CHAPTER 3
MATERIAL AND METHODS

The early detection of healthy carriers of thalassemia (heterozygotes) makes it possible to provide genetic counseling, which may lead to a reduced incidence of homozygous status and its fatal outcome. Therefore, in a prevention and control program, rapid, accurate and inexpensive screening protocols to identify carriers of thalassemias and its variants, especially in population and families at risk for Hb disorders are essential. Screening and genetic counseling is required to the individuals with β-thalassemia trait and α0-thalassemia trait to be identified. When, such abnormalities are found appropriate testing of that individual’s relatives is also required for successful preventive programs.

In societies in which most couples are unrelated, genes for recessive disorders usually run in families for many generations without manifesting themselves through the birth of an affected child. By contrast, in communities with a cultural preference for consanguineous marriage, as in many communities in India, when a gene for a recessive disorder is present in kindred, there is likely to be an affected child in at least one branch of the extended family. In turn, the diagnosis of disease in a child serves as a marker of an extended family that is at increased genetic risk. Therefore, in such communities, studies of extended families beginning with the first child with a diagnosis may offer an alternative to population screening for identifying present and future couples at risk for producing affected children (Alwan & Modell, 1997). This study was designed to test this hypothesis, using hemoglobin disorders as a model.

3.1 Subjects

The study was undertaken to determine the prevalence of β-thalassemia major and traits and its associated hemoglobin variants in Dhule and Nandurbar districts. Extended family screening was carried out for carrier identification, for this; 242 β-thalassemia major patients (receiving regular blood transfusion at Government Civil Hospital, Dhule) were followed retrospectively. After explaining the importance and need for extended family screening to the key members of affected families, information regarding their relatives and other family members both maternal and paternal and addresses was obtained. Criteria for selection were voluntary participation and not mandatory and the availability of many family members. 1702 family members and close relatives were enrolled in this study, a total of 1944 (242 +
1702) individuals of different communities and castes were underwent through
different hematological examinations. Screening of school children were also carried
out on 3214 school students for detection of hemoglobinopathies and iron deficiency
anemia. These two methods of population screening (extended family screening and
school children screening) were compared to determine the effectiveness and
feasibility of carrier identification.

3.1.1 Extended family screening

A total of 181 out of 242 β-thalassemia major patient’s families were
requested for testing to identify carriers, remaining 69 β-thalassemia major patients
and their families were not able to approach. Informed consent was obtained from
their parents. Details of racial origin, caste, sub-caste and family history (income,
income source and education, knowledge regarding thalassemia and any other
hereditary disease rather than thalassemia) were also recorded. When family
representatives agreed for screening, a two or three-generation pedigree was drawn up
(depends on families and consanguineous marriages) and arrangements were made for
testing family members at their homes. Results were given and explained to head of
the family (and/or directly to the carriers if more than 18 years) about the carrier
status and its importance in family planning. If one or both the partner was found to
be carrier, the availability of prenatal diagnosis and for those not yet married the
possibility taking screening test results into consideration when planning to marriage,
were explained orally.

Carrier testing

All the samples were collected during the period of study and were
immediately analyzed for different hematological tests for detection of thalassemia
carrier. Initial screening to identify carriers (of families with β-thalassemia major
index cases) was performed by the strategy described by Silvestroni & Bianco,
(1983). The samples undergone through initial screening like NESTROFT (Naked
Eye Single Tube Osmotic Fragility Test) and red cell morphology. The NESTROFT
was performed with different concentrations of hypotonic saline solutions. Red cell
morphology was examined on a freshly made blood films in such a way that it
included a monolayer of well spread and distinct erythrocytes; this made it possible to
detect anisocytosis, poikilocytosis, red cell fragments and target cells, as well as
hypochromia and an opacity that contrasts with the refractile appearance of normal
erythrocytes. The blood specimens in which abnormalities were found according to the above criteria were processed through a further series of studies. These include determination of red cell indices with an electronic cell counters. Individuals who showed hypochromic microcytosis with mean corpuscular volume (MCV) values less than 80fl and mean corpuscular hemoglobin (MCH) values less than 27pg, further testing using cellulose acetate electrophoresis and/or automated HPLC system (Bio-Rad Variant-II β-thalassemia short program) for HbA2 estimation were performed (The Thalassemia Working Party, 1994 and A Working Party of the General Haematology Task Force, 1998). Beta thalassemia trait was diagnosed when the percentage of HbA2 was 3.5 percent or higher (Steinberg & Adams, 1991). The analysis of globin gene mutations was performed by amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) (Old, 1996) in some cases.

**Follow-up of families**

The parents of the child with index cases and families who were at risk of having affected children were followed and asked at interval about engagements, marriages and births. However, it was not possible to keep track of family members, who lived far away, but when affected children were born, they were suggested to any nearer center for further testing.

**3.1.2 Screening of school children**

Here the study reported experience with a systematic school screening for hemoglobinopathies and iron deficiency anemia that took place from November, 2012 to August, 2013 in primary, secondary and higher secondary schools of Muslim community in Dhule city. To assess the impact of this preventive program, several variables were investigated reflecting the influence of screening.

