Screening of Beta Thalassemia with the Use of Hplc Technique

Anil Kumar
Department of Biotechnology Engineering and Technology,
Rama University, Uttar Pradesh, Kanpur, India.
anil.biotech@gmail.com

***Abstract*-Thalassemia hemoglobinopathies create severe health difficulty posses to cruel mortality in Indian population and globally. With the study of hemoglobin variants this trait can be identified in the populations. In the study hemoglobin variants have been studied with the use of HPLC. We have screened a total of 400 cases randomly from the different locations of Himanchal Pradesh especially at the adult stage persons. We have focused mainly on the beta thalassemia traits.**

Special consideration has been given to the homozygous/heterogygous HbA2 and HbF2 level whereas HbE, HbD and HbS level mildly studied during the study. We have found 2, 6, 10 person homogygous, intermediate and heterogygous thalassenic respectively. The aim of this study was to detect the thalassemia traits in the population as a random to create awareness to the deadly disease with the use of cheaper reliable technique.

Keywords-Thalassemia trait, intermediate, retention factor, reverse phase and hemoglobin variants.

I INTRODUCTION

Hemoglobin is the oxygen-carrying molecule of RBC. RBC is a polypeptide tetramer, globular in structure, and possessing of two pairs of unlike globin chains (α and β, δ, or γ), which form a shell around a central cavity containing four oxygen-binding heme groups each covalently linked to a globin chain. In healthy adults person around 95% of the Hb is Hb A ($\alpha_2\beta_2$) with small amounts (3.5%) of Hb A_2 and around 2% Hb F found. During embryonic development, globin chains contribute to embryonic HbF.

The α-globin chain is determined in duplicate on chromosome 16, and the non-α chain (β, δ, γ) are fixed in a cluster on chromosome 11. A diploid cell therefore has four α-globin genes and two β-like genes. The β chains consist of 141 and 146 amino acid residues, respectively. There is some sequence homology between the two chains (64 individual amino acid residues in identical positions), and the β chain differs from the δ and γ chains by 39 and 10 residues, respectively.

Although hemoglobinopathies and thalassemias are two genetically distinct disease groups, the clinical manifestations of both include anemia of variable severity and variable pathophysiology. The thalassemias are characterized by a reduction in the amount of the normal globin chain produced. This diminution in globin chain production may result from gene deletion or from mutations that adversely affect the transcription or stability of mRNA products. The manifestations range from mild anemia with microcytosis (thalassemia trait) to fatal severe anemia (Hb Barts hydrops fetalis or β-thalassemia major). The hemoglobinopathies, or structural Hb variants, are attributable to amino acid substitution in either α or non-α chain. More than 700 hemoglobinopathies have been described to date the majority of which are clinically benign and fortuitously discovered. The clinically significant hemoglobinopathies are attributable to amino acid substitutions, primarily in the non-α chain, that bring about changes in the secondary and tertiary structure of the Hb tetramer. These substitutions are most common at positions in close proximity to either heme group or globin chain attachment sites.

Hemoglobinopathy nomenclature is an assortment of letters (i.e., Hb S, C, and D), place names denoting the site of first discovery or residence of the propositus (e.g., Hb Edmonton), and family names of the index case. A systematic nomenclature that is both logical and informative identifies the chain, the location, and the amino acid substitution on the involved globin chain. Thus Hb Alberta (β101 Ala→Pro) is a substitution of proline for alanine (the normal amino acid) in the 101st position of the β chain. In the heterozygous state, the normal Hb is placed first, followed by the variant, e.g., AS trait.

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 similar to the traditional column chromatography, except for that the solvent is driven through the column by applying positive pressure. This allowed most separations to be performed in less than 20 minutes, with improved separations compared to the old method [1]. Modern flash chromatography systems are sold as pre-packed plastic cartridges, and the solvent is pumped through the cartridge. Systems may also be linked with detectors and fraction collectors providing automation. The introduction of gradient pumps resulted in quicker separations and less solvent usage[2].

In expanded bed adsorption, a fluidized bed is used, rather than a solid phase made by a packed bed. This allows omission of initial clearing steps such as centrifugation and filtration, for culture broths or slurries of broken cells [3].

Phosphocellulose chromatography utilizes the binding affinity of many DNA-binding proteins for phosphocellulose. The stronger a protein's interaction with DNA, the higher the salt concentration needed to elute that protein [4].

