Chapter

An Early Diagnosis of Thalassemia: A Boon to a Healthy Society

Nitu Nigam, Prithvi Kumar Singh, Suhasini Bhatnagar, Sanjay Kumar Nigam and Anil Kumar Tripathi

Abstract

The β-thalassemia is a hereditary blood disorders, characterized by reduced or absent synthesis of the hemoglobin beta chain that cause microcytic hypochromic anemia. An early diagnosis, economical test, awareness programs and prenatal screening will be a milestone for the eradication of this genetic disorder and to reduce burden of the health sector of a country subsequently the economics. Initially, the diagnosis of β-thalassemia depends on the hematological tests with red cell indices that disclosed the microcytic hypochromic anemia. Hemoglobin analysis shows the abnormal peripheral blood smear with nucleated red blood cells, and reduced amounts of hemoglobin A (HbA). In severe anemia, the hemoglobin analysis by HPLC reveals decreased quantities of HbA and increased the level of hemoglobin F (HbF). The decrease level of MCV and MCH are also associated with β-thalassemia. There are various different molecular techniques such as ARMS PCR, allele-specific PCR, Gap PCR, denaturing gradient gel electrophoresis, reverse dot blotting, DGGE, SSCP, HRM, MLPA, sequencing technology and microarray available to identify the globin chain gene mutations. These molecular techniques can be clustered for detection by mutation types and alteration in gene sequences.

Keywords: β-thalassemia, Microcytic Hypochromic Anemia, HPLC, Mutation, Molecular techniques

1. Introduction

There are many tests available for the diagnosis of thalassemia and hemoglobinopathies. However, diagnosis of these conditions that is sufficiently accurate for most of the clinical conditions can usually be established from complete family history (pedigree analysis) and a complete clinical and hematological examination of the patient and their family members.

Generally, doctors everywhere in the world diagnose thalassemia using blood tests which include a complete blood count and special tests for hemoglobin abnormalities. Initially, the primary screening of the thalassemia depends on the complete blood count (CBC), detection of carriers is done by hematological tests with red cell indices and microcytic hypochromic with mild anemia. The high

performance liquid chromatography (HPLC) or capillary zone electrophoresis is used for the identification of qualitative and quantitative assessment of hemoglobin (Hb) components. HbA2 test is the most significant identification test for β thalassemia minor, but it can be vary by the presence of defective δ-thalassemia. In earlier days various molecular techniques e.g. amplification refractory mutation specific-polymerase chain reaction ((ARMS-PCR), GAP PCR have been used for the detection of mutations in β- and α-thalassemia, which may help in the prenatal diagnosis of thalassemia hemoglobinopathy in a limited time. Recently the evolution of Next- Generation Sequencing (NGS) has taken an important place for the diagnosis of thalassemia as a confirmatory diagnostic test. The NGS has been introduced for the characterization of both α- and β-thalassemia genes. It gives an accurate diagnosis of thalassemia, although NGS predicts much higher carrier frequencies. The molecular analysis is fundamental to foresee the severe blood transfusion-dependent thalassemia cases to the mild or no blood transfusion. The prenatal diagnosis based on DNA by amniocentesis and chorionic villus sampling (CVS) is equivalent required to detect the genetic abnormalities of the foetus as per expertise. Now a day's NIPT (Noninvasive prenatal testing) is a technique to identify the genetic abnormalities of the foetus. This testing examines the small remains of foetus DNA that are circulating in a pregnant mother's blood. An appropriate lab conclusion is urgent for describing the various types of thalassemia hemoglobinopathy with a significant association for prevention and treatment.

This chapter will explain all of these tests, the information of which will be useful for those who are working and interested in the diagnosis of thalassemia hemoglobinopathy.

2. Diagnostic strategies for thalassemia

In many diagnostic labs, the diagnostic strategies have been established for the diagnosis of thalassemia from the simple PCR to NGS for the detection of common, less common and rare thalassemia mutations [1]. Although there are lots of PCR technologies available in the laboratory but most of the diagnostics labs are using the simple and robust technique on allele- specific oligonucleotide hybridization or allele-specific priming, e.g. reverse dot-blotting or ARMS-PCR for the identification of the beta-thalassemia carrier [2]. This approach helps in identifying the common and less common mutations in 90% of cases. The rare mutations will be identifying by secondary screening. The mutation which remains unidentified after these two screenings will be then characterized by DNA sequencing [3]. DNA sequencing is a technique used to identify the specific arrangement of nucleotide bases (A, C, G, and T) in a DNA. The DNA carries the information a cell needs to collect protein and RNA molecule. DNA grouping data is imperative to researchers examining the elements of qualities. DNA sequence information is mandatory to researchers examining the functions of genes [4].

