Review Article

Application of diagnostic methods and molecular diagnosis of hemoglobin disorders in Khuzestan province of Iran

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BACKGROUND: The hemoglobinopathies refer to a diverse group of inherited disorders characterized by a reduced synthesis of one or more globin chains (thalassemias) or the synthesis of structurally abnormal hemoglobin (Hb). The thalassemias often coexist with a variety of structural Hb variants giving rise to complex genotypes and an extremely wide spectrum of clinical and hematological phenotypes. Hematological and biochemical investigations and family studies provide essential clues to the different interactions and are fundamental to DNA diagnostics of the Hb disorders. Although DNA diagnostics have made a major impact on our understanding and detection of the hemoglobinopathies, DNA mutation testing should never be considered a shortcut or the test of first choice in the workup of a hemoglobinopathy.
MATERIALS AND METHODS: A careful three-tier approach involving: (1) Full blood count (2) Special hematological tests, followed by (3) DNA mutation analysis, provides the most effective way in which to detect primary gene mutations as well as gene-gene interactions that can influence the overall phenotype. With the exception of a few rare deletions and rearrangements, the molecular lesions causing hemoglobinopathies are all identifiable by PCR-based techniques. Furthermore, each at-risk ethnic group has its own combination of common Hb variants and thalassemia mutations. In Iran, there are many different forms of α and β thalassemia. Increasingly, different Hb variants are being detected and their effects per se or in combination with the thalassemias, provide additional diagnostic challenges.
RESULTS: We did step-by-step diagnosis workup in 800 patients with hemoglobinopathies who referred to Research center of Thalassemia and Hemoglobinopathies in Shafa Hospital of Ahwaz Joundishapour University of medical sciences, respectively. We detected 173 patients as iron deficiency anemia (IDA) and 627 individuals as thalassemic patients by use of different indices. We have successfully detected 75% (472/627) of the β-thalassemia mutations by using amplification refractory mutation system (ARMS) technique and 19% (130/627) of the β-thalassemia mutations by using Gap-PCR technique and 6% (25/627) as Hb variants by Hb electrophoresis technique. We did prenatal diagnosis (PND) for 176 couples which had background of thalassemia in first pregnancy. Result of PND diagnosis in the first trimester was 35% (62/176) affected fetus with β-thalassemia major and sickle cell disease that led to termination of the pregnancy.
CONCLUSION: Almost all hemoglobinopathies can be detected with the current PCR-based assays with the exception of a few rare deletions. However, the molecular diagnostic service is still under development to try and meet the demands of the population it serves. In the short term, the current generation of instruments such as the capillary electrophoresis systems, has greatly simplified DNA sequence analysis.

Key words: α and β- thalassemia; hemoglobin variants; hemoglobinopathies, iron deficiency anemia, polymerase chain reaction, prenatal diagnosis.

Iran, a country 1,648,000 km^2 wide, has a large number of thalassemia major patients like many other countries in the region.[1] β-thalassemia is very rare in Iran. The gene frequency of β-thalassemia, however, is high and varies considerably from area to area, having its highest rate of more than 10% around the Caspian Sea and Persian Gulf. The prevalence of the disorder in other areas is between 4-8%. In Isfahan, a city built around the river Zayandeh-Rood in the central part of Iran, the frequency rises again to about 8%. In the Fars Province, in southern Iran, the gene frequency is also high and reaches 8-10%.[1] Hemoglobinopathies are inherited disorders of globin, the protein component of hemoglobin (Hb). Thalassemias are the commonest monogenic diseases worldwide. They are, however, heterogeneous at the molecular level. More than 23 different molecular defects have been identified for β-thalassemia to date. The case of β-thalassemia, with over

