DIAGNOSIS OF BETA THALASSEMIA WITH HPLC

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Abstract-Thalassemia is a disease to create severe health difficulties and cause of many deaths in Indian population and worldwide. The study of hemoglobin variants is essential to know this trait in the populations. During the research hemoglobin variants have been examined by the use of HPLC. The total cases and controls were examined around 300 randomly from the different locations of Kanpur population especially. Our study was paying consideration chiefly on the beta thalassemia traits. In the Kanpur population has been given to the homozygous/heterogygous HbA2 and HbF2 level while HbE, HbD and HbS level slightly studied during the study. We have found 1, 4, 6 person homogygous, intermediate and heterogygous thalassemic correspondingly. The aim of this study was to diagnose the thalassemia traits in the population as a random to produce awareness to the lethal disease with the use of HPLC.

Keywords-Thalassemia trait, retention time, curve area, reverse phase and hemoglobin variants.

I INTRODUCTION

Blood hemoglobin is the oxygen transporting molecule of RBC. RBC is a complex polypeptide tetramer, globular in structure, and containing of two pairs of unlike globin chains (α, β and δ), which form a covering around a central cavity possessing four oxygen-binding heme groups each covalently associated to a globin chain. In strong adults person around 95% of the Hb is Hb A (α2β2) with small amounts (3.5%) of Hb A2 and around 2% Hb F established. Throughout embryonic development, globin chains give to embryonic HbF. The α-globin chain is resolute in reproduction on chromosome 16, and the non-α chain (β, δ, γ) are permanent in a cluster on chromosome 11. A diploid cell consequently has four α-globin genes and two β-like genes. The β chains formed of 141 and 146 amino acid residues, correspondingly. There is some sequence homology between the two chains (64 individual amino acid residues in indistinguishable positions), and the β chain dissociate from the δ and γ chains by 39 and 10 amino acid, correspondingly. Even though hemoglobinopathies and thalassemias

are two genetically dissimilar disease groups, the clinical investigations of both contained anemia of changeable harshness and uneven pathophysiology. The thalassemias are characterized by a decline in the amount of the normal globin chain shaped. This diminution in globin chain construction may result from gene removal or from mutations that unfavorably involve the transcript or stability of mRNA products. The diagnosis varies from mild anemia with microcytosis (thalassemia trait) to deadly cruel anemia. The hemoglobinopathies structural Hb variants are contributed to amino acid replacement in α chain. More than 700 hemoglobinopathies have been presented to date the majority of which are clinically caring and accidentally exposed. The clinically important hemoglobinopathies are attributable to amino acid substitution, principally in the non-α chain, that convey about changes in the secondary and tertiary structure of the Hb tetramer. These changes are the majority normal at positions in close closeness to either heme group or globin chain accessory position.

Hemoglobinopathy taxonomy is an collection of letters (Hb S, C, and D), position names representing the site of first detection or dwelling of the porosities (Hb edmonton), and family names of the catalog case. A methodical nomenclature that is both logical and educational identifies the chain, the location, and the amino acid substitution on

the concerned globin chain. Thus Hb alberta (β101 Ala→Pro) is a replacement of proline for alanine (normal amino acid) in the 101st position of the β chain. In the heterozygous state, the normal Hb is positioned first, followed by the variant. The sample is the matter analyzed in chromatography. It may consist of a single component or it may be a mixture of components. When the sample is treated in the course of an analysis, the phase or the phases containing the analytes of interest is/are referred to as the sample whereas everything out of interest separated from the sample before or in the course of the analysis is referred to as waste. Chromatography is based on the concept of partition coefficient. Any solute partitions are found between two immiscible solvents. When we make one solvent immobile (by adsorption on a solid support matrix) and another mobile it results in most common applications of chromatography. If matrix support is polar (e.g. paper, silica etc.) it is forward phase chromatography, and if it is non-polar (C-18) it is reverse phase. It is evident from above equation that the resolving power of a chromatographic column increases with column length and the number of theoretical plates per unit length. However, there are practical limits to the length of column owing to the problem of peak broadening. As the number of theoretical plates in the column is related to the surface of stationary phase, it follows that the smaller the particle size of stationary phase, the better the resolution, in part because it reduce the equilibration time of the analyte b/w the stationary and mobile phase. Many commercially available HPLC systems are microprocessor controlled to allow dedicated, continuous chromatographic separations.

