}. Serum iron, total iron binding capacity and transferrin saturation were estimated as per Ramsay et al (1958)\textsuperscript{125}. Serum ferritin was done using pharmacia kits\textsuperscript{126}. Hemolysate was prepared from EDTA blood. Hemoglobin electrophoresis was done
on paper as per Black et al (1966)\textsuperscript{104}. On the next day the band of different hemoglobin fractions were observed. Sickling test was done using 2% sodium metabisulphite\textsuperscript{128}. HbA2 was estimated from the same hemolysate by microchromatography as per Efremov et al (1974)\textsuperscript{106}. Fetal hemoglobin was estimated as described by Betke et al (1959)\textsuperscript{127}.

Survey of cases of Lohana Community

5 ml of blood was collected from 201 volunteers who agreed for the screening programme. History of Iron therapy (Oral or parenteral) within 3 months was enquired and noted.

Hemoglobin \%, PCV and total RBC counts was done in electronic counter. Naked eye single tube RBC osmotic fragility test (NESTROFT) was done on all samples. HbA2 was estimated by paper electrophoresis and elution in all cases with positive NESTROFT and in 23 samples where NESTROFT was negative. In 51 samples HbA2 was estimated by microchromatography method.

A comparative study of hemoglobin A2 quantitation by paper electrophoresis with elution\textsuperscript{104} and microchromatography with DEAE cellulose\textsuperscript{106} was done in 185 individuals. They were divided into 3 groups. Group-I i.e. thalassemia trait whose HbA2 level was >3.5\% by paper electrophoresis. Group-II- Iron deficiency anemia whose transferrin saturation was \(<16\% or ferritin was \(<10 \text{ ug/1} \) and Group-III who did not belong to
group I & II. Same hemolysate from each individual was used for HbA2 estimation using two methods.

OSMOTIC FRAGILITY AT 0.4 CONCENTRATION OF BUFFERED SALINE AS PER PARAPART ET AL (1947)123.

Reagents :-

(1) Stock solution of buffered Sodium chloride (10%) at pH 7.4.
   Sodium chloride - 90 gm.
   Disodium hydrogen phosphate NaHPO₄ - 13.65 gm.
   Sodium Hydrogen Phosphate NaH₂PO₄, 2H₂O - 2.43 gm.
   The ingredients were dissolved in Dist. water and made the volume to 1 litre.

(2) Working solution :-
   1% buffered saline was prepared by diluting 1 ml of 10% stock solution to 10 ml with 9 ml Dist. water.
   0.4 concentration was prepared by mixing 3 ml of Dist. water to 2 ml of 1% buffered saline.
   0.36 buffered saline was prepared by diluting 36 ml of 1% buffered saline to 100 ml with Dist. water.

(3) Blood with EDTA anticoagulant 1.5 mg per ml of blood.
Fig. No. 3.
Naked eye single tube red cell osmotic fragility test.

1. White paper
2. One mm thick black line on white paper.
3. Black line visible through tube with dist. water and a drop of blood (Control).
4. Black line not seen through the tube with 0.36% buffered saline and a drop of blood (positive).
5. Blackline visible through the tube with buffered saline and a drop of blood.
**Procedure:**

Two tubes were taken (10 x 1 cm). To one 5 ml of 0.4% buffered saline and 5 ml of Dist. water were added to 2nd tube. 0.05 ml of EDTA blood was mixed to the two tubes. They were mixed 2-3 times by inverting and kept for 30 minutes in room temperature (20 - 25°C). After remixing the tubes were centrifuged at 1500 for 5 minutes. The hemolysis was compared with control i.e. 100% with Dist. water using the spectrophotometer at wave length setting at 540 nm. Dist. water was used as blank.