However worldwide laboratories, it has been noticed that a result of the migration of the natives with different ethnicity has led to increasing the variety of hemoglobinopathy and thalassemia mutations that need to be identified. Because of migration the genetic makeup of the population has mixed, these Hb variations are presently seen everywhere. Thus, the compound heterozygous conditions (Hb D–β thalassemia, Hb E–β thalassemia and Hb Sβ thalassemia) are seen in various places [5]. Molecular indicative research centers in such nations should have the specialized skill, equipment, and diagnostic approach to distinguish an enormous variety of mutations rapidly for prenatal diagnosis, and these labs use DNA sequencing as the principle evaluating strategy for the diagnosis of β-thalassemia point mutations.

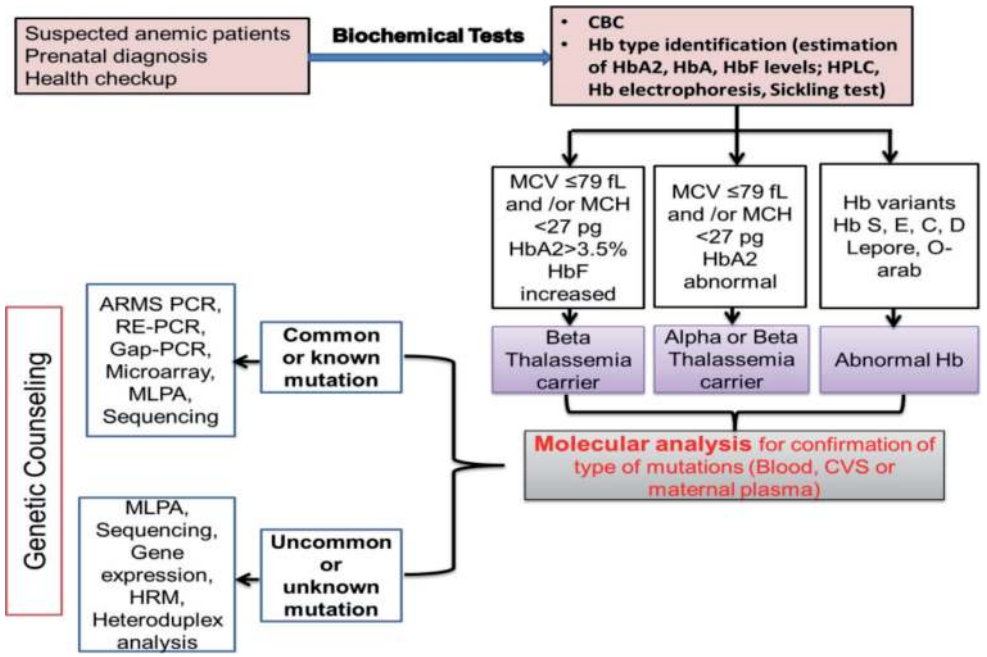

Figure 1.
Flow chart explain the diagnosis of thalassemia from primary to secondary screening.

The **flow chart** shows the step wise diagnostic model for thalassemia (**Figure 1**). The hematological and biochemical tests such as complete blood count (CBC) and Hb type identification were performed for suspected anemic patients. The altered or abnormal biochemical findings will be further confirmed by molecular analysis. The Allele-specific oligonucleotide (ASO), reverse dot-blot (RDB), ARMS-PCR, RE-PCR, Gap-PCR, MLPA and sequencing will be performed for known and unknown mutations. Finally prenatal will be done followed by genetic counseling in the affected family.

The government of the country should make various screening and diagnosis mandatory for the eradication of thalassemia. They are indicated as under mentioned [6]. The country Greece has followed this rule now Greece is a thalassemia free country.

- Premarriage screening
- Antenatal screening
- Preconception screening
- Neonatal screening
- Preoperative/pre-anesthesia screening
- Genetic counseling

2.1 Premarriage screening

Premarriage screening should be implemented to detect β thalassemia carriers and hemoglobinopathies such as sickle cell trait. In developing country, it is not frequently acceptable because of social stigma and reasons in the general public.

The premarriage screening should be possible in universities and colleges, schools, or public places such as theater, shopping mall etc. Earlier it was performed in where the predominance of thalassemia is high. But nowadays due to the mixing up of the gene pool this screening is recommended in all colleges where students are of marriageable age. Our lab has performed this screening in the Tharu tribal area of the Kheri Lakhimpur, Uttar Pradesh, India. We have collected more than 700 samples from the college and carrier screening of sickle cell disease has been performed. It is a belt of HBS [7].