A representative HPLC unit may contains (A) Solvent reservoirs, (B) Solvent degasser, (C) Gradient valve, (D) Mixing vessel for delivery of the mobile phase, (E) High-pressure pump, (F) Switching valve in "inject position", (G) Switching valve in "load position", (H) Sample injection loop, (I) Pre-column (guard column), (J) Analytical column, (K) Detector, (L) Data acquisition, (M) Waste or fraction collector [5].

High-performance liquid chromatography (HPLC formerly referred to as high-pressure liquid chromatography), is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It relies 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 (e.g. during the production process of pharmaceutical and biological products), legal (e.g. detecting performance enhancement drugs in urine), research (e.g. separating the components of a complex biological sample, or of similar synthetic chemicals from each other), and medical (e.g. detecting vitamin D levels in blood serum) purposes [7].

Chromatography can be described as a mass transfer process involving adsorption. HPLC relies on pumps to pass a pressurized liquid and a sample mixture through a column filled with adsorbent, leading to the separation of the sample components. The active component of the column, the adsorbent, is typically a granular material made of solid particles (e.g. silica, polymers, etc.), 2–50 micrometers in size. The components of the sample mixture are separated from each other due to their different degrees of interaction with the absorbent particles [8]. The pressurized liquid is typically a mixture of solvents (e.g. water, acetonitrile and/or methanol) and is referred to as a "mobile phase". Its composition and temperature play a major role in the separation process by influencing the interactions taking place between sample components and adsorbent. These interactions are physical in nature, such as hydrophobic (dispersive), dipole–dipole and ionic, most often a combination [9].

HPLC is distinguished from traditional ("low pressure") liquid chromatography because operational pressures are significantly higher (50–350 bar), while ordinary liquid chromatography typically relies on the force of gravity to pass the mobile phase through the column. Due to the small sample amount separated in analytical HPLC, typical column dimensions are 2.1–4.6 mm diameter, and 30–250 mm length. Also HPLC columns are made with smaller sorbent particles (2–50 micrometer in average particle size). This gives HPLC superior resolving power (the ability to distinguish between compounds) when separating mixtures, which makes it a popular chromatographic technique [10].

The schematic of an HPLC instrument typically includes a sampler, pumps, and a detector. The sampler brings the sample mixture into the mobile phase stream which carries it into the column. The pumps deliver the desired flow and composition of the mobile phase through the column. The detector generates a signal proportional to the amount of sample component emerging from the column, hence allowing for quantitative analysis of the sample components. A digital microprocessor and user software control the HPLC instrument and provide data analysis. Some models of mechanical pumps in a HPLC instrument can mix multiple solvents together in ratios changing in time, generating a composition gradient in the mobile phase. Various detectors are in common use, such as UV/Vis, photodiode array (PDA) or based on mass spectrometry. Most HPLC instruments also have a column oven that allows for adjusting the temperature [11].

High Performance Liquid Chromatography (HPLC) is a form of column chromatography that pumps a sample mixture or analyte in a solvent (known as the mobile phase) at high pressure through a column with chromatographic packing material (stationary phase). The sample is carried by a moving carrier gas stream of helium or nitrogen. HPLC has the ability to separate, and identify compounds that are present in any sample that can be dissolved in a liquid in trace concentrations as low as parts per trillion. Because of this versatility, HPLC is used in a variety of industrial and scientific applications, such as pharmaceutical, environmental, forensics, and chemicals [12].

Sample retention time will vary depending on the interaction between the stationary phase, the molecules being analyzed, and the solvent, or solvents used. As the sample passes through the column it interacts between the two phases at different rate, primarily due to different polarities in the analytes. Analytes that have the least amount of interaction with the stationary phase or the most amount of interaction with the mobile phase will exit the column faster [13].

Conventional columns used for HPLC are generally made of stainless steel and are manufactured so that they can withstand pressure of up to $5.5x10^7$pa. The columns are 3-50cm long and approximately 4 mm internal diameter, with flow rates of 1-3 cm 3 min micro bore or open tubular columns have an internal diameter of1 to 2 mm and are generally 25 -50 cm long. They can sustain flow rates of 5-20 mm^3 min^{-1}. Micro bore columns have three important advantages over conventional columns: that reduces eluent consumption owing to the slower flow rates. Ideal are interfacing with a mass spectrometer owing to the reduced flow rate. Increased sensitivity owing to the higher concentration of analyte can be used [14].