II REVIEW AND LITERATURE

The HPLC technique is very comparable to the established column chromatography; apart from for that the solvent is driven through the column by applying positive pressure. This allowed most separations to be conducted in less than 15 minutes, with enhanced separations compared to the old method (1). Modern flash chromatography systems are bringing as packed plastic cartridges, and the solvent is pumped through the cartridge. Systems may also be connected with detectors and fraction collectors as long as automation. The introduction of gradient pumps resulted in quicker separations and less solvent usage (2). In prolonged bed adsorption, a fluidized bed is used, rather than a solid phase made by a packed bed. This permits omission of initial clearing steps such as centrifugation and filtration, for culture broths or slurries of broken cells (3). Phosphocellulose chromatography consumes the binding affinity of many DNA-binding proteins for phosphocellulose. The stronger a protein's interaction with DNA, the higher is the salt concentration desirable to elute that protein (4). A delegate HPLC unit may contains solvent reservoirs, solvent degasser, gradient valve, mixing vessel for liberation of the mobile phase, high-pressure pump, switching valve in inject position, switching valve in load position, sample injection loop, pre-column, analytical column, detector, data acquisition, waste or fraction collector (5). HPLC previously referred to as high-pressure liquid chromatography, is a technique in analytical chemistry used to break up, identify, and quantify each component in a mixture. It confers on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column (6). HPLC has been used for manufacturing, legal, research, and medical purposes (7). Chromatography can be identified as a mass transfer process in relation to adsorption. HPLC relies on pumps to overtake a pressurized liquid and a sample mixture through a column filled with adsorbent, important to the partition of the sample components. The active component of the column, the adsorbent, is characteristically a granular material completed of solid particles, 1–100 micrometers in size. The components of the sample mixture are divided from each other due to their dissimilar degrees of communication with the absorbent particles (8). The pressurized liquid is characteristically a mixture of solvents and is suggested to as a mobile phase. Its composition and temperature play a chief role in the separation process by influencing the communications enchanting place between sample components and adsorbent. These connections are physical in nature, such as hydrophobic, dipole-dipole and ionic, most often a combination (9). HPLC is illustrious from traditional low pressure liquid chromatography since operational pressures

are considerably higher (50–450 bar), while ordinary liquid chromatography classically operate on the force of gravity to pass the mobile phase through the column. Due to the small sample amount estranged in analytical HPLC, characteristic column dimensions are 2.0–4.8 mm diameter, and 25–280 mm length. HPLC columns are completed with smaller sorbent particles (1–60 μm in average particle size). This gives HPLC superior resolving power when unscrambling mixtures, which forms it a popular chromatographic technique (10). The schematic of an HPLC instrument characteristically contianes a sampler, pumps, and a detector. The sampler brings the sample mixture into the mobile phase stream which performes it into the column. The pumps transport the preferred flow and composition of the mobile phase through the column. The detector performs a signal proportional to the amount of sample component promising from the column, hence allowing for quantitative analysis of the sample components. Microprocessor and user software manage the HPLC instrument and provide data analysis. A number of models of mechanical pumps in a HPLC instrument can mix multiple solvents together in ratios varying in time generate a composition gradient in the mobile phase. A variety of detectors are in ordinary use, such as UV/Vis, photodiode array (PDA) or dependent on mass spectrometry. The majority HPLC instruments also have a column oven that performs for adjust the temperature (11). HPLC is a form of column chromatography that pumps a sample mixture or analyze in a solvent at high pressure through a column with chromatographic stuffing material. The sample is approved by a touching carrier gas stream of helium or nitrogen. HPLC has the ability to separate, and recognize compounds that are present in any sample that can be soften in a liquid in trace concentrations as low as parts per trillion. Because of this adaptability, HPLC is worn in a mixture of industrial and scientific applications, such as pharmaceutical, environmental, forensics, and chemicals (12). The retention time will contrast depending on the interface between the stationary phase, the molecules being analyzed, and the solvent used. As the sample moves through the column it interacts between the two phases at different rate, first and foremost due to dissimilar polarities in the analytes. Analytes that have the less amount of interaction with the stationary phase or the most amount of interaction with the mobile phase will outlet the column faster (13).

Conformist columns worn for HPLC are normally made of stainless steel and are artificial so that they can survive pressure of up to 5.0x107pa. The columns are 2-50 cm long and about 4 mm internal diameter, with flow rates of 1-2 cm 3 μm bore columns have an internal diameter of1 to 2.5 mm and are usually 20-50 cm long. They can maintain flow rates of 5-25 ml/min. Micro bore columns have three important compensations over conventional columns that minimges eluent consumption due to the slower flow rates. Ideal are interfacing with a mass spectrometer owing to the summary flow rate. Increased sensitivity owing to the higher concentration of analyte can be worn (14). Thalassemia is an inherited disorder of autosomal recessive gene disorder caused by impaired synthesis of one or more globin chains. The impairment alters manufacture of hemoglobin (Hb). Thalassemia causes unreliable degrees of anemia, which can range from important to life intimidating. People of Mediterranean, Middle Eastern, African, and Southeast Asian drop are at higher risk of carrying the genes for thalassemia. This hereditary anemia is caused by mutations that decrease hemoglobin synthesis and red cell survival. These hereditary anemia caused by decreased or absent manufacture of one type of globin chain either or globin chain. These hematologic disorders variety from asymptomatic to severe anemia that can cause important morbidity and mortality. It was first documented clinically and explained a syndrome of anemia with microcytic erythrocytes (15). Then it was called Cooley's anemia. presently Wipple and Bradford renamed this disease as "Thalassemia". Thalassemias can cause important evils because these are inherited disorders, newborn screening and prenatal diagnosis are significant in management of patients. This topic will appraisal the clinical features of thalassemia while focus on pathophysiology, clinical features, complication, management, screening and diagnosis. Previously the sharing of thalassemia had been mainly incomplete to the areas from the Mediterranean basin through the middle East and Indian subcontinent up to Southeast Asia so called thalassemia belt. Though, fresh migrations of people have increase thalassemia genes throughout the world (16). Pathophysiology hemoglobin (Hb) is the molecule that carries and transports oxygen all