Calculation

\[
\frac{\text{Optical density of test}}{\text{Optical density of control}} \times 100 = \% \text{ of hemolysis.}
\]

**PROCEDURE FOR NAKED EYE SINGLE TUBE RED CELL OSMOTIC FRAGILITY TEST (NESTROFT) AS DESCRIBED BY KATAMIS ET AL (1981).**

0.36% of buffered saline was prepared by mixing 36 ml of 1% buffered saline to 64 ml of Dist. water. To one tube (10 x 1 cm) 2 ml of 0.36% buffered saline was taken and to another tube (10 x 1 cm) 2 ml of Dist. water was added. To both tubes one drop blood was added and mixed by inverting the tubes. It was kept at room temperature for \(\frac{1}{2}\) hr. At the end of half hour the tube was held against a white paper with 1 mm
thick black line on it. If the line was visible through the solution the test was read as negative while if the line was not visible the test was positive (Fig.No.3) for beta thalassemia trait. Second tube was taken as control (Negative) reading.

STUDY OF PERIPHERAL SMEAR AS DESCRIBED BY DACIE & LEWIS (1975)

Reagents :

(1) Giemsa Stain (Stock Solution)

Giemsa Powder - 1 gm
Glycerol - 56 ml
Methanol - 56 ml

1 gm of Giemsa powder was mixed with 56 ml of glycerol and crossed in a mortal pastle for 1/2 hr. It was kept in the bottle and put in the incubator at 56°C for 2 hrs. It was cooled in room temperature. Then 56 ml of Methanol was mixed and shaken vigorously. It was kept in the room temperature for 7 days for maturation. Then the solution was filtered and kept as stock solution.

Working Stain Solution:

1 part of stock solution + 9 part (volume) of buffer water. It was prepared all the time before staining the blood smear.
Buffer water:

66 mmol/litre Sorensen's phosphate buffer (stock)

(a) $\text{KH}_2\text{PO}_4$ 9.1 gm mixed in a litre of Dist. water
(b) $\text{Na}_2\text{HPO}_4$ 9.5 gm mixed in a litre of Dist. water.

To obtain pH 6.8, 50.8 ml of (a) and 49.2 ml of (b) were mixed.

Working buffer:

50 ml of stock Sorensen's phosphate buffer was made 1 litre with Dist. water.

Procedure:

Before adding to anticoagulant a drop of blood was taken on cleaned greasefree glass slide and smear was prepared. The smear was air dried and fixed in methanol for 10 minutes. Then the slide was dried and kept in the staining tray. Freshly prepared working diluted Giemsa solution was added and kept for 15 minutes. Then the slide was washed with running tap water, dried and examined the ideal part of the smear under oil-immersion.


Serum iron estimation:

Reagents:

(1) 2.2 Dipyridyl 0.2%

2.2 Dipyridyl 100 mg

glyceral acetic acid 1.5
Diluted to 50 with Dist. Water.
It was kept in the 2-8°C.

(2) Sodium sulphite Na₂SO₃ 0.2 M
2.52 gm anhydrous Na₂SO₃ dissolved in 100 ml of Dist. water.

(3) Standard Iron Solution
Stock solution :-
0.498 gm of ferrous sulfate (FeSO₄) was dissolved in 1 ml of Conc. sulfuric acid.
The volume was made upto 100 ml.
Working solution :-
3 ml of stock solution was diluted to 100 ml to make 3 mg/ml.

(4) Chloroform.

(5) Iron free glass ware washed in chromic acid and glass dist. water.

(6) Glass doubled dist. water.

Procedure :-
To 1 ml of serum 1 ml of dist. water was added.
Then 0.5 ml of sodium sulphite (Na₂SO₃) and 0.5 ml dipyridyl solution was added and kept in the boiling water bath for 5 minutes to precipitate protein. It was cooled and 1 ml of chloroform (CHCl₃) was added and shaken vigorously. Then it was centrifuged for
15 minutes at 3000 rpm. The supernatant was filtered in Whatman 44 No. To 2 ml of Dist. water 1.5 ml of filtrate was added.