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150 various known mutations, is even more perplexing.[2] Each population-at-risk, however, has its own spectrum of common mutations, usually from five to ten; a finding that simplifies mutation analysis and thus, determines the origin of the mutant genes. Thalassemia is found in some 60 countries with the highest prevalence in the Mediterranean region, parts of North and West Africa, the Middle East, the Indian subcontinent, southern Far East and southeastern Asia, together composing the so-called thalassemia belt. In western countries, thalassemia affects mostly individuals whose ancestry is traceable to high-prevalence areas.[3-6] As an example, there are around 1,000 cases of β-thalassemia major in the United States, most of whom are descendants of Mediterranean, Asian Indian, South Asian or Chinese ancestors.[3] This figure is even less than half of the number of β-thalassemic patients in Fars Province, a region only 120,000 km^2 large in southern Iran.[7] Mutations in genes' coding for the globin proteins that alter protein output produce the thalassemia syndromes. Mutations in the globin genes that lead to abnormal proteins are called variant Hbs.[8] Hemoglobinopathies are the commonest genetic defect worldwide with an estimated 269 million carriers.[9] Certain populations are particularly at risk of having a hemoglobinopathy, for example, in South East Asia, there are 90 million carriers, about 85 million in sub-Saharan Africa and 48 million in the West Pacific region.[9] The thalassemia syndromes and some of the Hb variants are inherited as autosomal recessive conditions. Very rarely, β-thalassemia demonstrates an autosomal dominant inheritance pattern. Some Hb variants also have an autosomal dominant inheritance pattern, while others can occur spontaneously. There are many genes' coding for the globins. They are found on Chromosome 11 (β globin cluster) or Chromosome 16 (α globin cluster) [Figure 1]. For the purpose of this review, the important globin genes in humans are the two β globin genes and the four α globin genes. Despite the different numbers of α and β globin genes, the net α/β globin protein output from these genes is balanced to give a ratio of 1.[8]

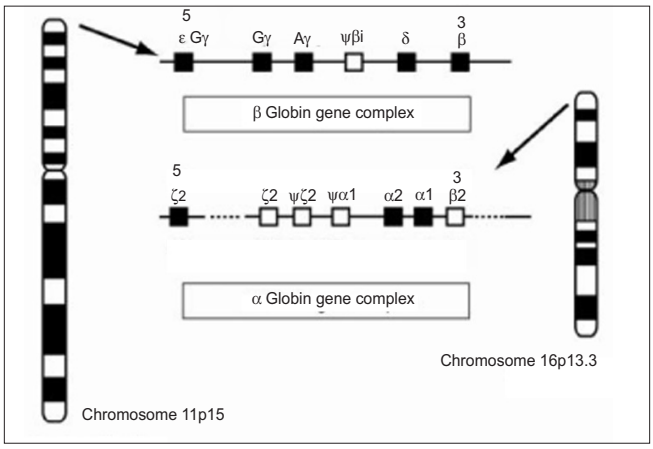

Figure 1: The ancestral globin gene duplicated into α and β globin genes about 500 million years ago.[10] Subsequently, the α and β globin gene clusters have evolved by various duplication events and are now represented by two clusters

Materials and Methods

Family studies

In view of the molecular heterogeneity found in the hemoglobinopathies, it is important to characterize each particular underlying abnormality so that appropriate advice can be given about the health implications to individuals, offspring and other family members. Since the hemoglobinopathies are usually inherited as autosomal recessive traits, there is a one in four (25%) risk of inheriting a potentially severe disorder in children of parents who are carriers of hemoglobinopathies. The severity of the hemoglobinopathy would depend on the type of disorder inherited by the children as well as a number of other factors.[8] These include genetic factors such as the ability to produce excessive HbF and co-inheritance of α-thalassemia (which would make β-thalassemia less severe) or co-inheritance of triplicate α gene (making β-thalassemia more severe). Non-genetic factors that can influence the hemoglobinopathy phenotype include access to and compliance with treatments such as blood transfusion and iron chelation.