through the body. Normal human hemoglobin is a tetramer shaped by two pairs of globin chains emotionally occupied to heme. The hemoglobin type is strong-minded by the combination of tetra-globin chains and chains). Each globin chain is structurally dissimilar and thus has different oxygen affinity, electrical charge, and electrophoretic mobility. Normal adult hemoglobins are spoken as A2, A and F (fetal). Ninety-five to ninety-eight percent of adult hemoglobin is A the major hemoglobin, which consists of two and two chains. Hemoglobin A2, the remainder of hemoglobin in adults is a minor component, and 1% or less of F. Advances in the study of genetic disorders main hemoglobin found only throughout fetal expansion. The equal manufacture of and non globin chains is required for normal red blood cell (RBC) function (17). The failure in hemoglobin synthesis is a main cause of microcytosis and anemia in many population groups around the world. Hb variants are characterized by the gene mutation of the globin chains form hemoglobin (i.e., the replacement of different amino acids at a certain position). Thalassemia occurs when there is decreased or absent production of one of the types of globin chains (most commonly either), that cause insufficeient amount of normal structure globin chains. This results in an imbalance between chains and causes the clinical features of thalassemia. It can be separated into two major types such as thalassemia and thalassemia. Red blood cell morphology is altered in patients with all forms of thalassemia. Hypochromic microcytes and target cells are the main features in asymptomatic individuals. Patients with more severe forms of thalassemia have the anisocytosis and poikilocytosis, hypochromic microcytic, target cells, ovalocytes, occasional fragmented red blood cells The absence or decreased of normal production of globin chains results in a relative excess of globin chains in the fetus and newborn, and globin chains in children and adults. When globin chains are not produced in equal amounts, any excess chains accumulate and precipitate damaging the RBC and accelerating its destruction. The absence of normal production of α-chains results in a relative excess of globin chains in the fetus and newborn, and globin chains in children and adults. Further, the globin chains are capable of forming soluble tetramers (Hb-H); yet this form of hemoglobin is unstable and tends to precipitate within the cell forming insoluble inclusions (Heinz bodies) that damage the red cell membrane. Thalassemia is generally less severe because the excess unpaired chains that accumulate are less damaging to RBCs than the unpaired chains. Furthermore, diminished hemoglobinization of individual red blood cells results in damage (18).

Thalassemia Syndrome is erythrocyte precursors and ineffective erythropoiesis in the bone marrow, as well as hypochromia and microcytosis of circulating red blood cells. In thalassemia, reduced amount or absence (0) of globin chains excess chains accumulate in the RBC and precipitate because they are highly insoluble. These precipitated globin chains occur in erythroid precursors in the bone marrow and circulating RBCs. The destruction of precursor RBCs results in ineffective erythropoiesis, increased erythropoietin, and proliferation of the bone marrow. This expanded bone marrow can result in the characteristic bony abnormalities of thalassemia if the process is not prevented by transfusion therapy. Prolonged and severe anemia and increased erythropoietic drive also result in hepatosplenomegaly and extramedullary erythropoiesis, leading to their premature death and hence to ineffective erythropoiesis. The degree of globin chain reduction is determined by the nature of the mutation at the globin gene located on chromosome. Peripheral hemolysis contributing to anemia is more prominent in thalassemia major than in thalassemia intermedia, and occurs when insoluble α-globin chains induce membrane damage to the peripheral erythrocytes. Genes that regulate both synthesis and structure of different globins are organized into separate clusters. The globin genes are encoded on chromosome and globin genes are encoded on chromosome as demonstrated. Each individual normally carries a linked pair of globin genes, from the paternal chromosome, and from the maternal chromosome (19). Therefore, each diploid human cell has four copies of the alpha globin gene. The four α-thalassemia syndromes thus reflect the disease state produced by deletion or no-function of one, two, three, or all four of the α-globin genes. The silent carrier state of α-thalassemia represents a mutation of one copy of the α-globin gene and results in no hematologic abnormalities. Schematic represent of the globin gene loci. The upper panel shows the α-globin locus that resides on chromosome. Each of the four alpha globin genes contributed to the synthesis of the α-

globin protein. The lower panel shows the β-globin locus that resides on chromosome. The two γ-globin genes are active during fetal growth and produce hemoglobin F. Geographical distribution of thalassemias and the malaria hypothesis It is a widely accepted conclusion that the high frequency of thalassemias and sickle cell anemia observed in some tropical and subtropical areas of the world. This due to the resistance against malignant malaria (Plasmodium falciparum) conferred by these inherited defects to the heterozygous carriers. According to the malaria hypothesis, the heterozygous for HbS or a thalassemic are resistant to malaria and have a selective advantage over both homozygotes which have a higher chance of dying during the first years of life because of either malaria or anemia. The preferential survival of the heterozygote thus makes possible the persistence at polymorphic frequencies of the abnormal genes in the population, provided that the selective agent (malaria) remains present and active (20).