2.2 Antenatal screening

In antenatal screening irrespective of gestational age of all pregnant women should be screened for carrier status of thalassemia and hemoglobinopathies. The spouse of the affected female should be test for mutation such as α-, β thalassemia and hemoglobinopathies (HbS trait, Hb E trait, Hb D trait etc.). The Prenatal diagnosis have to be encouraged if the foetus is in danger for the having thalassemia mutations e.g. α-, β thalassemia and hemoglobinopathies (HbS trait, Hb E trait, Hb D trait etc.). If the couple found positive for thalassemia during antenatal screening they can decide for prenatal diagnosis and subsequent pregnancy [8–11].

2.3 Preconception screening

While troublesome circumstances are in the developing country like India, this ought to be done but mostly females frequently do not enroll in antenatal centres before 12 weeks of gestation. A similar methodology with respect to antenatal screening ought to be followed. The preconception screening is important for all couples coming to IVF in infertility clinics. If the female is a thalassemia carrier then her husband or sperm donor should be screened and vice versa [8–11].

2.4 Neonatal screening

Infant screening is mainly suggested in haemoglobinopathies such as sickle cell diseases, prevalent in tribal and urban populations. If possible, neonatal screening has to be implemented universally where infants are at high-risk for homozygous β-thalassemia and all instances of HbS–β thalassemia. This methodology will miss a couple of instances of sickle β-thalassemia when the mother is a β-thalassemia carrier and the father is a carrier of HbS. All babies with significant hemoglobinopathy should be re-tested using molecular technology to confirm the diagnosis within three months after birth [8–11].

2.5 Preoperative/pre-anesthesia screening

Preoperative/pre-anesthesia screening of patients is preventive measure where the prevalence of HbS is high. As the presence of sickle hemoglobin might interfere during preoperative and postoperative procedures [8–11].

2.6 Genetic counseling

Genetic counseling should be given by an expert to affected thalassemia families. The advice can likewise be given by a trained genetic counselor, a hematologist, or a pediatrician [8–11].

3. Screening methods for thalassemia

3.1 Hematological screening

The primary screening of thalassemia is based on (MCV, MCH) values, Hb A2 levels, and F levels by automated blood analyzer, Hb electrophoresis and HPLC, respectively. The secondary screening encompasses further hematological studies to detect suspected variant such as sickle solubility, iron levels and heat instability tests. The **complete blood count** (CBC) measures the exact amount of hemoglobin and different types of blood cells. As it is clear that when there are mutations in the genes which are responsible for the hemoglobin synthesis that can lead to hemoglobinopathies. It can be further categorized based on of the type of mutations on the globin genes (α, β, δβ) and abnormal structural variants such as Hb Lepore and Hb E. These discrepancies in the mutations on the globin chains led to the different phenotypes of thalassemia. When there is a substantial increase of HbF in adults caused by the γ-globin chains synthesis after birth without any important clinical or hematological manifestations, this condition is known as Hereditary Persistence of Foetal Hemoglobin (HPFH) [12].

The variations from the typical hematological phenotypes of β-thalassemia carrier include:

- Decrease level of MCV and MCH with marginal or normal levels of Hb A2 when person must consider α -thalassemia, heterozygosity for mild β-thalassemia mutations, iron deficiency, heterozygosity for eγδβ-thalassemia.

- Normal/Borderline MCV and MCH levels with higher Hb A2 when person must deliberate co-inheritance of alpha and beta-thalassemia.

- Normal Hb A2 with Normal or decreased red cell indices but raised Hb F level when person should consider heterozygous δβ -thalassemia or HPFH.

- The deranged hematological and biochemical value after primary and secondary screening is confirmed by molecular analysis.

Red cell distribution width (RDW) test is an estimation of the range in the volume and size of the RBC (Red blood cell, Erythrocytes). The RBC move oxygen from the lungs to each cell in the body which helps in normal development. If the RBC's are bigger than the normal size, it could show a health problem. The RDW test is normally used to investigate anemia. It is a condition wherein the RBC cannot carry sufficient oxygen to the body. The RDW test may likewise be utilized to diagnose thalassemia [13]. This change in RDW is very remarkable in thalassemia from HbH sickness to thalassemia minor [14]. In thalassemia, RBC count is increased with microcytic anemia in compared to iron deficiency anemia (IDA) and iron deficiency where RBC count is relatively decreased. That's why in any hemoglobin disorder RBC count and RDW level should not be considered as only evaluating methods.

3.2 Biochemical screening

As per the International Committee for Standardization in Hematology (ICSH) in 1978, has recommended three kinds of lab tests for the diagnosis of thalassemia and hemoglobinopathies [15]. In that rule, the screening research facility ought to have the option to perform the alkaline electrophoresis. The reference lab approved

by ICSH needed to perform challenging tests like globin electrophoresis and citrate agar electrophoresis. It is necessary to have manual strides all through hemoglobin investigation from reagent availability, electrophoresis, and information examination, and accordingly, the experience of the research centre proficient was a key to fruitful recognizable proof. Late improvement of lab strategies and expanded information on hemoglobinopathy and thalassemia has determined the distribution of refreshed rules [16]. The British Committee for Standards in Hematology suggests possible recognizable proof of hemoglobins on at least two procedures and gets conclusive ID as that dependent on DNA examination, protein sequencing or mass spectrometry.