Diagnostic workup

The detection and characterization of a hemoglobinopathy involves a three-tier workup: (1) Full blood count (2) Special hematological tests and (3) DNA testing.

Full blood count

The key to successful detection and characterization of the hemoglobinopathies, particularly the thalassemias, is the initial hematological data. The clue for a thalassemia comes with a low mean corpuscular volume (MCV) or mean corpuscular hemoglobin (MCH). Although iron

deficiency is the other explanation for a low MCV or MCH, it is likely that this finding will point to thalassemia in regions of countries with at-risk ethnic populations. The first step after the initial abnormal blood count as described above, is to exclude iron deficiency and if present, to treat it. The blood count is then repeated and if the MCV/MCH remains low, a thalassemia is most likely. We studied 800 patients with hemoglobinopathies referred to the Shafa hospital, Research Center of Thalassemia and Hemoglobinopathies of Ahwaz Joundishapour University of medical sciences. Therefore, ferritin levels (and if necessary serum iron, iron binding capacity and percentage saturation) are sought. This is recommended because at times, particularly during pregnancy, it is possible that iron stores will be low or, in the presence of iron deficiency, it is possible that the MCV or MCH are influenced by the iron deficiency. It is also occasionally seen that the HbA_2 level can be falsely lowered by iron deficiency. We attempt to differentiate β-thalassemia from IDA with the help of discriminate indices including Mentzer Index,[11] England and Fraser Index,[12] Srivastava Index,[13] Green and King Index,[14] Shine and Lal Index,[15] red blood cell (RBC) count, red blood cell distribution width index (RDWI),[16] Mean Density of Hemoglobin per Liter of blood (MDHL) and mean cell hemoglobin density (MCHD).[17] If iron deficiency is present, it is essential to correct this and then repeat the full blood count and all other investigations. We detected 173 patients as IDA and the rest as thalassemia disorders.

Special hematological tests

Once a hemoglobinopathy is suspected, the next tier of investigation requires a number of special hematological tests. These are often described as a "thalassemia screening". The tests are listed in Table 1. We screened all patients by those tests and compared all data in order to classify them. The results of the screening are listed in Table 2. It is appropriate to order all tests simultaneously since they take time to perform and often information can be obtained from tests that might not have been seen to be relevant when first starting off the investigation of a hemoglobinopathy. Some of the tests are technically demanding and so the person ordering the tests should have some knowledge of the laboratory's technical skills as well as experience in interpreting results. Other special hematological tests are possible, particularly when investigating the more uncommon variant Hbs. These include tests for oxygen affinity, hemoglobin stability and detection of methaemoglobin. Mass spectrometry has been used to characterize various variant Hbs.[18] The latter approach might be very valuable for population screening, but for detection involving individual cases, DNA-based approaches remain the methods of choice. We compared values of HbA, HbA_2, HbF and Hb variants to confirm our finding, respectively. We found that all of the 627 patients (130 patients suspected to have α-thalassemia and 472 patients suspected to have β-thalassemia) were having thalassemia. Also, we detected 23 individuals who had HbS, HbD^{panjub} and two cases were suspected to have Hb Lepore [Table 2]. Later on the entire β globin gene was sequenced using direct mutation analysis by nucleotide base sequencing to confirm Hb Lepore status. Finally, we found that for both cases the presences of Hb Lepore was ruled out.

Sources of DNA

The main source of DNA is peripheral leucocytes obtained from blood anticoagulated, preferably with ethylene diamine tetra acetic acid (EDTA). Fetal DNA is mainly isolated from chorionic villi obtained through