Thalassemia is an inherited disorder of autosomal recessive gene disorder caused by impaired synthesis of one or more globin chains. The impairment alters production of hemoglobin (Hb). Thalassemia causes varying degrees of anemia, which can range from significant to life threatening. People of Mediterranean, Middle Eastern, African, and Southeast Asian descent are at higher risk of carrying the genes for thalassemia. These hereditary anemia's are caused by mutations that decrease hemoglobin synthesis and red cell survival. These hereditary anemia caused by decreased or absent production of one type of globin chain either or globin chain. These hematologic disorders range from asymptomatic to severe anemia that can cause significant morbidity and mortality. It was first recognized clinically in 1925 by Dr. Thomas Cooley, who described a syndrome of anemia with microcytic erythrocytes [15]. Then it was called Cooley's anemia. Later Wipple and Bradford renamed this disease as "Thalassemia". Thalassemias can cause significant problems because these are inherited disorders, newborn screening and prenatal diagnosis are important in management of patients. This topic will review the clinical features of thalassemia while focusing on pathophysiology, clinical features, complication, management, screening and diagnosis. Formerly the distribution of thalassemia had been mainly limited to the areas from the Mediterranean basin through the Middle East and Indian subcontinent up to Southeast Asia so called "thalassemia belt". However, recent migrations of people have spread 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 formed by two pairs of globin chains attached to heme. The hemoglobin type is determined by the combination of tetra-globin chains and chains). Each globin chain is structurally different and thus has different oxygen affinity, electrical charge, and electrophoretic mobility. Normal adult hemoglobins are expressed 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 (less than 3.3%), and 1% or less of F (Nathan & Oski, 1993.). Advances in the Study of Genetic Disorders predominant hemoglobin found only during fetal development. The equal production of and non globin chains is necessary 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 both

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 (80 to 90 million people) are carriers of thalassemia, with about 60,000 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].

Common thalassemia deletions that spare the embryonic gene allow for the production of functional embryonic www.intechopen.com Thalassemia syndrome hemoglobin early in gestation. In contrast, the large deletions (severe) lack the benefit of embryonic hemoglobin. Non-deletion mutations may have a more severe phenotype than most of the deletional mutations. The most common non-deletional thalassemia mutation is Hemoglobin Constant Spring; this mutation of the stop codon results in 31 amino acids being added to chain. Depending on the production of α-globin chains, α-thalassemia determinants can be classified into two groups: α0 and α. In α-thalassemia the production of α-chains by the affected chromosome is completely abolished; α+-thalassemia is defined by the variable amounts of α polypeptide chains which can still be expressed in cis to the thalassaemic cluster. This nomenclature, which describes α-thalassemias in terms of α-globin chain expression/haplotype, has replaced the previous classification of these defects into severe (α-thalassaemia-1) and mild (α-thalassaemia 2) forms. In the past, genetics of these syndromes were more confusing [24]. This was because the adult carriers of α-thalassemia do not produce large amounts of either Hb-H or Hb Bart's. Although the relatives of the affected individuals do not have a readily defined phenotype, it was eventually shown that the offspring of individuals with Hb-H disease have raised levels of Hb Bart's (γ4) in the neonatal period, and the parents of individuals with Hb-H disease and the Hb Bart's hydrops fetalis syndrome have mildly hypochromic, microcytic red cell indices; sometimes Hb-H inclusions could be demonstrated in occasional red cells. By 1969 it had been shown that HbH disease results from the inheritance of α-thalassemia-1 x α-thalassemia-2 and the Hb Bart's hydrops fetalis syndrome results from α-thalassemia-1 x α-thalassemia. The structural organization of the α-globin genes revealed by blot hybridization analysis, Normal individual have two α-genes on each chromosome 16 or four copies of the -globin gene (αα/αα) and carriers for α-thalassemia have either three (α/αα) or two (αα) α genes. Thus, the most frequently encountered genotype of Hb-H disease is α and Hb Bart's hydrops fetalis is. Thus by 1980 the molecular genetics of α-thalassemia was understood. The four thalassemia syndromes thus reflect the disease state produced by deletion or nonfunctional of one, two, three, or all four of the globin genes [25].