Because there is a loss of both normal and abnormal genes, equilibrium between their frequencies will be reached in a period of time which depends on the extent of the selective advantage (balanced polymorphism). The malaria hypothesis is supported by the overlapping geographical distribution of these disorders and endemic malaria and by clinical and epidemiological studies showing a positive correlation between malaria endemicity and frequency of abnormal alleles. Because of migration, hemoglobinopathies are improved into the area where malaria has never been endemic the thalassemias are most prevalent in Asian and African populations. Persons of Mediterranean and African descent have the highest incidence of thalassemia. Thalassemic mutations have maintained a high frequency, particularly in these areas, because the heterozygous state confers some protection against malaria (Weatherall, 1987). Other abnormalities of hemoglobin also occur with increased frequency in these populations: therefore, thalassemia may coexist with other disorders of hemoglobin such as the sickle cell syndromes, hemoglobin E (Hb-E), or hemoglobin C (Hb-C). www.intechopen.com Thalassemia Syndrome 105 Syndrome Molecular basis Laboratory values Clinical Feature α- Thalassemia α- Thalassemia silent carrier One α- gene deletion (α/αα) Heterozygous α-thalassemia No anemia or RBC morphology abnormalities; Asymptomatic may have l-2 % Hb Bart's at birth Asymptomatic α-Thalassemia trait (minor) Two α-gene deletion (αα) Heterozygous α-thalassemia- 1 Two α-gene deletion (α/α) Homozygous α-thalassemia 2 Mild anemia, microcytosis, and hypochromia; 4-6% Hb Bart's at birth Asymptomatic Hb H disease (Hb variants related to mutation in α- globin chain) Three α-gene deletion (α) α-thalassemia-1/α- thalassemia-2 Hb Constant Spring α-thalassemia-1/Hb Constant Spring Moderate anemia, microcytosis, hypochromia, RBC fragments; Hb Bart's prominent at birth α- chain has extra 31 amino acids Jaundice, gallstones, splenomegaly, occasionally need transfusion; antioxidant drugs can precipitate hemolysis Hb Bart's Hydrops fetalis Four α-gene deletion. Homozygous α-thalassemia Severe anemia, nucleated RRCs; only Hb H, Bart's, and Portland present Death in utero or shortly after birth β- Thalassemia β-Thalassemia trait (minor) Point mutations Heterozygous β0-thalassemia Heterozygous β+-thalassemia Mild anemia, hypochromia, and microcytosis; RBC morphologic abnormalities; Hb A2, and F often elevated Asymptomatic β-Thalassemia intermedia Point mutations - β0-thalassemia/β+ thalassemia HbEβ+-thalassemia Moderate anemia, microcytosis, and hypochromia; RBC morphologic abnormalities; Hb A, and F increased; Hb A decreased to absent Maintain Hb of 7 g/dL without transfusion; clinical phenotype between β- thalassemia trait and thalassemia major www.intechopen.com Advances in the Study of Genetic Disorders Syndrome Molecular basis Laboratory values Clinical Feature β-Thalassemia major point mutations homozygous β0 thalassemia HbEβ0 thalassemia (Thalassemia intermediate or thalassemia major) (21). Severe anemia, microcytosis, and hypochromia; RBC fragments and striking morphologic abnormalities; Hb A2, and F ncreased; Hb A decreased to absent Require chronic transfusion; develop iron overload resulting in endocrine abnormalities and chronic organ damage RBC red blood cell; Hb H hemoglobin H. Characteristic of the Thalassemia Syndromes β-thalassemia is prevalent in Mediterranean countries, the Middle East, Central Asia, India, Southern China, and the Far East as well as countries along the north coast of Africa and in South America. The highest carrier frequency is reported in Cyprus (14%), Sardinia (10.3%), and Southeast Asia (Flint et al., 1998). The high gene frequency of β-thalassemia in these regions is most likely related to the selective pressure from

Plasmodium falciparum malaria. Population migration and intermarriage between different ethnic groups has introduced thalassemia in almost every country of the world, including Northern Europe where thalassemia was previously absent. It has been estimated that about 1.5% of the global population are carriers of thalassemia, with about 70,000 symptomatic individuals born annually, the great majority in the developing world. The total annual incidence of symptomatic individuals is estimated at 1 in 100,000 throughout the world and 1 in 10,000 people in the European Union. According to Thalassemia International Federation, only about 200,000 patients with thalassemia major are alive and registered as receiving regular treatment around the world. Molecular basis and classification the thalassemia syndromes are one of the most thoroughly studied diseases at the molecular level. Consequently, some explanation for the clinical heterogeneity seen in patients can be explained at the molecular level (22). Alpha thalassemias the major clinical syndromes resulting from α-thalassemia were first recognized in the mid 1950s and early 1960s through the association of the abnormal hemoglobins (Hb-H and Hb Bart's) with hypochromic microcytic anemia in the absence of iron deficiency. Alpha thalassemia is divided into deletional and nondeletional types. There are at least 40 different deletions. The size of the deletion is important and affects the clinical phenotype of hydrops fetalis. Over 95% of thalassemia is caused by large deletions involving one or both of the globin genes. The globin gene cluster occurs on the short arm of chromosome 16, band 16 p 13.3 and includes the globin genes as well as the embryonic genes (23). The diagnosis of thalassemia trait is considered when the patient has the appropriate RBC abnormalities, when iron deficiency and thalassemia trait have been excluded, and when family studies (CBC, hemoglobin profile, and review of the peripheral smear) are consistent with the diagnosis. To make the diagnosis with complete certainty requires characterization of gene deletions with restriction endonuclease mapping or globin chain synthesis studies showing a decreased (ratio. However, this confirmation rarely is indicated clinically. Hemoglobin H disease Hemoglobin H (Hb-H) disease is the most severe non-fatal form of thalassemia syndrome, mostly caused by molecular defects of the globin genes in which globin expression is decreased, causes a moderate anemia with hypochromia, microcytosis, and red cell fragmentation (24).