Table 1: Hematological data of study groups

	IDA				Beta- TT			
	< 10 Years		> 10 Years		> 10 Years		> 10 Years	
Hermatological data	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**
Hb (8r/dL)	8.7-11.4	10.12 (0.72)	8.7-12.9	10.25 (1.30	9.1-12.25	10.45 (0.92)	8.7-15.4	11.6 (1.42)
RBC	3.7-5.49	4.45 (0.56)	3.57-5.94	4.48 (0.97)	4.3-6.95	5.66 (0.58)	4.38-7.71	(5.94 (0.7)
MCV (fl)	S1-82	69.7 (7.36)	57.1-84.8	71.64 *8.56)	50-75	58.25 (5.26)	54-80	61.41 (5.21)
MCH (pg)	14.7-26.7	22.12 (3.31)	16.27.6	22.16 (3.47)	16.4-24	18.59 (1.82)	15-26	19.45 (2.00)
MCHC (gr/dL)	22-35.1	31.14 (3.46)	24-40.4	30.23 (9.19)	27-39.4	32.6 (2.9)	24.1-47.3	36.12 (4.370
RDW (%)	12.5-24.2	14.20 (4.21)	11.4-30.2	15.60 (6.11)	105.-21.1	11.79 (6.75)	10.1-25.1	15.12 (2.37)

Beta-TT - Beta thalasemia trait, Hb - Hemoglobin, IDA - Iron deficiency anemia, MCH - Mean corpuscular hemoglobin, MCHC - Mean corpuscular hemoglobin concentration, MCV - Mean corpuscular volume, RBC - Red blood cells, RDW - Red blood cell distribution, SL - Resum iton, SIBC - Serum iron binding capacity, TS - Transferrin saturation.

Table 2: Special hematology tests requested once a hemoglobinopathy is suspected based on family history and/or full blood count. Often these tests are ordered by asking for a "thalassemia or hemoglobinopathy screen". There are other reasons why the HbA_2 might be elevated. These are very uncommon and include: some unstable Hbs, hyperthyroidism and megaloblastic anemia. However, in most cases it should be possible to distinguish a thalassemia from the above. Ψ. See reference[19] for more information on the various techniques listed in this table.

Test	What does it measure or detect Y	What does it mean
HbEPG	Electrophoresis of globin proteins. Different techniques possible from gel or membrane-based kits to HPLC. Abnormal bands apart from the usual HbA, HbF and HbA_2 peaks can be detected	(1) Gives some idea of the HbA_2 level but more importantly (2) identifies if there are any variant Hbs - particularly Hbs such as HbE and HbS.
HbA_2	Globin electrophoresis and quantization of the HbA_2 peak. Different techniques used from membrane or column-based kits to the more universally suited HPLC are in use.	A raised HbA_2 is the key parameter indicating the presence of β-thalassemia. It is said that variant Hbs can raise the HbA_2 but this must be a rare event. More of an issue is the borderline normal-raised HbA_2 because this might indicate silent β-thalassemia. A low HbA_2 is also important to note as this might indicate δ thalassemia
HbF	Globin electrophoresis and quantization with different methods available for the latter.	A slightly raised HbF to 2-3% (normal is <1% in an adult) might indicate heterocellular HPFH or may be a subtle pointer to an underlying silent β-thalassemia. HbF levels 5% and above are more likely to be due to δβ thalassemia or HPFH (heterocellular or pancellular). In the case of δβ thalassemia or deletional HPFH one would expect the HbA_2 level to be low.
Kleihauer	Red blood cells are stained to detect HbF. This test is used to distinguish heterocellular from pancellular HPFH.	Not a particularly useful test for distinguishing the types of HPFH because these are very rare and most laboratories are not sure how to interpret the results. The only practical value for a Kleihauer stain might be in fetal blood sampling to confirm that maternal blood has not contaminated a fetal sample (the latter would be homogeneously stained for HbF). Any cells not staining for HbF would represent maternal blood.
HbH inclusions	Red blood cells are stained to detect HbH inclusions (aggregates of β globin protein)	Requires patience and skill to find the HbH inclusions and even with a 2-gene deletion α thalassemia, only 1-2 such inclusions might be found after a search lasting many minutes. There fore, HbH inclusions are easy to miss if the labora tory is inexperienced or the individual looking down the microscope does not spend enough time searching for these inclusions.
Sickle solubility and instability tests	Various tests ranging from biochemical to immunoassay are used to detect HbS and unstable variant Hbs	HbS diseases as well as interactions of HbS with β-thalassemia are increasingly being detected in many Iranian cities. Therefore, efficient and accurate tests for sickling (sickle solubility, HbEPG) are important components of the hemoglobinopathy workup.