A preliminary meeting was designed to provide information. During this educational session, the following points were covered: a clinical presentation of the disease, the implication of the carrier status and the opportunity for a carrier couple to seek prenatal diagnosis. Then later, samples were collected and analyzed. A total of 3214 students were participated in this campaign. All blood samples were undergone through the strategy described by Silvestroni & Bianco, (1983). The samples who gives positive test result in one or both (NESTROFT and/or red cell morphology) tests undergone through hematological tests (red cell indices, hemoglobin electrophoresis) and biochemical examinations (serum ferritin, transferrin saturation,
total iron binding capacity (TIBC)) were conducted for discrimination of iron deficiency anemia (IDA). The HbA2 more than 3.5% was considered as a cut-off for determining carriers of β-thalassemia. If the test showed a carrier status, Principal of the school reported results to the participant. The families were advised to arrange for a specialized consultation, so that a family study might be carried out.

Furthermore the efficacy of general population screening (school children) and extended family screening were compared for better establishment of preventive programs of thalassemia in the present study area.

3.2 Screening and diagnostic tests

As stated above different tests were applied and evaluated for the diagnosis of carriers of β-thalassemia.

Collection of blood specimen

Before proceeding to collect the blood from patients, all the necessary materials were assembled viz. blood collection containers, syringes, tourniquet, methylated spirit or 70% alcohol, cotton and gloves.

Containers for blood collection were prepared before collecting the blood. Readymade EDTA containers were used and then reuse for another collection by thoroughly cleaned with no trace of detergent and disinfected. Specific amount of anticoagulants were added and dried inside the container. A 10% solution of dipotassium salt of EDTA was prepared by taking 10gm of the salt in about 80ml of water in a 100ml volumetric flask and then made the volume of the solution up to 100ml mark. Then specific amount of EDTA solution was poured into the container (0.05ml for 2.5ml of blood collection and 0.1ml for 5ml of blood collection).

At the time of blood collection, the requisite amount of blood collected into the containers and mixed gently and thoroughly by swirling. It was kept in mind that the concentration of anticoagulant should be 1.5 ± 0.3mg/ml. If the concentration of anticoagulant is more resulting in distortion of blood cells, on the other hand if the blood is more and anticoagulant is in less concentration clotting of blood might be occur.

The blood was collected by venipuncture of the median cephalic vein of the working forearm because the veins are more prominent in working arm. After collection the blood was poured into the containers; the containers were gently swirl
to mix the anticoagulant with blood. The containers were labeled with patient’s initials and specific numbers.

The most effective and feasible mass screening method for highly populated countries like ours is preventive genetics and population screening. Individuals at risk need to be identified by simple and inexpensive methods as described by Silvestrini & Bianco, (1983).

3.2.1 Naked Eye Single Tube Red cell Osmotic Fragility Test (NESTROFT)

Initial screening was performed by NESTROFT and red blood cell morphology to discriminate between thalassemic and non-thalassemic. Thalassemic red cells are resistant to lysis when placed in hypotonic solutions. This abnormal osmotic resistance was used in the diagnosis of thalassemia trait and suggested for clinical laboratories in developing countries (Fucharoen, et al., 2004). The osmotic fragility (OF) test with 0.36% saline solution is an attractive alternative to identify carriers of α- and β-thalassemias (Silvestrini & Bianco, 1983 and Thool, et al., 1998).

Materials

1. NaCl (Sodium chloride)
2. Na₂HPO₄ (Disodium hydrogen phosphate)
3. NaH₂PO₄.2H₂O (Sodium dihydrogen phosphate)
4. Distilled water
5. Glass wares (test tubes, pipette, measuring cylinder, beaker, conical flask etc.)

Method

A 10% stock solution of buffered saline with pH7.4 was prepared by dissolving 90gm NaCl, 13.655gm Na₂HPO₄ and 2.4gm NaH₂PO₄.2H₂O in 1,000ml of distilled water. From this 10% stock solution, 1% buffered saline was prepared by 1:10 dilution with distilled water. Three buffered saline solutions with concentrations 0.32%, 0.34% and 0.36% were prepared by further diluting 32ml, 34ml and 36ml of 1% buffered saline solution with 68ml, 66ml and 64ml of distilled water, respectively, to make 100ml of each solution. The test was performed using different concentrations of buffered saline solution. Two ml of each of three concentrations of buffered saline were taken in three separate test tubes (10 × 1cm diameter) each and 2ml of distilled water was taken in another tube, so that there was a set of four test tubes. EDTA blood (20μl) was added to each of these four tubes. The tubes were
shaken well and left undisturbed for half an hour at room temperature. After 30 minutes, the contents of all these tubes were shaken and the tubes were held against a white paper on which a thin black line was already drawn. The line was clearly visible through the contents of the tube containing distilled water (control). If the line was similarly visible through the contents of the tubes with buffered saline, then the test was considered as negative; if the line was not visible, then the test was considered positive. In the case of a blurred line, the test was considered as doubtful. The interpretation of this doubtful result was taken as being positive. In the case of carriers, if the test was positive, it was called true positive (TP) and if negative, it was called false negative (FN). On the other hand, in case of normal samples, if the test was negative, it was called true negative (TN) and if positive, it was called false positive (FP). The principle of NESTROFT is based on the limit of hypotonicity, which the red cells can withstand. There is a pronounced decrease in osmotic fragility of red cells in β-thalassemia.