Thalassemia trait occurs with deletion or nonfunction of two globin genes. The two genes are deleted from the same chromosome (cis-

deletion) or one gene is lost from each chromosome 16 (transdeletion). The cis-deletion is most common in Asian and Mediterranean populations, whereas individuals of African descent usually have the trans-deletion. Both varieties of -thalassemia trait produce an asymptomatic, mild anemia associated with microcytosis. Hemoglobin H (Hb-H) disease, a three-gene deletion, usually results from inheritance of the cis-thalassemia trait from one parent and the one gene deletion from the other parent. Therefore, this abnormality is rare in the black population because the cisdeletion is uncommon. Hydrops fetalis results from deletion of all four globin genes and generally causes death in utero because no physiologically useful hemoglobin is produced beyond the embryonic stage. Although the thalassemia syndromes also are of varying clinical severity, these differences cannot be explained by the number of deleted or nonfunctional genes. One of the most frequent thalassemia mutations is the SEA deletion, which deletes both globin genes but spares the embryonic gene [26]. Homozygosity for this deletion (SEA) is the most common cause of hydrops fetalis. The sparing of the embryonic gene allows enough functional embryonic haemoglobin to allow gestation to continue and the www.intechopen.com 108 Advances in the Study of Genetic Disorders phenotype of hydrops fetalis to develop. In contrast, other common thalassemia mutations (FILTHAI) also lack the entire embryonic globin cluster, and therefore do not produce the functional embryonic Hemoglobin Portland. These embryos may terminate unnoticed early in gestation. Over 5% of individuals in the Philippines are carriers for the SEA or FIL mutation. Hydrops fetalis, while most common in Southeast Asia, is found worldwide among many ethnic groups; MED is a common 0-thalassemia mutation in Mediterranean regions, particularly Greece and Cyprus. It has resulted in hydrops fetalis. Non-deletional thalassemia is found throughout the world. Up to 8% of Southeast Asians are carriers of Hemoglobin Constant Spring. In the Middle East, Hemoglobin TSaudi is a common thalassemia non-deletional mutation. It is a mutation of the polyadenylation signal sequence of the 2 gene, resulting in decreased expression of structurally normal chains. Hemoglobin Koya Dora, another structural non-deletional mutation, is found in India. Other structural mutations, such as hemoglobin Quong Sze found in Southeast Asia, are highly unstable and result in defects in the hem pocket. α Thalassemia trait α-Thalassaemia trait is usually caused either by the interaction of the normal haplotype with a α°- or a α+-thalassaemia determinant or by the homozygosity for two α+ haplotypes. Much less frequently this phenotype can be the result of compound heterozygosity for a deletional α+- thalassaemia and a, α+ determinant caused by a point mutation or even homozygosity for the latter kind of determinant. Depending on the nature and localization of the mutation, the phenotype of the trait can thus range from the silent carrier to individuals showing very pronounced haematological abnormalities. Patients with -thalassemia trait have microcytosis, hypochromia, and mild anemia. Small amounts of hemoglobin Bart's may be noted on a newborn screen. Individuals with this disorder are asymptomatic and do not require transfusions or any other treatment [27].

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 [28].

The β-thalassemias are widespread throughout the Mediterranean region, Africa, the Middle East, the Indian subcontinent and Burma, Southeast Asia including southern China, the Malay Peninsula, and Indonesia. Estimates of gene frequencies range from 3 to 10 percent in some areas. Within each population at risk for β-thalassemia a small number of common mutations are found, as well as rarer ones; each mutation is in strong linkage disequilibrium with specific arrangements of restriction-fragment length polymorphisms, or haplotypes, within the β-globin cluster. A limited number of haplotypes are found in each population, so that 80 percent of the mutations are associated with only 20 different haplotypes. This observation has helped demonstrate the independent origin of β- thalassemia in several populations. There is evidence that the high frequency of β-thalassemia throughout the tropics reflects an advantage of heterozygotes against. Plasmodium falciparum malaria, as has already been demonstrated in α-thalassemia [29]. The globin gene cluster is located on chromosome and is not duplicated like the globin genes. Therefore, each diploid cell contains only two globin genes. Mutations are described that affect every step in the process of gene expression from transcription and translation to post-translational stability of the globin chain. The variable clinical severity of the thalassemia syndromes depends on how significantly these different mutations affect globin synthesis. Although over ninety such mutations are known, a given mutation generally is found in one ethnic group and not another. Nearly 200 different mutations have been described in patients with β-thalassemia and related disorders. Although most are small nucleotide substitutions within the cluster, deletions may also cause β-thalassemia [30].