Electrophoresis is a strategy used to isolate atoms or mixtures dependent on their movement design in a gel and electrical field. It is more commonly utilized in diagnostics labs for protein electrophoresis and the separation of some isoenzymes. Manual planning of gel and electrophoresis is infrequently utilized in evolved nations as further developed and computerized methods, for example, narrow electrophoresis are accessible.

In early long stretches of finding cellulose acetic acid derivation electrophoresis is an agent custom electrophoresis strategy. It gives distinguishing proof of Hb A, F, S/G/D, C/E, and H and different variations [16].

High Performance fluid chromatography (HPLC) is a technique in which isolate mixtures of atoms dependent on their substance qualities. Numerous division standards like affinity, segment and size are accessible; for hemoglobin, molecule exchange chromatography is proficient and frequently utilized. The technique can be additionally physically worked, yet as of late completely mechanized frameworks are accessible. Those frameworks might be devoted to hemoglobin examination for haemoglobinopathies and thalassemia (**Figure 2**).

HPLC is useful for the finding of β-thalassemia carrier since that HbA2 can be correctly quantitated [16]. Similar other HPLC methods, a wary control of insightful conditions like segment temperature, stream rate, and backup conditions are important.

Mass spectrometry is a procedure to recognize molecules dependent on their mass (sub-atomic weight) to charge proportion. In this procedure, the molecule of the interest required minimal specific binding reagents. The simple analytical norm allows less interfering but rather more precise detection. In this technique, the examination of hemoglobin is not easy as it required specialized ability for the investigation of proteins and costly instruments. Other than detecting of hemoglobin based on the intact molecular weight, it can likewise examine the amino acids sequence. Mass spectrometry is helpful tool for the detection of new confirmation and DNA sequencing variations [17].

3.3 Advance molecular techniques for the characterization of thalassemia

There are various different molecular techniques available to identify the globin chain gene mutations. These molecular techniques can be clustered for detection by mutation types such as structural variations (Gene deletion, duplication, or triplication) and alteration in gene sequences (Insertion, substitution, or short insertion/deletions) [18, 19]. Over 90% of α-thalassemia patients are caused by gene deletion. Approximate 10% of the α-thalassemia cases are due to the alteration in the gene sequence such as single nucleotide insertion, deletion or substitution [20]. The α-globin gene group encode identical protein which consists of exceptionally homologous genes as well as 2 HBA genes. The gene deletions in α-thalassemia are mainly cause due to imbalanced crossing over between these homologous regions during meiosis [21]. The most well-known deletion of 3.7 kb and 4.2 kb has been