ultrasound-guided transcervical aspiration or ultrasound-guided transabdominal aspiration. Fetal DNA can also be prepared from amniotic fluid cells directly or after culture. It is prudent to set aside a few milliliters for culture as a back-up as the DNA yield from amniotic fluid cells is often minimal but sufficient for PCR-based analysis. Noninvasive methods of prenatal diagnosis utilize DNA from fetal cells in maternal circulation[20] or free circulating fetal DNA in maternal blood.[21] The noninvasive methods, however, are still under development and are not offered routinely for the hemoglobinopathies.

DNA Tests

The third diagnostic tier involves DNA testing. This is requested in two circumstances: (1) a hemoglobinopathy cannot be confirmed by the special hematological tests. At times it is possible to suspect a hemoglobinopathy but the hematology tests (as well as the family studies) cannot determine which gene is likely to be involved. (2) The underlying mutation is being sought in a confirmed hemoglobinopathy. This is usually required as part of a prenatal workup. A detailed description of the various

approaches used to detect DNA mutations is beyond the scope of this review. Readers interested in more technical aspects of DNA mutation testing should read the monograph edited by Cotton, Edkins and Forrest.[22] In general, α-thalassemias are caused by gene deletions although increasingly non-deletional forms of β-thalassemia are being sought in difficult cases. In contrast, the β-thalassemias and Hb variants are, in most cases, the result of point mutations. Thus, DNA mutation strategies need to be developed with some knowledge of the likely underlying defect that will need to be detected.

Overview of techniques and methodology

Almost all the methods for DNA analysis of the hemoglobinopathies used today are based on the polymerase chain reaction (PCR).[23] Therefore whether a mutation is a deletion, a rearrangement or a point mutation, a similar test will be performed with the variability and specificity coming from the primers used. The sensitivity and specificity of PCR has revolutionized the molecular diagnostic field. It has almost eliminated the use of radioactive isotopes for detecting sequences and has enabled diagnosis to be made on much smaller quantities of DNA. The PCR-based techniques used in hemoglobin diagnostics include allele-specific oligonucleotide (ASO) hybridization or dot-blot analysis, reverse dot-blot analysis, allele-specific priming or amplification refractory mutation system (ARMS), restriction enzyme analysis, amplification created restriction analysis, mutagenically separated PCR and gap-PCR. These PCR-based techniques are useful for identifying a known mutation; PCR-based approaches for scanning or screening for unknown mutations take advantage of altered conformation of single-stranded DNA and include denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP) and heteroduplex analysis. The characteristic altered patterns of migration and their position in the scanning methods act as a guide for the location of the mutation, targeting the region for identification by other means. The ultimate method of mutation identification is by direct sequence analysis of specifically amplified DNA. In the last decade, the use of automated sequencers has led to nonradioactive, more robust and more rapid sequencing, making it suitable as a routine diagnostic tool. Direct sequencing analysis is particularly applicable to the globin genes which are compact and relatively small (1.2-1.6kb) with the majority of the point mutations within the gene or its flanking sequences. Southern blotting[24,25] is probably one of the few non-PCR based molecular techniques that still has a significant role to play in the molecular diagnosis of the hemoglobinopathies. It is very useful in the screening for large deletions or rearrangements and is essential in the characterization of novel deletions. The more common deletions can be detected by gap-PCR once the deletion break points have been defined and specific primers flanking the deletion designed. Each of these techniques has its own limitations; the particular repertoire chosen by a laboratory for molecular diagnosis of the hemoglobinopathies depends on the spectrum of mutations encountered in their catchment area and the technical expertise available in the diagnostic laboratory. It is good practice for any DNA diagnostic laboratory to have at least two alternative methods for detecting each mutation.