All the mutations result in either the absence of the synthesis of β globin chains (β0-thalassemia) or a reduction in synthesis (β+ thalassemia). Mutations in or close to the conserved promoter sequences and in the 5' untranslated region down-regulate transcription, usually resulting in mild β+ -thalassemia. Transcription is also affected by deletions in the 5' region, which completely inactivate transcription and result in β0 -thalassemia. Both splicing of the messenger RNA (mRNA) precursor and ineffective cleavage of the mRNA transcript are result in β- thalassemia. In some mutations, no normal message is produced, whereas other mutations www.intechopen.com Thalassemia Syndrome only slightly reduces the amount of normally spliced mRNA. Mutations within invariant dinucleotides at intron–exon junctions, critical to the removal of intervening sequences and the splicing of exons to produce functional mRNA, result in β0 -thalassemia. Mutations in highly conserved nucleotides flanking these sequences, or in "cryptic" splice sites, which resemble a donor or acceptor splice site, result in severe as well as mild β+-thalassemia [31]. Substitutions or small deletions affecting the conserved AATAAA sequence in the 3' untranslated region result in ineffective cleavage of the mRNA transcript and cause mild β+ thalassemia. Mutations that interfere with translation involve the initiation, elongation, or termination of globin-chain production and result in β0 thalassemia. Approximately half of all β-thalassemia mutations interfere with translation; these include frame-shift or nonsense mutations, which introduce premature termination codons and result in β0-thalassemia. A more recently identified family of mutations, usually involving exon 3, results in the production of unstable globin chains of varying lengths that, together with a relative excess of α-globin chains, precipitate in red-cell precursors and lead to ineffective erythropoiesis, even in the heterozygous state. This is the molecular basis for dominantly inherited (β+) thalassemia. In addition, missense mutations, resulting in the synthesis of unstable β-globin chains, cause β-thalassemia. β-thalassemia includes three main forms: Thalassemia Major, variably referred to as Cooley's Anemia and Mediterranean Anemia, Thalassemia Intermedia and Thalassemia Minor also called " β-thalassemia carrier", "β-thalassemia trait" or "heterozygous β-thalassemia". According to Thalassemia International Federation, only about 200,000 patients with thalassemia major are alive and registered as receiving regular treatment around the world [32]. The most common combination of β-thalassemia with abnormal Hb or structural Hb variant with thalassemic properties is β-thalassemia/Hb-E which is most prevalent in Southeast Asia where the carrier frequency is around 50%. 4.6 β-Thalassemia trait Carriers of thalassemia, individuals with this disorder are heterozygous for a mutation that affects globin synthesis. They are mildly anemic with hypochromic, microcytic RBCs. Targeting and elliptocytosis are often seen. As with thalassemia trait, one must exclude iron deficiency to make the diagnosis. In general, patients with thalassemia trait have a lower mean corpuscular volume (MCV) and a higher red cell count for the degree of anemia than seen in iron deficiency. Thus, the Mentzer index (MCV/RBC) is useful as a screening test to differentiate thalassemia from iron deficiency [33].

If the Mentzer index is < 13, thalassemia is more likely; if > 13, iron deficiency is more common. Hb electrophoresis is normal with iron deficiency, but with thalassemia trait the hemoglobin A2, (Hb A2) is often e1evated. Globin chain synthesis studies show an excess of chains. These patients need no treatment, but should receive genetic counseling regarding the potential for having a child with thalassemia major or a combination of thalassemia trait and sickle hemoglobin (S-thal). When both parents are carriers there is a 25% risk at each pregnancy of having children with homozygous thalassemia. Within the first months of life, adult hemoglobin containing 2 pairs of α and β- chain (Hb-A: $\alpha_2\beta_2$) physiologically replaces fetal hemoglobin (HbF: $\alpha_2\gamma_2$). In thalassemia, deficient of production structurally normal chain lead to anemia, largely as a consequence of ineffective hemopoiesis These thalassemia patients who clinically are between the extremes of thalassemia trait and thalassemia major have milder anemia and by definition do not require or only occasionally require transfusion. The regular transfusion therapy is not required initially. These patients usually maintain a hemoglobin level of 7 g/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. Its

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 [34].