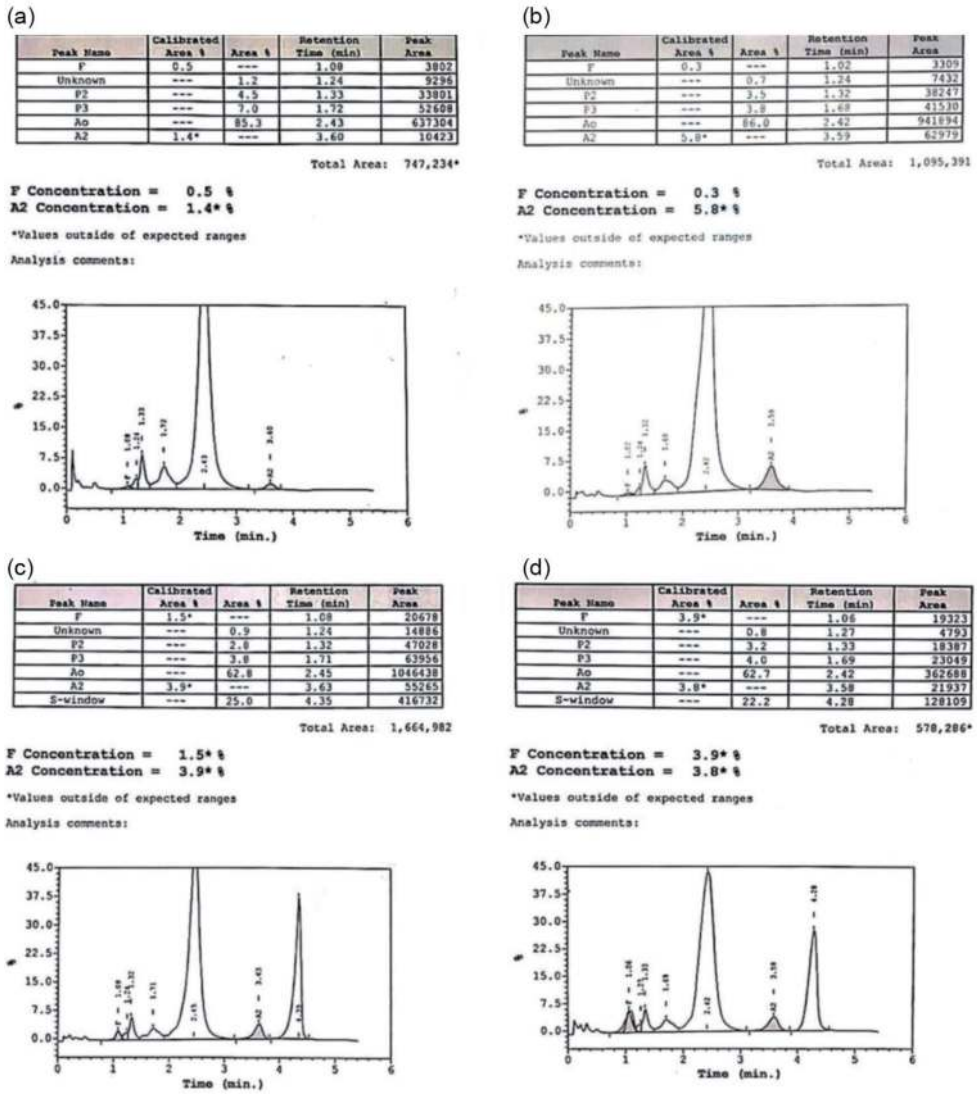

Figure 2.
HPLC of various types of haemoglobinopathies and thalassemia; (a) Normal hemoglobin (Hb); (b) β-thalassemia trait with A2 fraction on 5.8%; (c) and (d) compound heterozygous HbS and β-thalassemia trait (Nigam et al., 2020).

reported [22]. More than 90% of β-thalassemia cases are caused by the alteration in the gene sequence as compared to α-thalassemia. Approximately, 280 gene sequence alterations are related with β-thalassemia in which some mutations are caused by the deletion of gene including the HBB gene [22].

Gap polymerase chain reaction (PCR) technique is mainly used for the detection of deletion. The southern blotting may be used for unknown deletions with the help of using probes. The MLPA (multiplex ligation-dependent probe amplification) technique can identify both known and unknown deletions. It is commonly used in diagnostics labs for its highly sensitive and is easy to use. Common mutation or alteration in gene sequences can be detected by using techniques such as amplification refractory mutation specific (ARMS) PCR, allele-specific PCR, denaturing gradient gel electrophoresis, reverse dot blotting, DGGE, SSCP, HRM (High resolution melting), sequencing technology and microarray in a cost-effective manner.

Allele-specific oligonucleotide (ASO) hybridization and reverse dot-blot (RDB) techniques are used for the detection of known mutations. In this technique, the PCR products (amplified target DNA sequences) are hybridize with two

oligonucleotide probes; one complementary to mutant sequence and the other to a normal sequence. The normal probes hybridized with normal individuals [23, 24]. The ASO's vary from one another by the changes in a single nucleotide. The analysis of the change in a single nucleotide in DNA using hybridization with ASO probes that have been bound to a nylon membrane in the form of the dot after restriction endonuclease digestion and electrophoresis [23, 25–26]. The ASO probes are specific and complementary for the several alleles, to detect known mutation or single nucleotide polymorphism. This dot blot technique is used for one or two major mutations [27]. This was surprised by the development of the reverse dot-blotting technique, in which, the panel of mutation-specific probes is hybridized with amplified DNA, fixed to a nylon membrane. This method is viable with the ideal procedure for screening β-thalassemia mutations, using a panel of common known mutations for the primary screening and a panel of uncommon for the second screening [28].

Primer Specific Amplification– amplification refractory mutation specific (ARMS) PCR is the most commonly used technique for the detection of β-thalassemia mutations [29, 30]. This technique is a simple, quick screening assay; does not require the latest technology [30]. The ARMS primers have been made for the detection of common mutations of β-thalassemia [31]. In various countries like India and Pakistan, this technique quit well-known for screening and prenatal diagnosis due to its quick and low cost [32, 33]. The ARMS PCR is able to detect multiple known mutations in a single assay [29, 34, 35]. It is also identified the change in target DNA is heterozygous or homozygous. With the help of ARMS primers (common forward primer and two reverse one mutant and other to the normal primer sequence), ARMS PCR is differentiated homozygote or heterozygote.