Sensitivity, specificity, positive and negative predictive values and efficiency of the test were calculated as validity statistics by the following formulae:

Sensitivity: $$\frac{TP}{TP + FN} \times 100$$
Specificity: $$\frac{TN}{TN + FP} \times 100$$
Positive predictive value: $$\frac{TP}{TP + FP} \times 100$$
Negative predictive value: $$\frac{TN}{TN + FN} \times 100$$
Efficiency of test: $$\frac{TN + TP}{TP + FP + TN + FN} \times 100$$

3.2.2 Red blood cell morphology

The present study followed wedge method (Push) which provides a sufficiently large area for microscopic examination. Morphologic abnormalities of peripheral blood cells were studied by microscopic examination with high magnification microscope of well-prepared films of peripheral blood stained with Leishman stain. For appropriate interpretation of the morphology of erythrocytes, the smear areas where the red cells appear singly and have central pallor were concentrated. Examination of erythrocytes far out on the feathered edge discloses erythrocytes lacking central pallor, whereas in thick areas of the slide the morphology of the erythrocytes is distorted by contact between cells.
Materials
1. Microscopic slides (clean, grease-free and scratch free), cover slips and glass wares
2. High magnification microscope (Olympus CH20i)
3. Racks for staining and drying slides
4. Absolute methanol and 70% ethanol or ether
5. Romanowsky stain (Leishman stain)
6. Na₂HPO₄.2H₂O (Disodium hydrogen phosphate) and Na₂HPO₄ (Disodium hydrogen phosphate anhydrous)
7. KH₂PO₄ (Potassium dihydrogen phosphate anhydrous)
8. Distilled water
9. Digital camera (Nikon D-3100)

Lieshman stain

Reagents
1. Buffered solution was prepared by taking 3.8gm Disodium hydrogen phosphate (Na₂HPO₄.2H₂O) and 2.1gm Potassium dihydrogen phosphate (KH₂PO₄) anhydrous in 1000ml distilled water. The pH was adjusted to 7.0–7.2.
2. Stock Leishman stain was prepared by taking 1.5gm Leishman powder dissolved in methanol (CH₃OH) and makes it 1000ml.
3. Diluted Leishman stain was made by taking one part of stock stain and three parts (1:3) of buffered water, mixed well and ready for use (Chakravarthy & Dierolf, 2011).

Procedure
1. Labeling of pre-cleaned slides with patient’s name date and time of collection.
2. The slides were cleaned with 70 to 90% alcohol and allow drying. The surface of the slide where the blood smear will be made was kept away from dust.
3. One or two drops of blood were taken on one side of clean slide.
4. A clean spreader slide was held at a 45° angle, towards the drop of blood on the specimen slide and waited until the blood spreads along the entire width of the spreader slide.
5. While holding the spreader slide at the same angle, pushed it forward rapidly and smoothly.
6. The smear was dried in air.
7. The smear was fixed for at least 2–3 min in absolute methanol. Methanol was removed by tilting the slide.
8. Diluted Leishman stain was applied for 7–10 min.
9. The buffer water then added for washing the stain for 2–3 min to differentiate the film. The time taken for differentiation depends on the stain and the pH of the buffered water.
10. The slides were kept in an upright position in drying rack, keeping smeared surfaces of the slide facing down for shaking off all adhering water to the slide. The slides were ready for examination.
11. The slides were examined under 40X magnification with Olympus CH20i microscope and photographs were captured.

3.2.3 Red cell indices

Automated cell counters are widely used in routine practice and are easily available at every diagnostic center, so screening can be done without additional costs. The most consistent finding in carriers of β-thalassemia is the combination of a relatively high or normal RBC count with a low MCV and MCH along with normal Hb and hematocrit values (England & Fraser, 1973 and Aghai, et al., 1986). In fact, the MCV alone can identify a high number of thalassemia carriers both in adults and children (Borgna-Piagnatti, et al., 1983).

The subjects who were positive in osmotic fragility test and red cell morphology (i.e. abnormal osmotic fragility of red blood cell in various saline concentration and abnormal red cell morphology) were undergone through complete blood count. The automated cell counter (Coulter AcT 5diff, Beckman Coulter) was used for obtaining complete blood count.

3.2.4 Discriminant function analysis

*Discrimination between β-thalassemia trait and iron deficiency anemia*

The thalassemia trait requires a differential diagnosis from iron deficiency anemia (IDA), both being microcytic and hypochromic. The statistical technique used to distinguish between the two microcytic groups according to routine hematological results is discriminant analysis.

The present study evaluated the validity of discriminant functions, which would best differentiate between the two microcytic groups (β-thalassemia traits from
iron deficient patients). This study intended to evaluate the diagnostic reliability of 12 published DFs in differentiating βTT and IDA in north Maharashtra region.