Direct sequence analysis

For the β globin gene, which is relatively small (covering 1.6 kb of genomic DNA including 5' and 3' untranslated region), the majority of mutations can be detected in two sequence reads, whereas for α globin, each gene (α1 or α2) can be sequenced after specific amplification in a single read. This allows detection and identification of the mutations in a single procedure, as a consequence of which screening techniques such as SSCP and DGGE are not as cost-effective for hemoglobinopathy mutation analysis. The ease with which the β globin gene can be sequenced is taken advantage of in prenatal diagnosis cases where the mother has sickle trait and the father is unavailable for testing. If the fetus is also found to carry the sickle mutation then the fetal β globin gene is sequenced to be sure that the fetus does not have SCD or coexisting β-thalassemia because of a compound heterozygous state. The only pitfalls of using sequencing as a routine diagnostic technique are the cost and the time of analysis, which is increased compared with standard PCR assays. Sequencing is a multistage procedure requiring PCR amplification, cycle sequencing and precipitation before the sequence can be determined. After this the sequence must be analyzed and checked and any changes noted.

Although sequence analysis software is available it is not 100% efficient at detecting heterozygotes. This is an important consequence for the hemoglobinopathies as most cases involve carrier testing, therefore sequence traces frequently require checking by eye.

Allele-specific priming - ARMS-PCR

Primer-specific amplification is based on the principle that a perfectly matched primer is much more efficient at annealing and directing primer extension than a mismatched primer. The most widely used method is the ARMS,[26,27] in which allele-specific amplification relies on the specificity of the 3' terminal nucleotide. To enhance allele specificity, it is common practice to deliberately incorporate a second mismatch at position -2 or -3 from the 3' end. The target DNA is amplified in two separate reactions using a common forward primer and either one of two reverse allele-specific primers, one complementary to the mutant sequence, the other to the normal DNA sequence. Presence of the mutant allele will generate a PCR product in the tube containing the mutation-specific primer and vice versa. To monitor false-negative results because of failure of the amplification reaction itself, an internal PCR control which amplifies another region of the genome should be included in the reaction. The ARMS-PCR has the advantage that it is theoretically possible to detect any known mutation.[26] Recently, the methodology has been improved by development of a single tube assay,[28] where both the mutant and the wild type alleles are detected simultaneously in the same reaction with an internal positive control, referred to as tetra primer ARMS-PCR. We analyzed all 472 patients who were suspected of having β-thalassemia by direct mutation analysis of β-globin gene by ARMS and found the type of mutation of 385 of them, which are listed in Table 3. We couldn't detect the mutation of the remaining 87 patients by this technique, which means the technique helped to detect approximately 75% (472/627) of the mutations. The most predominant mutation that we have found was IVS II - 1 (G → C) (20%) followed by other many less frequent known mutations for the Southern part of Iran, listed in Table 4.

Gap-PCR

The PCR primer pairs are designed to flank a known

Table 3: Hb electrophoresis data of 23 out of 513 β-thalassemic patients showing Hb variant.