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 [35]. 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 [36].

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 [37].

III MATERIALS AND METHODS

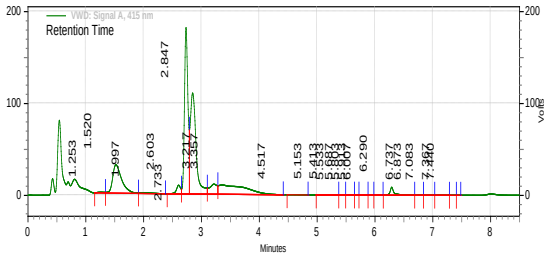
The following materials were used to perform thalassemia screening.

1. HPLC instrument- Agilent Technologies 1220 Infinity LC, Software version**:** EZchrome Elite.
2. Thalassemia prepared 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 (2 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 sample was loaded in the HPLC for the analysis.
6. Refrigerator- We have used refrigerator for the storing the samples.

The study has groups included were: A) the relatives of known thalassemia major Patients. B) Antenatal patients coming to Gynecology and Obstetrics Department of our Hospital (Rama Medical College Kanpur, India). Screening for thalassemia is compulsory for antenatal cases in our hospital; C) Thalassemia screening camps held in high risk communities in our area. A total of 400 subjects (age<30 years) with confirmed microcytic anemia were included in the study. Anemia was defined 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 did not test for serum ferritin levels since we do not have facilities to perform this assay. 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 400 samples for the thalassemia, out of 400 samples we have got 40 samples positive and remaining samples were thalassemia negative. The thalassemia screening required the blood CBC report. In the CBC report we have focused mainly at MCH, MCHC, Hb and RDW-SD and from the HPLC chromatograms we have collected the values of HbA2 and HbF. On the basis of above mentioned values we have prepared report for the thalassemia. Some reports are mention here.



(Fig. 1, HPLC chromatogram of normal person)

(HPLC Report)

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 expected ranges

Chromatogram of beta thalassemia trait is showing 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)

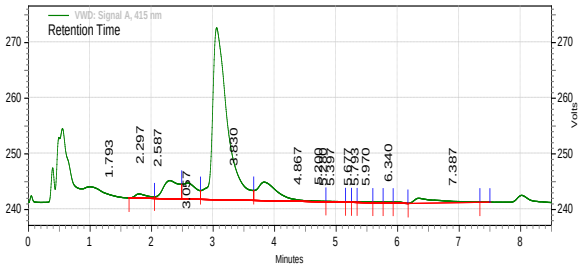
MCH: 29 pg (Normal value 27-33 pg)

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.



(Fig. 2, HPLC chromatogram of thalassemic person)

Test: Whole Blood Examination

(HPLC Report)

Detection of Beta Thalassemia Trait

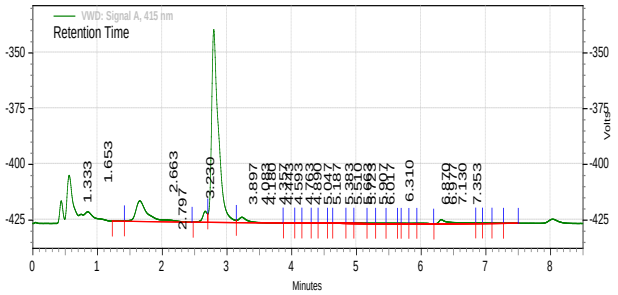
Quantification of Hb A2: 10.49% (Normal value up to 3.8%)

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)
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 [39]. 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 [40].

V CONCLUSIONS

Premarital testing should be introduced in the nation. In other Asian countries such as India, Pakistan and Chinait is already in place. If a silent carrier is suspected on the basis of borderline red cell indices and/or borderline HbA2 levels, definitive diagnosis may be obtained using characterization of the mutation by DNA analysis. Because of their silent phenotype these carriers may escape identification in a routine screening programme.