Beta thalassemia minor and iron deficient subjects are the most common encountered conditions and are usually associated with microcytosis and/or hypochromasia (Olivieri, 1999). The differentiation between thalassemic and non-thalassemic microcytosis has important clinical implications (Hallberg, 1992). Determination of red cell indices by electronic cell counters has been used as first indicator of possible β-thalassemia minor in population or mass screening. The second step in screening schemes is exclusion of microcytosis and/or hypochromasia due to iron deficiency. An effective, but costly, approach to this exclusion step is by measuring serum ferritin level and quantitation of HbA₂, in which HbA₂ > 3.5% indicates β-thalassemia minor (Esposito, 1992).

The exclusion of iron deficiency could be achieved mathematically using the RBC parameters. Since the early 1970s, different indices and formulae of CBC parameters have been proposed. As simple and inexpensive tool to determine whether the blood sample is more suggestive of β-thalassemia minor or iron deficiency (England & Fraser, 1973; Mentzer, 1973; Srivastava & Bevington, 1973; Shine & Lal, 1977; Ricercuca, et al., 1987; Green & King, 1989; Jayabose, 1999; Ehsani, et al., 2005 and Sirdah, et al., 2008). These indices were derived from several simple red blood cell (RBC) indices, like RBC count, Hb, MCV and RDW and can be effective for use as preliminary screening tools. Youden’s index provides an appropriate measure of validity of a particular technique or question by taking into account both sensitivity and specificity (Pekkanen & Pearce, 1999).

In this study, the diagnostic reliability of 12 RBC indices and formulae in the differentiation of βTT from IDA by calculating their sensitivity, specificity and Youden’s index were evaluated. The Hb level lower than normal range in males and females, in respect with their ages and low level of serum ferritin indicated IDA; while an HbA₂ >3.5% and normal serum ferritin were indicative of βTT. In both groups, MCV and MCH were lower than 80fl and 27pg respectively and in mixed cases βTT + IDA the Hb level were lower than 9g/dl. Sensitivity, specificity, efficacy, positive likelihood ratio (+LR), negative likelihood ratio (–LR), positive predictive value (PPV), negative predictive value (NPV), construction of receiver operative characteristic (ROC) curves to calculate the area under the curve (AUC) (Bangdiwala, et al., 2008) and YI were calculated and measured for each index.
- Ehsani Index (EI): \((MCV - 10 \times RBC)\)
- England & Fraser Index (E&F): \(MCV - RBC - (5 \times Hb) - 3.4\)
- Green & King Index (G&K): \((MCV^2 \times RDW / Hb \times 100)\)
- MCV
- Mentzer Index (MI): \(MCV / RBC\)
- RBC count
- RDW
- RDW Index (RDWI): \((MCV \times RDW / RBC)\)
- Ricerca Index (RI): RDW / RBC
- Shine & Lal Index (S&L): \((MCV^2) \times MCH / 100\)
- Srivastava & Bevington Index (S&B): \(MCH / RBC\)
- Sirdah Index (SI): \((MCV - RBC - 3 \times Hb)\)

**Calculation**

Sensitivity = \((TP) / (TP + FN)\)

Specificity = \((TN) / (TN + FP)\)

PPV = \((TP) / (TP + FP)\)

NPV = \((TN) / (TN + FN)\)

Efficacy = \((TP + TN) / (TP + TN + FP + FN)\)

+\(LR = TP\) rate / \(FP\) rate or Sensitivity / 1-Specificity

-\(LR = FN\) rate / \(TN\) rate or 1-Sensitivity / Specificity

\(YI = (\text{Sensitivity} + \text{specificity}) - 100\)

Where TP = true positive, TN = true negative, FP = false positive and FN = false negative. The collected data were treated using the SPSS 22.0 software (IBM, USA), Microsoft office Excel program 2007 and ROC curves were constructed by SPSS.

**3.2.5 Cellulose acetate electrophoresis**

Different hemoglobins have different net charges and according to those charges and the amount, hemoglobins move at different speeds on the cellulose acetate paper in alkaline pH.
Apparatus and reagents

Apparatus

1. For hemolysate preparation: Centrifuge machine (Bioera micro centrifuge model-II, Bioera Pune and Remi centrifuge machine, Remi instruments, Mumbai), graduated conical centrifuge tubes, Pasteur pipette, glassware.
2. For electrophoresis: a variable type of power pack capable of supplying direct current up to 10mA at 200 volts, an electrophoretic horizontal tank, cellulose acetate strips 3 × 15 cm, spectrophotometer (Spectro-106, Systronics, Ahmedabad) with a narrow wavelength band at 413nm is suitable instruments.

Reagents

1. For hemolysate preparation: NaCl, distilled water and toluene
2. For electrophoresis: Tris-hydroxymethyl-aminomethane, ethylene diamine tetra-acetic acid, boric acid and distilled water. Tris buffer (pH 8.9) was prepared by taking Tris-hydroxymethyl-aminomethane 14.5gm, EDTA 1.5gm and boric acid 0.9gm in 1000ml distilled water, and Ponceau-S stain.