The patients usually maintain a hemoglobin level of 7g/dL without transfusions. At the severe end of the clinical spectrum, patients present between the ages of 2 and 6 years and although they are capable of surviving without regular blood transfusion, growth and development are retarded. At the other end of the spectrum are patients who are completely asymptomatic until adult life with only mild anemia. Therefore, pregnant or older patients are less able to tolerate the anemia and may need transfusion support. Hypertrophy of erythroid marrow with the possibility of extra-medullary erythropoiesis, a compensatory mechanism of bone marrow to overcome chronic anemia, is common. consequences are characteristic deformities of the bone and face, osteoporosis with pathologic fractures of long bones and formation of erythropoietic masses that primarily affect the spleen, liver, lymph nodes, chest and spine (25). Enlargement of the spleen is also a consequence of its major role in clearing damaged red cells from the bloodstream. 4.8 β-Thalassemia major Thomas Cooley, first described this disorder in 1925 after noticing similarities in the appearance and clinical findings in several anemic children of Greek and Italian immigrants. Prior to the advent of routine transfusion therapy, thalassemia major patients did not survive beyond the first few years of life. Survival is now improved with hypertransfusion regimens, iron chelation therapy, and bone marrow transplantation. Serious thalassemia is associated with iron overload, tissue damage, and increased risk of cardiovascular complications. Thalassemias are the most important among the thalassemia syndromes with an average trait prevalence of 7% in Greece, 15% among Cypriots, and 4.8% in Thailand. Clinical presentation of thalassemia major occurs between 6 and 24 months. Affected infants fail to thrive and become progressively pale. Feeding problems, diarrhea, and irritability, recurrent bouts of fever, and progressive enlargement of the abdomen caused by spleen and liver enlargement may occur. In some developing countries, where due to the lack of resources patients are untreated or poorly transfused, the clinical picture of thalassemia major is characterized by growth retardation, pallor, jaundice, poor musculature, genu valgum, hepatosplenomegaly, leg ulcers, development of masses from extramedullary hematopoiesis, and

skeletal changes resulting from expansion of the bone marrow. Skeletal changes include deformities in the long bones of the legs and typical craniofacial changes (bossing of the skull, prominent malar eminence, depression of the bridge of the nose, tendency to a mongoloid slant of the eye, and hypertrophy of the maxillae, which tends to expose the upper teeth). In thalassemia major, severity of anemia requires initiation of blood tranfusions during infancy (26). If a regular transfusion program that maintains a minimum Hb concentration of 9.5 to 10.5 g/dL is initiated, growth and development tends to be normal up to 10 to12 years. Transfused patients may develop complications related to iron overload. Complications of iron overload in children include growth retardation and failure or delay of sexual maturation. Later iron overloadrelated complications include involvement of the heart liver, and endocrine glands. Hemoglobin E (Hb-E) The most common combination of β- thalassemia with abnormal Hb or structural Hb variant with thalassemic properties is Hb-E/β-thalassemia which is most prevalent in an area stretching from northern India and Bangladesh, through Laos, Carmbodia, Thailand, Vietnam, Malaysia, the Philippines, and Indonesia where the carrier frequency is around 50%. Hb-E is caused by a mutation of the 26th amino acid of a normal β-chain, glutamine, is replace by lysine. This mutation also activates a cryptic synthesis of the globin chain and leads to a thalassemic phenotype. Furthermore, the hemoglobin E gene, which can interact with thalassemic alleles and cause a broad phenotypic spectrum, reaches a frequency of up to 50% in Thailand. These Hb-E/beta-thalassemias may be identified to three categories depending on the severity of symptoms: 4.10 Mild Hb-E/β- thalassemia (27). It is observed in about 15% of all cases in Southeast Asia. This group of patients maintains Hb levels between 9 and 12 g/dl and usually does not develop clinically significant problems. No treatment is required. 4.11 Moderately severe Hb-E/β-thalassemia. The majority of Hb-E/β-thalassemia cases fall into this category. The Hb levels remain at 6-7 g/dl and the clinical symptoms are similar to thalassemia intermedia. Transfusions are not required unless infections precipitate further anemia. Iron overload may occur. 4.12 Severe Hb-E/β-thalassemia The Hb level can be as low as 4-5 g/dl. Patients in this group manifest symptoms similar to thalassemia major and are treated as thalassemia major patients. Hb-E thalassemia is more frequent than homozygous thalassemia in Thailand because of the high frequency of Hb-E. It is the most common severe thalassemia syndrome in adults. There are two types of Hb-E-thalassemia, classified based on the presence or absence of Hb- A, Hb-E+-thalassemia and Hb-E thalassemia (28). III