Case	HbA (%)	HbA2 (%)	HbF (%)	Hb D ^{panjub} (%)	Hb S (%)	Hb C
1	74.6	2.3	1.4	21.7	-	-
2	74.4	2.3	0.9	-	22.4	-
3	62.7	2.6	0.2	-	34.5	-
4	73.7	3.2	0.0	-	23.1	-
5	95.9	1.7	0.5	-	-	-
6	57.4	2.9	0.3	-	-	-
7	62.7	4.2	0.5	-	32.6	-
9	67.5	3.5	0.8	-	28.2	-
10	44.8	0.5	2.9	-	51.8	-
11	69.1	1.5	1.8	37.6	-	-
12	57.5	2.4	0.6	-	39.5	-
13	59.1	1.7	0.1	-	39.1	-
14	61.1	0.3	0.0	38.6	-	-
15	53.1	1.9	0.1	44.9	-	-
16	67.6	3	1.4	-	28	-
17	66.5	2.5	1.0	-	30	-
18	57.8	3.1	0.4	39.9	-	-
19	56	1.6	0.7	41.7	-	-
20	54.1	2.5	0.7	-	42.7	-
21	60.9	1.9	0.6	-	35.6	-
22	55.4	0.9	0.4	-	43.3	-
23	58	1.3	0.7	40	-	-
24	58.4	2	0.2	-	39.4	-
25	53	2	1	-	-	44

Table 4: Frequencies of α-thalassemia mutations in study group

Mutation	Frequency (%)	Number of patients	Phenotype
IVS II - 1 (G → C)	20	96	β⁰
CD 36/37 (-T)	13.5	63	β⁰
IVS I -110 (G → A)	9.5	45	β⁺
CD 6 (HbS)	7.5	36	β^S
CD 8/9(+G)	4.5	22	β⁰
IVS I-6(T → C)	4	21	β⁺
CD 8(-AA)	3	16	β⁰
CD 44(-C)	2.5	14	β⁰
IVS II-745 (C → G)	2.4	13	β⁺
CD 5 (-CT)	2.3	12	β⁰
IVS I-5 (G → C)	2.1	11	β⁰
CD 39 (C → T)	2.1	11	β⁰
IVS I-(3' end)-25bp	1.5	9	β⁰
IVS I-1 (G → A)	1.3	7	β⁰
CD 22 (GAA → TAA)	1	5	β⁰
IVS I - 2 (T → C)	0.8	4	β⁰
-88 (C → A)	0.6	3	β⁺
-30 (T → A)	0.4	2	β⁺
IVS I - 130	0.4	2	β⁰
IVS I - 116	0.4	2	β⁰
IVS II-2,3 (+11,-2)	0.2	1	β⁺
Unknown	19.8	87	-
Total	100	472	-

deletion generating a unique amplicon that will be smaller in the mutant sequence compared with the wild type.[29] For small deletions such as the 619bp deletion, a common cause of β-thalassemia in Asian Indians, differential amplification products are generated in the mutant and wild type. When the deletion is vast (>2 kb) it is technically difficult to generate a product in the wild type. As a control, a primer can be included that anneals within the deleted sequence to generate an additional product with one of the other primers flanking the deletion. In this way, false-negatives are monitored in the wild type and it indicates if an individual is heterozygous for the deletion. This principle has been used in the technique of gap-PCR for the common HPFH and δβ-thalassemias.[30] Unlike the β-thalassemias, deletions are a common cause of α-thalassemia. The common α-thalassemia deletions and rearrangements can be routinely detected using gap-PCR.[31-33] Gap-PCR is also routinely used to detect Hb Lepore, a variant created by deletion of 7 kb and results in a functional hybrid delta/beta globin product.[7] In contrast to β-thalassemia, in which mutations are predominantly caused by point mutations in the structural genes, α-thalassemia is more often caused by deletions involving one or both of the α-globin genes.[34] A small number of point mutations, usually within the α2 gene, have been characterized. Rare deletions removing the upstream α-globin regulatory element have also been described.