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VII REFERENCES

[1]. Hedlund B. Hemoglobins of human embryos, fetuses, and neonates. 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 hemoglobinopathies. 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 hemoglobin 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 hemoglobins 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. Recommendations 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 discriminating between thalassemic and non-thalassemic microcytosis. Am J Clin Pathol 1996; 106: 201–5.
[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

Ceme-nelum 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 determination 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]. Bisse´ E, 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 hemoglobins 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.
[31]. Vaysal E, Huisman THJ. Detection of common deletional a -thalassemia-2 determinants by PCR. Am J Hematol 1994; 46: 208-13.
[32]. Bowden DK, Vickers MA, Higgs DR. A PCR-based strategy to detect the common severe determinants of a -thalassemia. Br J Hematol 1992; 81: 104-8.
[33]. Bowie LJ, Reddy PL, Nagabhushan M, Serigny P. Detection of a -thalassemias by multiplex polymerase chain reaction. Clin Chem 1994; 40: 2260-6.
[34]. Labo RV, Saiki RK, Swanson K, Montano MA, Erlich HA, Globus MS. Prenatal diagnosis of a -thalassemia by polymerase chain reaction and oval restriction enzyme analysis. Hum Genet 1990; 85:293–9.
[35]. Lundeberg J, Wahlberg J, Uhlen M. Rapid colorimetric quantification of PCR-amplified DNA. Biotechniques 1999; 10:68-75.
[36]. Simsek M, Daar S, Ojek H, Bayoumi R. Improved diagnosis of sickle cell mutation by a robust amplification refractory polymerase chain reaction. Clin Biochem 1999; 32:677-80.
[37]. Newton CR, Graham A, Heptinstall LW, Markham AF. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res 1989; 17:2503–16.
[38]. Reddy PL, Bowie LJ. Sequence-based diagnosis of hemoglobinopathies in the clinical laboratory. Clin Lab Med 1997; 17: 85–96.
[39]. Huisman THJ. Combination of b chain abnormal hemoglobins with each other or with b-thalassemia determinants with known muta-tions: influence of phenotype. Clin Chem 1997; 43: 1850 – 6.
[40]. Krauss JS, Bures K, Kenimer E. Elution of hemoglobin a Montgomery Hb S2 hybrid tetramers by the Variant apparatus. Clin Chem 2000; 46: 139-40.
[41]. Olivieri NF. The b -thalassemias. N Engl J Med 1999; 341: 99-109.
[42]. Fairbanks VF. Thalassemias and related disorders. In: Fairbanks VF, ed. Hemoglobinopathies and thalassemias. New York: Brian C. Decker, 1980:18 –27.
[43]. Kutlar F. Diagnostic approach to hemoglobinopathies. Hemoglobin 2007; 31: 243-50.
[44]. Tyagi S, Saxena R, Choudhry VP. HPLC--how necessary is it for haemoglobinopathy diagnosis in India? Indian J Pathol Microbiol 2003; 46: 390-3.
[45]. Joutovsky A, Hadzi-Nesic J, Nardi MA. HPLC retention time as a diagnostic tool for hemoglobin variants and hemoglobinopathies: A study of 60000 samples in a clinical diagnostic laboratory. Clin Chem 2004; 50: 1736-47.
[46]. Bravo-Urquiola M, Arends A, Montilla S, Velásquez D, Garcìa G, Alvarez M, *et al.* Advantages in the use of high performance chromatography technique for screening hemoglobinopathies in Venezuela. Invest Clin 2004; 45: 309-15.
[47]. Sood SK, Madan N, Colah R, Sharma S, Apte SV. Collaborative study on Thalassemia 1993. Report of ICMR task force study. Indian Council of Medical Research.
[48]. Zeng YT, Huang SZ, Zhou LD, Huang HJ, Jiao CT, Tang ZG, *et al.* Identification of hemoglobin D Punjab by gene mapping. Hemoglobin 1986; 10: 87-90. 7. Kishore B, Khare P, Gupta RJ, Bisht S, Majumdar K. Hemoglobin E. disease in North Indian population: A report of 11 cases. Hematology 2007; 12: 343-7.
[49]. Desai DV, Dhanani H, Kapoor AK, Yeluri SV. HbQ-India in a Sindhi family: An uncommon hemoglobin variant. Lab Hematol 2004; 10: 212-4.
[50]. Kar BC, Devi S. Clinical profile of sickle cell disease in Orissa. Indian J. Pediatr 1997; 64: 73-7.
[51]. Rohe RA, Sharma V, Ranney HM. Hemoglobin D Iran alpha A2 beta 22 2-Glu leads to Gln in association with thalassemia. Blood 1973; 42: 455-62.