Procedure

Hemolysate preparation

1. 2ml of anticoagulated blood centrifuged approximately for 15–20min at 2500rpm in a graduated conical centrifuge tube. Plasma was removed.
2. The packed erythrocytes were washed 3–4 times with saline solution, mixed by inversion after each wash. The final supernatant should be colorless. This supernatant was removed with a Pasteur pipette.
3. The packed erythrocyte was measured, 1.4ml of distilled water was added for each 1ml of cells, mixed and placed in the freezer for 15–20min. Removed from the freezer and allowed to come to room temperature. 0.4ml of toluene was added for each 1ml of the original PCV. Covered and shake for 5min.
4. Centrifuge at 2500 rpm to 3000 rpm for 15–20min.
5. The toluene was removed with cotton tip swab. Pasteur pipette was inserted into clear hemoglobin solution and transferred it to clean test tube. This hemolysate was labeled and stored for several weeks by keeping it frozen or refrigerated.
6. The hemolysates needs to be diluted to a 1:6 dilution with one volume of hemolysate and five volume of distilled water.
Electrophoresis

Two ml blood was taken into EDTA tubes. Hemolysates were prepared by the method as described in clinical hematology (Turgeon, 2005). The cellulose acetate strips were immersed in the Tris buffer, in order to allow capillary action to draw the buffer up evenly through the membrane. When the entire membrane has become wet immersed it completely in the buffer by carefully agitating the tray. The membrane immediately removed from the buffer and blotted evenly between two sheets of Whatman no. 3 filter paper to remove excess moisture and mounted horizontally in the electrophoretic tank. The electrophoresis tank was prepared by placing equal amounts of TEB buffer in each of the outer buffer compartments. The strips were allowed to equilibrate in the closed unit for 10 minutes ensuring that the membrane lies evenly and that each end hangs freely in the opposite chambers containing the buffer.

Qualitative analysis

The qualitative separation of hemoglobins was performed on 1–2μl samples of the hemolysate. The test samples and controls were applied as 1 to 1.5cm bands and placed towards the cathode (negative side) of the midpoint of the strip. Several specimens may be run simultaneously. Power cords connected to the electrode terminal pins of electrophoresis unit and other to the power pack and after sample loading switched on the power supply. Optimal separation occurs in 45 minutes at 20 to 25volts/cm with a current of 0.3 to 0.5mA/cm. After the completion of electrophoresis the membrane were carefully removed from the bridge without allowing any buffer to run over the membrane. Immediately immersed the membrane in a pan containing Ponceau-S stain and were allowed to stain for five minutes. The membranes were then removed from the stain and were placed in a decolorizing solution, to rinse the excess stain off. Repeating it two three times in fresh decolorizing solution until all the excess stain was removed. The membrane became white color, with only the hemoglobin taking up the pink or red color. The migration patterns of various hemoglobins were determined.

Quantitative analysis

The quantitation analysis of the hemoglobins were performed in a similar manner to the qualitative procedure with the exception that approximately 30μl of the hemolysate was applied as three 5μl samples to each of two cellulose acetate strips.
The strips were removed after a 45-minute period of electrophoresis and cut midway between the areas of greatest concentration. The hemoglobin fractions to be quantitated, other than A₂, were placed in a plastic capped test tube and eluted in 20ml of Tris buffer by rotation on a blood-mixer for 30 minutes. It is necessary to elute the A₂ fractions in 4ml of buffer because of the smaller quantities involved. This approximates the intensities of the solutions to be compared. The eluates were measured in a spectrophotometer at a wavelength of 413nm and percentages calculated. Maximum absorption for hemoglobin solutions of A, A₂, D, S, C and H were recorded at this wavelength.

3.2.6 HPLC studies

Now a day, high-performance liquid chromatography (HPLC) has become a method of choice for the study of hemoglobin (Hb) abnormalities in most of clinical laboratories. This technique is used for quantitative analysis of the various Hb fractions by fully automated HPLC analyzers, which is now done in routine hospital laboratories.

The Bio-Rad Variant II β-thalassemia Short program (Bio-Rad laboratories Inc., Hercules, CA) apparatus is available at Shri Bhausaheb Hire Government Medical College, Dhule. The system is fully automated using double wavelength detection (415 and 690 nm). The β-thalassemia Short program is the most widely used system for measurement of area percentage of HbA₂ and HbF and to provide qualitative determinations of few abnormal Hbs within 5 to 6 min. Windows of retention time have been assigned for presumptive identification of the most commonly occurring Hb variants.