A 2nd tube for blank with 2 ml Dist. water and a third tube for standard with 1 ml of standard solution and 1 ml of Dist. water was started along with the 1st tube containing the serum 1 ml + Dist. water. All the steps were same and done simultaneously till the addition of 1.5 ml filtrate to 2 ml of Dist. water and reading was taken in spectronic 20 at wave length at 520 adjusting the blank to 100% transmission (0% optical density) and then % transmission was converted to optical density.

Calculations: -

\[
\text{Serum iron mg}\% = \frac{\text{Optical Density of test}}{\text{Optical Density of Standard}} \times 300
\]

TOTAL IRON BINDING CAPACITY (TIBC) AS PER RAMSAY ET AL (1955) \(^{125}\).

Reagents: -

(1) All the reagents required for Serum iron.
(2) Ferric chloride
Iron 5 mg/1 ml in 0.5 N Hcl.
(3) Light Magnesium carbonate Mg Co³.
Procedure:

To 1 ml of serum 2 ml of Ferric chloride (FeCl₂) working solution was added, mixed and waited for 5 minutes. Then 200 mg magnesium carbonate was added and shaken and rotated between palms intermittently for 30 minutes. After 30 minutes the tube was centrifuged for 20 minutes at 3000 rpm. Then 2 ml of supernatant was taken. Along with the other 3 tubes for serum iron, standard and blank, the steps were same and TIBC was done with same procedure simultaneously i.e. 0.5 ml of dipyridyl and 0.5 ml of sodium sulphite were added and boiled with the other tubes for 5 minutes. It was cooled and 1 ml of chloroform was added and shaken vigorously. The tubes were centrifuged for 15' at 3000 rpm. The supernatants were filtered and 1.5 ml of each filtrate was added individually to 2 ml of Dist. water and reading taken in 520 mm of spectronic 20.

Calculation:

\[
\text{Optical Density of TIBC} \times 450 = \text{mg%}
\]

\[
\% \text{ Transferritin saturation} = \frac{\text{Serum Iron}}{\text{Total Iron binding capacity}} \times 100
\]
Phadebas ferritin RRIST (R) is a direct radio immunoessay using special paper discs as the solid phase. Ferritin antibody covalently coupled to the paper discs, reacts during the first incubations with the patient's sample. After washing, a fixed amount of immuno absorbant purified \( ^{125}I \) labelled ferritin antibodies against ferritin molecules bound to the antibodies on the paper disc during the previous incubation. The radio-activity of this complex is then measured in a gamma counter. The more radio-activity bound, the more ferritin present in the sample. The kit contains the following components:

(a) In 20 ml vials
   Diluent colour coded yellow
   Ready for use.

(b) In 10 ml vials
   Antiferritin \( ^{125}I \) lyophized or \( \approx 0.5 \mu g, 130 \text{ kBq} \)
   \( (\approx 3.5 \mu Ci) \) at date of manufacture(Antibodies raised in rabbits) colour coded blue.

(c) In 5 ml vials
   Ferritin standards (Human spleen)
Ready for use 0.5, 2.5, 10, 25, 50 μg/l

Colour coded yellow

In cassettes: Antiferritin disc. 30 in buffer solution
( Antigodies raised in sheep ).

Preparation of reagents :-

For ferritin assay the reagents bearing the same lot number of the package was used. So only one standard curve needed to be prepared per run. All the preparations were mixed gently to avoid foaming.

Ferritin standard solutions :-

The standard solutions are ready for use. No further dilution was needed.

Diluent :-

The diluent is ready for use.

Anti-Ferritin ¹²⁵I Solution :-

The antiferritin ¹²⁵I solution was reconstituted by adding 5 ml dist. water to the vial.

Antiferritin disc :-

They were ready for use. Evaporation of buffer was avoided by keeping the cassette closed when it was not in use. The following points were noted while transferring the disc to the tubes i.e. Discs to be handled with clean forceps. Surplus moisture was removed by pressing a folded tissue paper against
the disc sheet in the lid in order to avoid adhesion of disc to the wall of the tube.