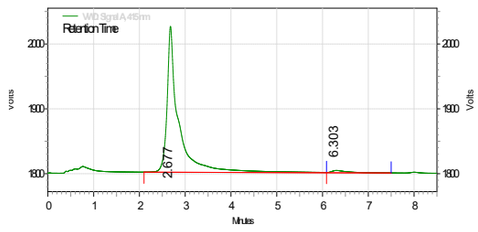
III MATERIALS AND METHODS

The subsequent materials were worn to perform thalassemia screening. 1. HPLC instrument- Agilent Technologies 1220 Infinity LC, Software version**:** EZchrome Elite. 2. Thalassemia equipped buffer we have obtained from Gordian health technology. The flow rate was 1 ml/min. The flow rate of buffer Aand B was 85:15. The total run time was 8 min and absorbance was set 415 nm. 3. Methanol- we have used for the washing of the HPLC, before the run and after the run. 4. Thalassemia samples (1 ml EDTA blood) we have collected blood from the patients in the EDTA vials. Samples were diluted with hemolysis buffer in the ratio of 990:10µl. 20 µl samples were loaded in the HPLC for the analysis.

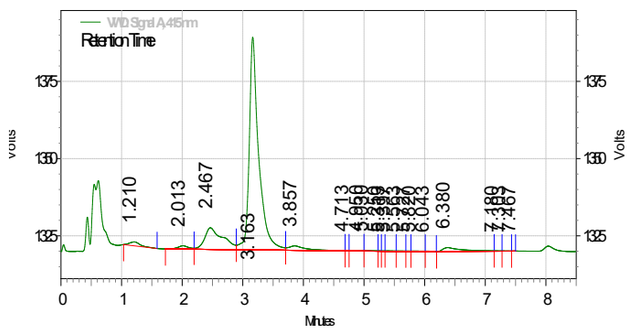
The study has groups incorporated were: A) the associations of known thalassemia major Patients. B) Antenatal patients pending to Gynecology and Obstetrics subdivision of our Hospital (Rama Medical College Kanpur, India). Viewing for thalassemia is required for antenatal cases in our hospital; C) Thalassemia screening camps held in high risk communities in our area. A total of 300 subjects (age<25 years) with established microcytic anemia were built-in in the study. Anemia was distinct as a hemoglobin concentration of at least 2 standard deviations lower than age and sex-specific average. IDA and BTT were diagnosed by the following tests: serum iron levels, serum TIBC levels and HbA2 levels performed by chromatography. We retested the patients having borderline HbA2 values and low serum iron levels after giving them iron replacement therapy. The following hematological data were obtained: Hemoglobin, Red cell count, Hematocrit, MCV, MCH, MCHC, RDW-CV, RDW-SD, Serum iron, HbA2 [38].

IV RESULTS AND DISCUSSIONS

We have screen about 300 samples for the thalassemia, out of 300 samples we have got 10 samples positive and remaining samples were thalassemia negative. The thalassemia screening there is a need of compulsory the blood CBC report. In the CBC report we have listen cautiously mainly at MCH, MCHC, Hb and RDW-SD and from the HPLC chromatograms we have composed the values of HbA2 and HbF. On the basis of above mentioned values we have equipped report for the thalassemia. Some reports are bringing up here. Detection of Beta Thalassemia Trait quantification of Hb A2: 3.80% (Normal value up to 3.8%) quantification of Hb F: 0.48% (Normal value up to 2%) HbA2 value is normal than predictable ranges chromatogram of beta thalassemia trait is presentation normal HbA2 3.80% (RT 3.217 min) and normal HbF 0.48% (RT 1.997 min). We have also obtained data from the CBC report which are essential for the screening the thalassemia. (Hematological Report) MCV: 96 fl (Normal value 80-96 fl) MCH: 29 pg (Normal value 27-33 pg) Hb (Hemoglobin): 13.0 g/dl (Normal value 12-16 g/dl) RDW-SD: 39.8fL (Normal value 29-46 fL) From the above report we have concluded taht person is normal with respect the thalassemia trait since the HbA2 and HbF2 are in the normal range and MCH value which is rather significance is in normal range and finally we conclude:

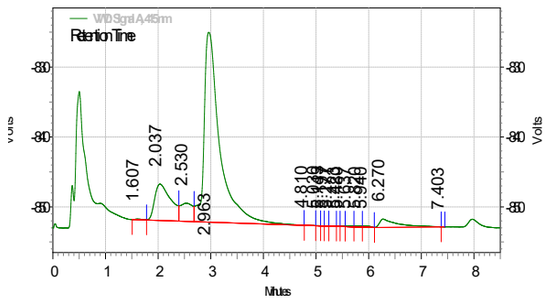


Impression:-NO EVEDENCE OF BETA THALASSEMIA HAEMOGLOBINOPATHY. Quantification of Hb F: 7.92% (Normal value up to 2%) Values outside of expected ranges Chromatogram of beta thalassemia trait showing elevated HbA2 10.49% (RT 3.830 min) and HbF 7.92% (RT 2.297 min). (Hematological Report) MCV: 93 fl (Normal value 80-96 fl) MCH: 25 pg (Normal value 27-33 pg) MCHC 28 g/dl (Normal value 33-35g/dl) Hb: 13.3 g/dl (Normal value 12-16 g/dl) RDW-SD: 40.8 fL (Normal value 29-46 fL)