The β-thalassemia Short program uses a 3.0 × 0.46cm nonporous minicartridge (cation-exchange column). In this automated system, the samples are mixed by the Variant II sampling station, diluted with the specific hemolyzing/wash (two phosphate elution buffers) buffer of different concentrations and are eluted at 32 ± 1°C, with a flow rate of 2 ml/min. Whole blood samples undergo an automatic two-step dilution process in the sampling station and then are introduced into the analytical flow path of the HPLC. A programmed buffer gradient of pH and an ionic strength made of two phosphate buffers delivered by dual pumps of Variant II Chromatographic Station (VCS) to the analytical cartridge, where HbA₂/F are separated based on their ionic interaction with the cartridge material. The separated
hemoglobin fractions (HbA₂/F) then pass through the flow cell of the filter photometer where the absorbance at 415nm is measured, an additional filter at 690nm corrects the background absorbance and a chromatogram/sample report is generated by Variant II CDM software.

The components of various Hbs and its concentration can give slight differences in elution time from one column to another and from one reagent batch to another. Thus to avoid this, a more accurate calibration elution time could be obtained by using HbA₂ as a reference instead of manufacturer's proposed. This Hb is present only between narrow concentration limits, which prevent any significant modification of its elution time (Riou, et al., 1997).

Reagents

1. Sodium phosphate elution buffers (Variant II β-Thalassemia Short Program Reorder Pack, Bio-Rad laboratories Inc., Hercules, CA)
2. Whole blood
3. HbA₂/F calibrator/diluent set (Variant II β-Thalassemia Short Program Reorder Pack, Bio-Rad laboratories Inc., Hercules, CA)
4. Wash buffer/deionized water (Variant II β-Thalassemia Short Program Reorder Pack, Bio-Rad laboratories Inc., Hercules, CA)
5. Control samples: normal (HbF: 1–2%, HbA₂: 1.8–3.2%) and abnormal (HbF: 5–10%, HbA₂: 4–6%) controls (Variant II β-Thalassemia Short Program Reorder Pack, Bio-Rad laboratories Inc., Hercules, CA)
6. Hemolysing reagent (Variant II β-Thalassemia Short Program Reorder Pack, Bio-Rad laboratories Inc., Hercules, CA)

Procedure

1. Hemolysates were prepared by taking 20µl of whole blood with 1ml hemolysing reagent and mixed well.
2. 20µl of hemolysates were injected into the HPLC system for analysis.
3. The samples prepared as above can be stored at ambient temperature for about 24 hours and at 2–8°C up to 36 hours
4. Both the calibrators (HbA₂/F), normal and abnormal controls were run at the beginning of each analysis.
Interpretation of reports/chromatogram

Under this experimental condition an excellent agreement is found between chromatographic measurement of HbA₂ and HbF. The generated reports and chromatograms were studied and interpreted by observing HbA₂ and F concentration for β-thalassemia (as the level of HbA₂ is high > 3.5%) and retention time and area percentage of other peaks and windows for structural variants. Each chromatogram shows peaks of HbA₀, HbA₂ and HbF along with C-window, D-window, S-window and two minor peaks P2 and P3. The most commonly occurring variants like HbS, HbC, HbE and HbD Punjab could be easily identify by using the retention time, area percentage and ethnic background of the patients as these Hb variants co-elute in the same window with some other Hbs.

The integrated peaks are assigned to manufacturer-defined windows derived from the retention time, i.e. the time in minutes from sample injection to the maximum point of the elution peak, of normal hemoglobin fractions and common variants (Table 3.1). If a peak elutes at a retention time not predefined, it is labeled as an unknown.

Table 3.1 Manufacturer-assigned windows for Bio-Rad Variant II HPLC system

<table>
<thead>
<tr>
<th>Peak name</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₁ window</td>
<td>0.63–0.85</td>
</tr>
<tr>
<td>F window</td>
<td>0.98–1.20</td>
</tr>
<tr>
<td>P₂ window</td>
<td>1.24–1.40</td>
</tr>
<tr>
<td>P₃ window</td>
<td>1.40–1.90</td>
</tr>
<tr>
<td>A₀ window</td>
<td>1.90–3.10</td>
</tr>
<tr>
<td>A₂ window</td>
<td>3.30–3.90</td>
</tr>
<tr>
<td>D window</td>
<td>3.90–4.30</td>
</tr>
<tr>
<td>S window</td>
<td>4.30–4.70</td>
</tr>
<tr>
<td>C window</td>
<td>4.90–5.30</td>
</tr>
</tbody>
</table>

During the study, samples were analyzed in the Department of Hematology, Shri Bhausaheb Hire Government Medical College, Dhule for quantification of hemoglobin fractions and screening for hemoglobin variants.
3.2.7 ARMS-PCR

In order to gain insight into molecular basis of β-thalassemia in some patients, ARMS-PCR for screening of common β-thalassemia mutation was used. The ARMS technique for detecting known point mutations was first described by Newton, et al. (1989). It has been developed for the diagnosis of all the common β-thalassemia mutations found in all main ethnic groups (Old, 1996). The technique is based on the principle of allele-specific priming of the PCR process, that is, a specific primer will permit amplification to take place only when its 3’ terminal nucleotide matches with its target sequence. Thus to detect β-thalassemia mutation IVS1–5 (G→C), the 3’ nucleotide of the ARMS primer is G in order to base pair with the substituted C in the mutant DNA. The primer forms the G–G mismatch with normal DNA, but this is a weak mismatch and do not prohibit extension of the primer by itself. Only strong mismatches (C–C, G–A and A–A) were found to reduce priming efficiency to 0 or below 5% and to prevent amplification, a further mismatch with the target sequence is introduced at second, third, or fourth nucleotide from the 3’ end of the primer. Generally, if the 3’ terminal mismatch is a weak one, a strong secondary mismatch is engineered. If it is a strong one, a weak secondary mismatch is introduced (Old, 2003).