**Preparation of samples:**

If low values were expected, serum sample could be run undiluted. If very high values was expected, then the serum had to be diluted 1:9 eg. 50 of unknown was mixed with 450 diluent.

**Preparation of standard Curve:**

A standard curve was prepared on every test occasion. Each standard curve was run in duplicate. The tubes were labelled arranged and pipetted according to the test scheduled.

**Test Procedure:**

The tubes were labelled and arranged in a rack according to the test scheduled. Each determinations were made in duplicate for standards and unknowns were put in sights. A standard curve was made every time the test was performed. Dispensing of the solutions on the walls of the tube was avoided. For our interest, the background absorption of anti-ferritin discs were also determined by running the diluent instead of serum as sample.

1. One antiferritin disc was added to the bottom of each tube except 1 and 2 (total activity tubes) which were set aside until step 6. The discs were more conveniently handled with a pair of forceps.
(2) 50 μl of standards 0.5, 2.5, 10.25, 50 μg/l were pipetted onto the discs in duplicate (3-12).

(3) 50 μl of unknown diluted or undiluted depending upon the expected values were pipetted onto the discs 13 onwards.

(4) The tubes were covered with aluminium foil or plastic film and agitated on a horizontal shaker for 1 hr at room temperature.

(5) The liquid from each tube was removed with Pasteur pipette. 2.5 ml of 0.9% of normal saline was added to the tubes and agitated for 2-3 minutes and the tubes were let undisturbed for 10 minutes. The liquid was discarded and washing with normal saline was repeated twice.

(6) 50 μl of anti ferritin 125I solution was pipetted onto the bottom of all tubes including 162 which contained only anti-ferritin 125I which was used to determine the total activity added (T1 & T2) which were stopped immediately and set aside until step 9.

(7) The tubes were covered with aluminium foil and agitated for 2 hrs on a horizontal shaker at room temperature.

(8) The liquid was removed and washed twice with 0.9% Normal saline according to step 5.
(9) The tubes were stoppered and their bound radio-activity in all tubes was determined using a gamma counter per minutes. The background was determined using an empty test tube.

Calculation of results:

(1) The counts \( B \) was expressed for each of the standards and unknowns as a percentage of the mean counts of the total activity \( T \). The background counts were subtracted if it was significant.

\[
\% \text{ activity bound} = \frac{B \text{ of standard or unknown}}{T} \times 100
\]

(2) The percentage values obtained for the ferritin standards against the ferritin concentration on a lin-log paper was plotted and a standard curve was made.

(3) The concentration of ferritin in \( \mu g/l \) was read directly from the standard curve for each of the unknown samples. The result was multiplied by the appropriate dilution factor.

If the test reagents are performing correctly, the mean counts of the standard and mean counts of total activity \( B 25/T \) should be \( \leq 20\% \).
Preparation of Hemolysate:

3 ml of EDTA blood was centrifuged to remove plasma. The cells were washed 3 times with normal saline. The cells were mixed with normal saline and centrifuged. The supernatent was discarded. Cells were mixed with normal saline and centrifuged. This was repeated thrice. After 3rd washing the supernatent was discarded. About 1/4 volume of Dist. water was added. About 0.5 ml of carbon tetrachloride was added and shaken vigorously for 1-2 minutes. Then it was centrifuged for 20 minutes at 3000 rpm. The supernatent was pure hemolysate. Hemoglobin concentration of the hemolysate was adjusted to 10 gm% using dist. water as diluent. The hemolysate was kept at -20°C and used for HbF, HbA2 estimation.

HbF% ESTIMATION AS DESCRIBED BY BETKE ET AL (1951)127.

Reagent:

1. Hemolysate
2. Drabkin's solution
3. 1.2 N NaOH
4. Saturated Ammonium sulfate \((\text{NH}_4)_2\text{SO}_4\).

Procedure:

Drabkin's solution converts all hemoglobin into cyanmeth hemoglobin. 0.25 ml of hemolysate was added to 4.75 ml of Drabkin's solution (Solution A).
Control:
To 6.75 ml of Dist. water, 0.4 ml of solution A was mixed.