Impression:- EVEDENCE OF BETA THALASSEMIA (MINOR) HAEMOGLOBINOPATHY. Note: This report was validated only when the patient blood transfusion had not been done recently. (Disclaimer: All investigations had done their own limitation which was imposed by the limits of sensitivity and specificity of individual assay procedures as well as the specimen received by the laboratory. Isolated laboratory investigations were never confirm the diagnosis of the disease. They only helped in arriving at a diagnosis in conjunction with clinical presentation and other related investigations). (Fig. 3, HPLC chromatogram of thalassemic person) Test: Whole Blood Examination (HPLC Report) Detection of Beta thalassemia Trait Quantification of Hb A2: 2.60% (Normal value up to 3.8%) Quantification of Hb F: 2.86% (Normal value up to 2%) Chromatogram of beta thalassemia trait showing HbA2 2.60 % (RT 3.230 min) and elevated HbF 2.86 % (RT 2.663 min). (Hematological Report) MCV: 96 fl (Normal value 80-

96 fl) MCH: 25 pg (Normal value 27-33 pg) Minutes 0 1 2 3 4 5 6 7 8 Volts -425 -400 -375 -350 Volts -425 -400 -375 -350 1.333 1.653 2.663 2.7973.230 3.897 4.083 4.180 4.357 4.443 4.593 4.763 4.890 5.047 5.187 5.383 5.510 5.6635.723 5.907 6.017 6.310 6.870 6.977 7.130 7.353 VWD: Signal A, 415 nm Retention Time MCHC 26 g/dl (Normal value 33-35g/dl) Hb: 11.5 g/dl (Normal value 12-16 g/dl) RDW-SD: 51.3 fL (Normal value 29-46 fL).



Impression:-EVIDENCE OF BETA THALASSEMIA (MINOR) HAEMOGLOBINOPATHY. From the above reports we have showed one negative and two positive patients are reported here. Both positive samples are beta thalassenic minor (heterogygous). None of the samples are found beta thalassenic intermediate (heterogygous), beta thalassenic major (homogygous) and sickle cell anemia.

Hemoglobin HPLC is a labor intensive and time consuming method and is efficient when quantifying low concentrations of HbA2 and HbF. The HPLC (High Performance Liquid Chromatography) method is a sensitive and precise method and has become the preferred method for thalassemia screening because of its simplicity, superior resolution, rapid assay time and accurate quantification of Hb fractions. Despite technical advances and the large amount of accumulated knowledge several problems in carrier identification remain. The most common problem is the presence of microcytosis with HbA2 and HbF concentrations within the reference range. This may be due to iron deficiency or α-thalassemia trait. Iron deficiency anemia produces a wide range of red cell abnormalities (reduction of MCV, MCH and hemoglobin levels and normal or lowered RBC) depending on the severity at the time of hematological analysis. For this reason iron deficiency anemia can be easily mistaken for some forms of heterozygous thalassemia. On the other hand a raised RBC with low MCV and MCH is more consistent with α thalassemia trait. It is mandatory that testing for iron deficiency accompany all requests for thalassemia analysis. There are a number of calculations based on the red cell indices that are helpful in differentiating iron deficiency from thalassemia (29). It is possible that with a very severe iron deficiency in β-thalassemia carriers the HbA2 levels can fall to within the normal range. In practice, if an individual has very severe iron deficiency anemia with normal HbA2, it is preferable to correct the anemia before repeating tests to determine HbA2 levels. Family studies may also be useful for distinguishing iron deficiency anemia from the thalassemia traits. An effective health education programme should aim to provide reliable, accurate and up-to date information on all aspects of the prevention and clinical care of thalassemia major, in a clear, accessible format. The key to successful control programmes is health education along with screening, genetic counseling and prenatal diagnosis (30).

V CONCLUSIONS

Premarital testing should be incorporated in the nation. In the Asian countries such as India, Pakistan and China it is already in place. If a silent transporter is suspected on the basis of borderline red cell index and borderline HbA2 levels, perfect diagnosis may be obtain using characterization of the mutation by DNA analysis. Since of their silent phenotype these carriers may get away recognition in a routine screening programme.

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VII REFERENCES

(1). Hedlund B. Hemoglobins of human embryos, fetuses, and neo-nates. In: airbanks VF, ed. Hemoglobinopathies and thalasse-mias. New York: Brian C. Decker, 1980: 14 –7.

(2). Lindeman NL, Ng VL. Clinical hematology. Check Sample No. CH 99-5. Chicago, IL: American Society of Clinical Pathologists, 1999.

(3). Rioux J, Godart C, Hurtrel D, Mathis M, Bimet C, Bardakdjian J, et al. Cation-exchange HPLC evaluated for presumptive identification of hemoglobin variants. Clin Chem 1997; 43: 34 –9.

(4). Eastman JW, Wong R, Liao C, Morales D. Automated HPLC screening of newborns for sickle cell anemia and other hemoglo-binopathies. Clin Chem 1996; 42: 704 –10.

(5). Mario N, Baudin B, Aussel C, Giboudeau J. Capillary isoelectric focusing and high performance cation-exchange chromatography compared for the qualitative and quantitative analysis of hemoglo-bin variants. Clin Chem 1997; 43: 2137– 42.

(6). Fucharoen S, Winichagoon P, Wisedpanichkiy R, Sae-Ngow B, Sriphanich R, Oncoung W, et al. Prenatal and post natal diagnosis of thalassemias and hemoglobinopathies by HPLC. Clin Chem 1998; 44:740-8.