This technique fits the main requirements of a PCR technology, i.e. speed, cost, convenience and ability to test multiple mutations simultaneously while providing a screening method without any form of labeling of primers or amplified DNA. The mutation specific ARMS primers used in this study to diagnose the common β-thalassemia mutation are listed in Table 3.2. All are 30 bases long so that they can all be used at a single high annealing temperature (Old, 2003).

A typical ARMS test for a single mutation consists of two amplifications in the same reaction mixture using the same genomic DNA as substrate. One amplification product results from the specific ARMS primer and its primer pair (when the mutation is present in the genomic DNA) and the other amplification results from two primers that generate a control fragment in all cases. The generation of control product indicates the reaction mixture and thermal cycler are working optimally (Old, 2003).
<table>
<thead>
<tr>
<th>Name of mutation</th>
<th>ARMS-Primer sequence (5’-3’)</th>
<th>Mutation detected</th>
<th>Amplified product size bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS1–1 N</td>
<td>GAT GAA GTT GGT GAG GCC CTG GGT AGG</td>
<td>IVS1–1nt G→T (N)</td>
<td>450 bp</td>
</tr>
<tr>
<td>IVS1–1 M</td>
<td>TTA AAC CTG TCT TGT AAC CTT GAT ACG AAA</td>
<td>IVS1–1nt G→T (M) Point mutation</td>
<td>450 bp</td>
</tr>
<tr>
<td>IVS1–5 N</td>
<td>CTC CTT AAA CCT GTC TTG TAA CCT TGT TAC</td>
<td>IVS1–5nt G→C (N)</td>
<td>285 bp</td>
</tr>
<tr>
<td>IVS1–5 M</td>
<td>CTC CTT AAA CCT GTC TTG TAA CCT TGT TAG</td>
<td>IVS1–5nt G→C (M) Point mutation</td>
<td>285 bp</td>
</tr>
<tr>
<td>Codon 8/9 N</td>
<td>CCT TGC CCC ACA GGG CAG TAA CGG CAC ACT</td>
<td>Cd 8/9 +G (N)</td>
<td>225 bp</td>
</tr>
<tr>
<td>Codon 8/9 M</td>
<td>CCT TGC CCC ACA GGG CAG TAA CGG CAC ACC</td>
<td>Cd 8/9 +G (M) Frame shift mutation</td>
<td>225 bp</td>
</tr>
<tr>
<td>Codon 41/42 N</td>
<td>GAG TGG ACA GAT CCC CAA AGG ACT CAA AGA</td>
<td>Cd 41/42 (−TCTT) (N)</td>
<td>439 bp</td>
</tr>
<tr>
<td>Codon 41/42 M</td>
<td>GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT</td>
<td>Cd 41/42 (−TCTT) (M) Deletion</td>
<td>439 bp</td>
</tr>
<tr>
<td>HbE N</td>
<td>TAA CCT TGA TAC CAA CCT GCC CAG GGC GTC</td>
<td>HbE Cd 26 G–A (N)</td>
<td>236 bp</td>
</tr>
<tr>
<td>HbE M</td>
<td>TAA CCT TGA TAC CAA CCT GCC CAG GGC GTT</td>
<td>HbE Cd 26 G–A (M) Point mutation</td>
<td>236 bp</td>
</tr>
<tr>
<td>Int. C1</td>
<td>CAA TGT ATC ATG CCT TTT TGC ACC</td>
<td>Internal control primer</td>
<td>861 bp</td>
</tr>
<tr>
<td>Int. C2</td>
<td>GAG TCA AGG CTG AGA GAT GCA GGA</td>
<td>Internal control primer</td>
<td></td>
</tr>
<tr>
<td>Rev. Pri. A</td>
<td>ACC TCA CCC TGT GGA GCC AC</td>
<td>Common reverse primer A</td>
<td></td>
</tr>
<tr>
<td>*Rev. Pri. B</td>
<td>CCC CTT CCT ATG ACA TGA ACT TAA</td>
<td>Common reverse primer B</td>
<td></td>
</tr>
</tbody>
</table>

*For IVS1–1 N only
Material

For DNA extraction
1. Blood samples
2. DNA extraction Kit (Merck Genei, Bangalore)
3. Distilled ethanol, ice-cold distilled water (Sigma Aldrich, Mumbai)