Test:
To 2.8 ml of solution A, 0.2 ml of 1.2 N NaOH solution was added and mixed thoroughly and left at room temperature. Exactly after 2 minutes 2 ml of saturated solution of Ammonium solution was added, mixed thoroughly and kept for 10 minutes. After 10 minutes, the mixture was filtered through Whatman paper No.1 and the reading of the test and control was taken. Blank was adjusted with Dist. water.

Calculation:
\[
\frac{\text{OD of test (HbF)}}{\text{OD of total Hb(Control)}} \times 10 = \% \text{ of HbF}
\]

HEMOGLOBIN A2 ESTIMATION BY PAPER ELECTROPHORESIS AND ELUTION AS PER BLACK ET AL (1966)104.

Reagent:
1. Hb pipette
2. Buffers
   A. Tris EDTA borate buffer pH 8.9
   B. Veronal buffer
3. Drabkin solution
   A. Tris-EDTA borate buffer
      (a) Tris-(Hydroxymethyl)-amino-methane 12.1 gm
      (b) EDTA - 1.56 gm
      (c) Boric acid 0.92 gm
Dissolved in Dist. water and made to 1 litre.

B. Veronal buffer:
(a) Sodium barbitone - 30.9 gms
(b) Barbitone - 5.52 gms
(c) Sodium azide - 0.840mg.

Dissolved in Dist. water - 2 litres.

Barbitone is insoluble in water. Barbitone was boiled in 500 ml of Dist. water and mixed with other ingredients.

Procedure:
Whatman paper No.3 was cut 14" long and 6" wide with mid line of 8.5". The paper was dipped in Tris buffer and blotted to remove excess of buffer. The wet paper was placed hanging in vertical tank containing veronal buffer. 20 µl hemolysate was spotted 3 cms from the midline. A control normal hemolysate was spotted along with all test samples. The tank was covered and electrophoresis was run at a voltage of 180 with 3 mA power for 18 hours. After 18 hrs the strip was dried in air. The bands A2 and (A+F) were cut separately. They were cut into small pieces and put them in tubes containing Drabkin's solution. For A2 4 ml of Drabkin's solution and for A+F 16 ml of Drabkin's solution were taken. If HbS band was found, the strip was cut into small pieces and put in 4 ml of Drabkin solution. The tubes containing the Drabkin solution with cut pieces Hb bands were kept in dark for 3 hrs shaking intermittently. The solutions were centrifuged for 2 minutes at
1000 rpm to allow the paper fibres to settle down. Reading was taken at 540 nm with Drabkin's solution taken as blank.

Control was not eluted as it is required to compare the abnormal hemoglobin bands if any.

Calculations:

% HbA2 = \frac{{OD \text{ of } A2}}{{3 \times OD \text{ of } (A+F) + OD \text{ of } A2}} \times 100

% HbS = \frac{{2 \times OD \text{ of } S}}{{3 \times OD \text{ of } (A+F) + OD \text{ of } \text{abnormal Hb(S)} + OD \text{ of } A2}} \times 100

HbA2 estimation by DEAE cellulose microchromatography as per Efremov et al. (1974) using Tris-HCl developer.

Reagents:

1. Anion exchanger
   Diethyl aminoethyl cellulose (DEAE-Cellulose)

2. Developer
   0.05 M Tris-HCl buffer
   Tris(Hydroxymethyl) aminomethane 6.57 gm
   KCN - 100 mg
   Dist. water - 1 litre
   The pH of developer 8.5, 8.3 and 7 was adjusted with 1 N-HCl.
Procedure:

The anion exchanger DEAE cellulose was equilibrated by mixing with an equal volume of 0.05 M Tris-HCl buffer and KCN 100 mg/litre with pH 8.5. After the cellulose had settled, the clear supernatant and particles if any floating were removed. The equilibration procedure was repeated at least 3 times. The resin was stored as a slurry in a covered container. The supernatent volume of buffer was about 0.7 of that settled anion. A small plug of cotton was placed in the constricted part of the pipette (Ordinary glass ware) and a 6 cm column was prepared by pipetting the DEAE cellulose slurry with a pasture pipette. The pipette with column of slurry were kept in a special pipette stand. One drop of hemolysate solution was diluted with 5 drops of dist water and applied on the top of the column. The column was developed with 0.05 M Tris-HCl developer pH 8.3 containing KCN. The HbA2 fraction was eluted as a sharp band in 6 to 8 ml and was collected in a tube graduated at 10 ml. The remaining hemoglobin was eluted with 2nd developer (0.05 M Tris-HCl with 100 mg KCN) pH 7.0 and collected in a tube graduated at 25 ml. The volume of the fractions were adjusted to 10 ml and 25 ml respectively and the optimal density was determined at 415 nm.
Calculation:

\[
\% \text{ of } \text{HbA2} = \frac{\text{OD of A2} \times 100}{\text{OD of A2} + 2.5 \times R \times \text{OD}}
\]

\( R \) = Remaining hemoglobin.

SICKLING TEST OF WHOLE BLOOD AS PER DACIE & LEWIS (1975)\(^{128}\).

Reagent:

1. Sodium metabisulphite - 2%
   Two gm of sodium metabisulphite was dissolved in 100 ml of Dist. water. Reagent was prepared fresh before each test.

Procedure:

One drop of EDTA blood was added to 5 drops of 2% sodium metabisulphite solution in a slide and covered immediately with cover slip and sealed with petroleum jelly or soft wax. Examined after 1 hr under microscope. Sickled or holy leaf shaped RBCs were seen in positive cases.

RETICULOCYTE COUNT AS PER DACIE & LEWIS (1975)\(^{128}\).

Reagent:

1. Brilliant Cresyl blue - 1 gm
2. Citrate-Saline - 100 ml.
   (1 part of Na citrate 3% + 4 parts of 0.9% Na cl)
   The mixture was filtered after the dye had dissolved and was ready for use.
Procedure:

To 2 drops of brilliant cresyl blue solution, 2 drops of blood was mixed and the mixture was kept at 37°C for 1/2 hr. In anemic blood more blood was added to get an optimal colour. The mixture was mixed at short interval. Smear was prepared from the mixture in a clean slide. After air dry reticulocyte count was done under oil immersion.

CALCULATION OF ABSOLUTE VALUES:

1. MCV was calculated based on the formula:

\[
\frac{PCV \text{ in litre} \times 1000}{\text{Total RBC per litre}} = \text{fl (Femtolitres)}
\]

2. Mean corpuscular hemoglobin (MCH). This was calculated with the formulae:

\[
\frac{\text{Hemoglobin per litre}}{\text{Total RBC per litre}} = \text{pg (Picogram)}
\]

3. Mean corpuscular hemoglobin concentration (MCHC).

MCHC was calculated with the formula:

\[
\frac{\text{Hemoglobin per litre}}{\text{Packed cell volume per litre}} = \text{x g/litre}
\]

Calculation of discriminant factors:

The following discriminant factors were calculated for differentiation of beta thalassemia trait and iron deficiency anemia.
(1) **DF1 - Discriminant factor-1**

DF = MCV - RBC - (5 x Hb) - 3.4.

A positive value indicates iron deficiency and negative value indicates beta thalassemia trait.

(2) **DF2 - Discriminant factor-2.**

DF2 = \( \frac{MCV}{RBC \text{ count}} \)

If the ratio was \( \geq 13.0 \) Iron deficiency was suspected

\( \leq 13.0 \) Beta thalassemia trait

(3) **DF3 Discriminant factor-3.**

DF3 = \( \frac{RBC \text{ count} \times Hb}{MCV} \)

If the ratio was \( \geq 0.8 \) - Thalassemia trait.

If the ratio was \( \leq 0.8 \) - Iron deficiency anemia.

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