(7). Fisher SI, Haga JA, Castleberry SM, Hall RB, Thompson WC. Validation of an automated HPLC method for quantification of Hb S. Clin Chem 1997; 43: 1667-8.

(8). Papadea C, Cate JC. Identification and quantification of hemoglo-bins A, F, S, and C by automated chromatography. Clin Chem 1996; 42: 57-63.

(9). Campbell M, Henthorn JS, Davies SC. Evaluation of cation-exchange HPLC compared with isoelectric focussing for neonatal hemoglobinopathy screening. Clin Chem 1999; 45: 969-75.

(10). Ou CN, Rognerud CL. Rapid analysis of hemoglobin variants by cation-exchange HPLC. Clin Chem 1993; 39: 820-4.

(11). British Committee for Standards in Haematology. Guidelines for haemoglobinopathy screening. Clin Lab Haematol 1988; 10: 87-94.

(12). International Committee for Standardization in Haematology. ICSH Expert Panel on Abnormal Haemoglobins. Recommenda-tions for neonatal screening of haemoglobinopathies. Clin Lab Haematol 1988; 10: 335-45.

(13). Lafferty JD, Crowther MA, Ali MA, Levine ML. The evaluation of various mathematical RBC indices and their efficacy in discrimi-nating between thalassemic and non-thalassemic microcytosis. Am J Clin Pathol 1996; 106: 201-215.

(14). Guidelines for the investigation of the and b thalassemia traits. The Thalassemia Working Party of the BCSH General Haematology Task Force. J Clin Pathol 1994; 47: 289-95.

(15). Hall RB, Haga JA, Guerra CG, Castleberry SM, Hickman JR. Optimizing the detection of hemoglobin H disease. Lab Med 1995; 26: 736-41.

(16). Lafferty J. College of American Pathologists hemoglobinopathy survey HG-B. Chicago, IL: College of American Pathologists, 1999.

(17). Turpeinen U, Siplia I, Antila P, Kayalainen U, Kuronen B, Kalteki-nen N, et al. Two a -chain variants, Hb Brousscis and Hb cemenelum characterized by cation-exchange HPLC, iso-electric focus-ing and peptide sequencing. Clin Chem 1995; 41: 532-6.

(18). Cotton F, Lin C, Fontaine B, Gulbis B, Janssens J, Vertongen F. Evaluation of a capillary electrophoresis method for routine deter-mination of hemoglobins A2 and F. Clin Chem 1999; 45: 237-43.

(19). Hempe JM, Graver RD. Quantification of hemoglobin variants by capillary isoelectric focussing. Clin Chem 1994; 40: 2288-95.

(20). White JM, Christie BS, Nam D, Daar S, Higgs DR. Frequency and Clinical Chemistry 46, No. 8(B), 2000 1289 clinical significance of erythrocyte genetic abnormalities in Oma-nis. J Med Genet 1993; 30:b396-400.

(21). Suh DD, Kruss JS, Bures K. Influence of hemoglobin S adducts on hemoglobin A 2 quantification by HPLC. Clin Chem 1996; 42:113- 4.

(22). Dash S. HbA2 in subjects with HbD. Clin Chem 1998; 44: 2381-2.

(23). Cotton F, Gulbis B, Hansen V, Vertongen F. Interference of hemoglobin D in hemoglobin A2 measurement by cation-exchange HPLC. Clin Chem 1999; 45: 1317-8.

(24). Zorn N, Eigel A, Lizama M, Huaman-Guillen P, Ma rzW,et al. Hemoglobin Rambam (b69[E13]Gly3 Asp), a pitfall in the assessment of diabetic control: characterization by electrospray mass spectrometry and HPLC. Clin Chem 1998; 44: 2172-7.

(25). Shackleton CHL, Witkowska HE. Characterizing abnormal hemo-globins by MS. Anal Chem News Features 1996; 68: 29A-33A.

(26). Honda S, Bhagavan N, Sugiyama C, Gallaty R, Dublin R, Flegal I,et al. Hemoglobinopathies detected by CBC analysis and HPLC hemoglobin A 1C analysis. Lab Med 1994; 25: 176-81.

(27). Chem D, Crimmins DL, Hsu FF, Lindberg FP, Scott MG. Hemoglo-bin Raleigh as the cause of a falsely increased hemoglobin A 1C in an automated ion-exchange HPLC method. Clin Chem 1998; 44: 1296-301.

(28). Watanabe T, Kato K, Yamada D, Midorikawa S, Sato W, Shiga M, et al. A nondiabetic case of hemoglobin variant (Hb Niigata) with inappropriately high and low HbA1C titers detected by different methods. Clin Chem 1998; 44: 1562-4.

(29). Schnedl WJ, Reisinger EC, Picker TR, Lipp RW, Schreiber F, Hopmeier P, et al. Hemoglobin Sherwood Forest detected by high performance liquid chromatography for hemoglobin A1C . Am J Clin Pathol 1995; 104: 444-6.

(30). Globin Chain Disorder Working Party of the BCSH General Haema-tology Task Force. Guidelines for the fetal diagnosis of globin genes disorders
. J Clin Pathol 1994; 47: 199-204.