For PCR and electrophoresis
1. ARMS-PCR buffer (Merck Genei, Bangalore): A 10X stock buffer contains 1M Tris HCl (pH 8.3 at room temperature), 2M KCl, 1M MgCl₂, gelatin and distilled water.
2. 10 mM dNTP mix (2'-deoxynucleoside 5'-triphosphates) (Merck Genei, Bangalore) containing each dNTP at 2.5mM concentration. 1.25mM dNTP stock solution was prepared by mixing at 1:1 ratio of 10 mM dNTP mix and distilled water and stored frozen.
3. Aliquots of primer stock solution were diluted to make a working solution of one OD (optical density) U/ml and stored it frozen.
4. Taq DNA polymerase (Merck Genei, Bangalore)
5. Tris-borate-EDTA buffer (pH 8.0) (Sigma Aldrich, Mumbai)
6. 100bp DNA Marker (pBR322 DNA/Hinfl I digest)(Merck Genei, Bangalore)
7. DNA loading dye(Merck Genei, Bangalore)
8. Agarose powder (Sigma Aldrich, Mumbai)

Apparatus
1. For DNA extraction: microcentrifuge (10,000 rpm) (Bioera, Pune), cooling centrifuge C-24 (Remi instruments, Mumbai), glassware, centrifuge vials
2. For ARMS-PCR: PCR machine (Peltier PCR processor, Model Neo, Bio Era, Pune), PCR tubes, micropipette
3. For gel preparation: Glassware, fine electronic weighing balance, gel casting tray, micro pipette and oven
4. For electrophoresis: gel electrophoresis tank, variable type of power pack
5. Gel visualization: Uvitech Gel doc system (Merck Millipore, Bangalore)

Methods

For DNA extraction
1. 3 ml blood was taken into EDTA-coated centrifugal vial and centrifuged it at 5000 rpm for 8 min at room temperature.
2. Discarded the serum i.e. the supernatant and the pellet was mixed with 10 ml of 1X Solution A (ice-cold) (provided with Kit) by inverting the vial and left for 5–7 min at room temperature and then centrifuged at 8000 rpm for 5 min.

3. The supernatant was removed (lysed RBCs) and the above procedure no. 3 was repeated for complete lysis of RBCs.

4. The lysed RBCs were carefully removed i.e. supernatant above the WBCs, which appears as a tiny white pellet.

5. To the WBC pellets, 6 ml of Solution B was added and centrifuged at 10,000 rpm for 10 min at room temperature.

6. The supernatant was collected in a fresh vial.

7. To this 9 ml of distilled absolute ethanol was added and mixed it by inverting the vial.

8. Centrifuged it at 10,000 rpm for 25 min at 4°C and discarded the supernatant.

9. Strands of DNA precipitated out, and it was washed twice with 5 ml of 75% ethanol by giving short spins at 10,000 rpm for 5 min at 4°C.

10. The supernatant was discarded and the DNA pellets were dried in air for 5 min only at room temperature.

11. To the DNA pellets, 0.5 to 1.0 ml of Solution C was added and incubated at 55°C for 10–15 min to improve the solubility and left it in Solution C at 4°C for 24 hrs.

12. Centrifuged it at 10,000 rpm for 2 min to remove any insoluble material and the supernatant was collected, which contains DNA and stored at –20°C for further use.

For ARMS-PCR and electrophoresis

1. A PCR reaction stock solution of 200 reactions (4ml) comprising of 0.5ml of 10X stock buffer, 0.8ml of 1.25mM dNTP stock solution and 2.7ml of distilled water were prepared.

2. 20μl of PCR reaction mixture was pipetted out into a PCR tube.

3. To this 1μl of each primer (viz. one normal or mutant, two internal controls and one reverse primer) were added (1OD U/ml).

4. 0.05μl of Taq DNA polymerase (5U/μl) was added.
5. When more than one test is being performed, a primer and enzyme can be mixed together in a separate tube before addition to the reaction mixture. This decreases pipetting errors, as larger quantities are used.

6. 1μl of genomic DNA (100ng/μl) was added.

7. Mixed, centrifuged and placed in the thermal cycler.

8. Amplification was carried out for 25 cycles as follows: 1min at 94°C, 1min at 68°C, 1.5min at 72°C with a final extension period of 3min at 72°C following the 25 cycles.

9. The tube was removed from thermal cycler and 5μl of blue dye (15% Ficoll/0.05% bromophenol blue) was added. Mixed and centrifuged.

10. 2% agarose gel was prepared in TBE buffer and ethidium bromide was added carefully before cooling of agarose gel.

11. Immediately after cooling up to 50–60°C, the gel was poured into gel casting tray, in which already desired quantity of comb was placed.

12. After cooling the gel, it became white opaque and inserted into the electrophoresis chamber without disturbing the comb.

13. The electrophoresis chambers were filled with TBE buffer up to 0.5 cm above the gel and safely removed the comb without damaging the wells.

14. In a separate PCR tube the DNA marker was mixed with 6X gel loading buffer (supplied along with DNA marker), sterile distilled water and the mixture was loaded in a separate well.

15. 20μl aliquot was loaded onto a 2% agarose gel and run at 100V for approximately 45 min in TBE.

16. After completion of electrophoresis (the blue dye reached 3/4th of the gel) the gel was visualized under UV light (312nm) and the photographs of the bands were taken from an electronic camera system fitted with in gel doc system and compared it with bands of DNA marker.