Recently, ARMS PCR technology has been improved for the detection of both normal and altered alleles with internal positive control are detected in a single tube assay [35], referred to as tetra primer ARMS-PCR. Two pairs of primers are used in tetra, ARMS-PCR in which one pair of primers for flanking regions and other pair primers are complementary to different strands. These primers amplify the two different bases that are located in a single position of the globin gene. The different alleles (mutant and wild type) can be detected on an agarose gel based on their sizes (**Figure 3**). This ARMS PCR technique has been useful in the diagnosis of β-thalassemia mutations. Multiplex ARMS PCR can be screened for more than one mutation a single reaction by multiplexing the ARMS primers attached with a common primer [36].

Restriction enzyme PCR (RE-PCR)/restriction fragment length polymorphism (RFLP) has had a restricted diagnostic role due to few β-thalassemia mutations restriction sites. Even though its use can be enlarged by the artificial formation of a restriction that includes the target mutation [37]. The fundamental use of RE-PCR has been for the examination of β-globin gene haplotypes to define the origin of mutations in the globin gene in various ethnic groups [38].

The target genomic DNA containing the mutation is amplified by PCR with the help of specific primers. The PCR products are digested by a specific restriction enzyme. The digested PCR products are separated on the agarose gel. The digested PCR products are separated according to their molecular weight (size). Based on restriction site (presence or absence) is determine the size or pattern of PCR products. This RE analysis is simple, relatively economical, and powerful prompting unequivocal outcomes; the RE-PCR-based technique is a precious molecular diagnostic tool. Be that as it may, it is restricted in its application as just an extent of the alpha-thalassemia, β-thalassemia mutation, and hemoglobin variations, naturally generate restriction sites [26, 39].

Gap-PCR is a rapid, simple and non-radioactive technique that identified the deletions of globin gene. Gap-PCR amplifies the deleted DNA sequence in target

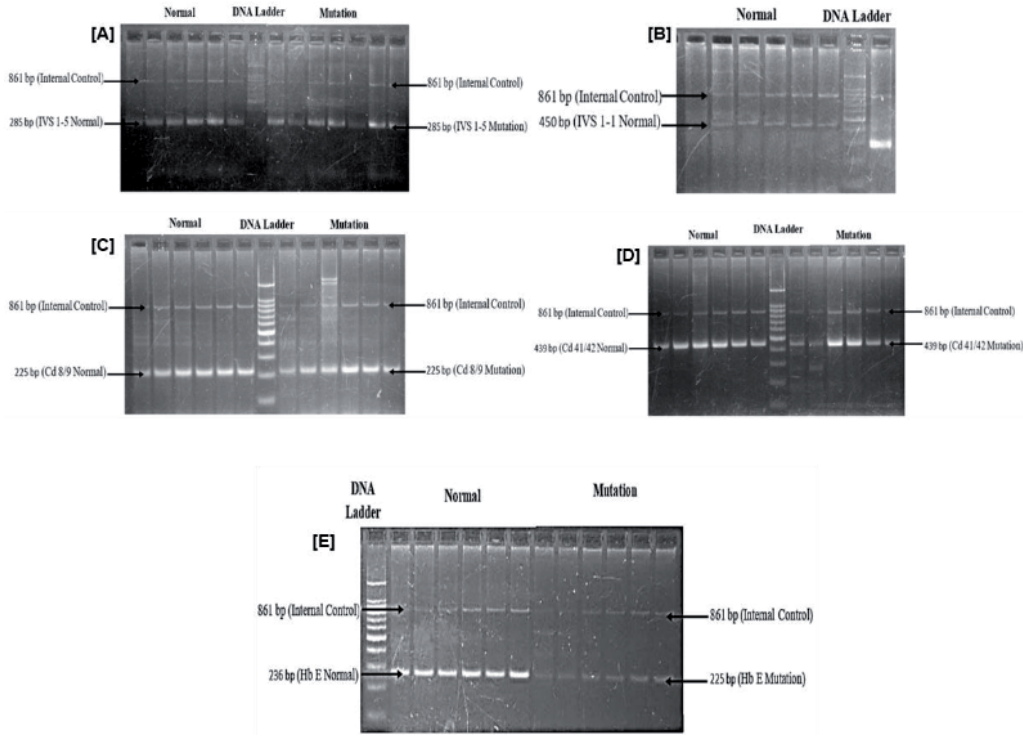

Figure 3.
Gel image shows the multiplex ARMS PCR for five common mutations: [A] IVS 1–5 mutation/normal; [B] IVS 1–5 mutation/normal; [C] Cd 8&9 mutation/normal; [D] Cd 41&42 mutation/normal; and [E] Hb E mutation/normal.

DNA by using the flanking primers for this region. This flanking primer pairs are making a unique PCR product, smaller for mutant sequence as compared to wild type sequence [40]. In alpha-thalassaemia mostly mutation are deletion types e.g. $-\alpha^{3.7}$, $-\alpha^{4.2}$ deletion, HbLepore and HPFH deletion etc. [41, 42]. In Asian Indians, 619 bp deletion is found in β-thalassaemia. The limitation of Gap-PCR technique is that the deletion endpoints must be known for primers designing.

Denaturing gradient gel electrophoresis (DGGE) and single-stranded conformation polymorphism (SSCP) are used for the screening of the unknown mutations. The DGGE technique allows the DNA fragments differing by single nucleotide base change according to its melting characteristics [43, 44]. In SSCP, single nucleotide substitutions can be identified but the efficiency is between 70 to 90%. In both techniques, the PCR products mobility on gel is altered in mutant sequence as compared to normal sequence and further confirmed by sequencing. Once the unknown mutation has been detected by DNA sequencing, these DNA can be used as a control to develop ASO and ARMS primer for its detection in further cases. The only disadvantage of these techniques is that it cannot be applied to hemoglobinopathies.

Heteroduplex analysis is another method utilizes non-denaturing gel electrophoresis. By annealing and amplified target DNA fragment with an amplified hetroduplex generator molecule, the unique heteroduplex pattern can be generated for each mutation of 130 bases in length [45].

High Resolution Melting (HRM) investigation is a moderately new, post-PCR examination technique used to recognize the changes in DNA sequences. The technique depends on distinguishing little contrasts in PCR melting (dissociation) curves. It is empowered by further developed dsDNA- binding dyes conjunction with real-time PCR instrumentation that has exact temperature ramp control and

advanced data capture capabilities. Data are analyzed and manipulated using software designed specifically for HRM analysis. The HRM is an analytical platform for rapid prenatal and postnatal diagnosis of β-thalassemia common among Southeast Asian population. The advantage of HRM is a simple, economical with fast workflow platform for the diagnosis of single gene disorder [46, 47].

Multiplex Ligation-Dependent Probe Amplification (MLPA) is versatile useful technique for the diagnosis of copy number variations (CNVs) from complete chromosomes to single exons associated with genetic disorders and tumors. To detect DNA methylation changes can be detected by Methylation-specific MLPA (MS-MLPA) which is sensitive enough to distinguish the changes in disease causing genes from highly similar pseudogenes [48].

The multiplex PCR technique that amplify up to 60 probes by using one pair of primer. The PCR amplicon with novel genomic target and length are fluorescently labeled and identified by capillary electrophoresis. The number of genomic sequence of interest is determined by comparing the peak pattern with reference sample [49].

Since, the varied molecular basis of α-thalassemia and β- thalassemia mutation are uncommon and difficult to detect. In addition to well-established methods, MLPA is known as an effective, simple and unambiguous technique for the identification and classification of deletions and duplications in thalassemia [48–50].

Direct DNA sequencing is a technique used to identify the specific arrangement of nucleotide bases (A, C, G, and T) in a DNA. The DNA carries the information a cell needs to collect protein and RNA atoms. DNA grouping data is imperative to researchers examining the elements of qualities. DNA sequence information is mandatory to researchers examining the functions of genes [51]. In beta globin gene, most of mutations can be identified in two sequence reads but for alpha-globin only single read required. The advantage of the simple sequencing method for beta globin gene play in important role in prenatal diagnosis where the mother is HBS carrier and father is unavailable [52]. If the developing embryo found HBS mutation then further analysis of beta globin sequences is must to assure that foetus does not have compound heterozygous state HBS and coexisting beta-thalassaemia [53].

This is a significant result for the haemoglobinopathies as most cases include carrier testing, subsequently arrangement follows much of the time require checking by eye. There are two diverse sequencing sciences accessible dependent on the Sanger technique [54–56], dye primer and dye eliminator; they differ from each other in the way wherein the fluorescent level is incorporated during linear cycling. Despite the fact that the dye eliminator is more straightforward to set-up the signal from each nucleotide is less dependable making the dye primer chemistry more suitable for heterozygote detection and it is friendlier with sequence analysis programming. The dye eliminator would have more application in X- linked diseases, for instance, G6PD transformations in which influenced males are hemizygous and will show up as a homozygous change. Taking everything together, the way toward getting a succession from whole blood and the examination may require 4–5 days, with certification using another PCR based test before the change is accounted for. The only drawbacks of using sequencing as a routine investigation technique are the cost and time taking examination compared to PCR. Sequencing is a multistage procedure requiring PCR intensification, cycle sequencing and precipitation before the sequence can be distinguished. After this the sequence ought to be researched and checked and any movements noted. Notwithstanding the grouping examination programming is available it is not 100% compelling at identifying heterozygotes.

Next-generation sequencing (NGS) allow the generation of immense measures of genomic data to uncover the genetic constitution of people and to evaluate potential health risks. NGS has been commonly utilized for non-invasive prenatal diagnosis and novel mutation detection in thalassemia [57, 58].

Microarray analysis procedures are utilized in deciphering the information produced from probes DNA (Gene chip examination), RNA, and protein microarrays, which permit scientist to explore the expression of large number of gene in many cases, an organism's whole genome in a single step experiments. The complementary sequences will bind to each other. The unknown DNA's are cut into pieces by restriction endonucleases and these DNA pieces are label with fluorescent markers. These are then allowed to react with tests of the DNA chip [59–61].

Microarray analysis of gene expression has formed into a great tool for the characterization of various pathophysiological processes. The fundamental idea is that RNA isolated from tissue is hybridized to probes for specific genes that are fixed in a grid in small microscopic spots. The microarray is a quick, simple to perform, and precise strategy for concurrent identification of α and β-thalassemias. But, this technique needs should be improved and approved in a bigger number of specimens with hemoglobinopathies before further routine laboratory use [62–64].

3.4 Prenatal screening

The prenatal diagnosis based on DNA by amniocentesis and CVS sampling is required to detect the genetic abnormalities of the foetus. Now a day's NIPT is a technique to identify the genetic abnormalities of the foetus. This testing examines the small remains of foetus DNA that are circulating in a pregnant mother's blood. An appropriate lab conclusion is urgent for describing the various types of thalassemia with a significant association for prevention and treatment. Because of population migration and mixing of the gene pool of different populations in many immigration countries as well as regions the hemoglobiopathies and thalassemia are more prevalent over their [65–68].

The importance of prenatal diagnosis comes in the diagnostic field as it helps and early diagnoses the growing foetus in the mother wombs for thalassemia and hemoglobinopathies. It plays impartment role in the eradication of these genetic disorders such as β-thalassemia major, sickle cell disease and hemoglobin Bart's nonimmunehydropsfetalis [69, 70]. The prenatal diagnosis includes the investigation of fetal material from chorionic villi, amniotic liquid, string blood, and fetal DNA in maternal dissemination. In spite of the way that examination of fetal hemoglobin types is successfully performed by means of robotized HPLC, it is assessable through assessment of fetal blood got by cordocentesis and the technique is inclined to mistake because of mixing of sample by maternal tissue [71].

Advances in molecular testing have worked with the assurance of complex thalassemias and hemoglobinopathies saw in ethnically varying population. Comprehensive screening programs highlighted recognizing carriers and offering prenatal diagnosis in pregnancies for thalassemia have been incorporated in Canada and European countries [72, 73]. Different procedures have been implimented to distinguish thalassemia like genotyping assay, genotyping measur next-generation sequencing and mass spectrometry [74–76]. The methods are as yet testing; consequently, more investigations are expected to create and approve them and eventually lead to proficient, exact and concrete non-invasive prenatal diagnosis of thalassemia and hemoglobinopathies [77].

4. Cost comparison for testing

	Low cost techniques	High cost techniques
Hematological and Biochemical Techniques	MCV and MCH	Mass spectrometry
	CBC	
	RDW	
	Electrophoresis	
	HPLC	
Molecular Techniques	ASO, RDB,	MLPA
	ARMS PCR	Direct sequence
	RE PCR/RFLP	Microarray
	Gap-PCR	HRM
	DGGE and SSCP	

5. Point of care (POC) testing for thalassemia

As there is a saying that prevention is better than cure. The thalassemia and hemoglobinopathies are genetic disorder and due to the migration of population in the different regions and endogamy the new combinations of thalassemia hemoglobinopaty are arising fast. For the eradication and further treatment and management of thalassemia disease an effective diagnostic test at the point of care (POC) is the need of hour.

As thalassemia has been spread worldwide an early diagnosis, economical test, awareness programmes and prenatal screening will be a milestone for the eradication of this genetic disorder and to reduce burden of the health sector of a country subsequently the economics.

The initial hematological, biochemical screening to the advance molecular testing under one roof will not only help to diagnose the thalassemia patients but shall be also helpful in the treatment and management of the disease (**Figure 1**). The objective of POC for testing thalassemia will be achieved if these quick testing methods like NESTROFT. This has to be in the approachable distance to the patients.

The government should include the thalassemia screening as a mandatory tool for screening. The best example Italy and Cyprus there with the initiation of prenatal diagnosis and screening of the carrier now this is a thalassemia free country.

Likewise in India, the Uttar Pradesh state government has initiated the task by giving free of cost of screening of thalassemia carrier, blood transfusion and iron chelators to several medical colleges. The Indian government has provided the HPLC machines to provide the screening of the carrier of thalassemia hemoglobinopathies. Basically the eradication, treatment and management of thalassemia are joint efforts of government, stake holder, policy makers, pediatrician, pathologist, transfusion medicine and geneticist.

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