α°-thalassemia, because of the loss of both α1 and α2 genes on the same chromosome (/- -), is found in the Mediterranean (/- $-^{MED}$ and/-$(\alpha)^{20.5}$) and in South-east Asia (/- $-^{SEA}$,/- $-^{FIL}$ and/- $-^{THAI}$). Although α°-thalassemia has also been described in individuals of Asian Indian and South African origin, it is extremely uncommon; the phenotype of two α gene deletions is usually because of a homozygous deletion of single α globin genes, i.e. -α/-α. α⁺-thalassemia (/-α) is prevalent in parts of Africa and Asia; the/-$\alpha^{3.7}$ is predominantly found in Africa, the Mediterranean and Asia while the/-$\alpha^{4.2}$ deletion is found in South-east Asia and the Pacific Islands. The gap-PCR technique is extremely useful in the identification of these common α° and α⁺-thalassemias. The primers for these deletions can be multiplexed in various combinations to capture the deletions that are most likely to be encountered in the different catchment areas.[31,35] The multiplex set encompassing gap-PCR primers for /- $-^{SEA}$,/- $-^{MED}$,/- -$(\alpha)^{20.5}$,/- $-^{FIL}$/-$\alpha^{3.7}$ and/-$\alpha^{4.2}$ is used by many laboratories supplemented by another set for identification of triple α complex (/ααα)[32,36] useful in the workup of thalassemia intermedia. Gap-PCR primers are also available for the Thai α° deletion (/- $-^{THAI}$) and can be included in the multiplex panels.[35] Multiplex gap-PCR requires careful optimization but once optimized, is highly efficient and rapid for the detection of these common α° and α⁺-thalassemia deletions, particularly when performed in large batches in microtitre plates. There were 130 patients who were suspected of having α-thalassemia based on hematological tests in our study. So for confirming that we analyzed the α-globin gene deletion by Gap-PCR technique and found six types of mutations in 98 individuals out of 130. The most predominant one was - $\alpha^{3.7}$ (62 %) followed by - - ^{MED}, - α^{5NT}, - - ^{MEDII}, - $\alpha^{4.2}$, - $\alpha^{PA\ and}$ - - ^{MED} /- $\alpha^{3.7}$ (HbH) for Southern part of Iran listed in Table 5.

Restriction enzyme analysis of PCR product

Restriction enzymes cut double-stranded DNA at specific recognition sequences; some mutations naturally create or abolish restriction enzyme sites. Computer

Table 5: Frequencies of alpha-thalassemia mutations in study group

Mutation	Number of patients	Frequency (%)
- $\alpha^{3.7}$	82	63
- α^{5NT}	5	4
- - ^{MED}	2	2
- - ^{MEDII}	1	0.8
- $\alpha^{4.2}$	4	3
- α^{PA}	1	0.8
- - ^{MED}/ - $\alpha^{3.7}$	3	1.4
Unknown	32	25
Total	130	100

programs are now available that allow sequences to be screened for putative recognition sites for restriction enzymes. Genomic DNA containing the mutation, the 'target', is amplified by PCR and the product is then digested by the diagnostic restriction enzyme and the resulting DNA fragments separated on gels. The presence or absence of the recognition site is determined from the pattern of the PCR digest; hence an alternative term for this technique is restriction fragment length polymorphism (RFLP) analysis. Restriction enzyme analysis is simple, relatively cheap and robust leading to unequivocal results; the technique is an invaluable molecular diagnostic tool. However, it is limited in its application as only a proportion of the hemoglobin variants, β-thalassemia and α-thalassemia mutations, naturally create or abolish restriction enzyme cutting sites.[35,37] Incomplete or partial digests can be a problem for some restriction enzymes leading to false negative or positives - thus positive and negative controls should always be included. Restriction endonucleases are usually used when there is some other evidence suggesting the causative mutation, for example HPLC, Hb electrophoresis analysis and ethnic origin of the individual or it is used as a secondary confirmation technique i.e., after sequence analysis. In some instances, although the mutations do not naturally create or abolish a restriction enzyme site, the sequence in the vicinity of the mutation allows one to artificially create restriction sites adjacent to the mutation sequence by introducing a single base mismatch in the amplification primer. This technique, referred to as amplification-created restriction site (ACRS) is routinely used to identify the Saudi nondeletional alpha thalassemia mutation in the α2-globin gene, AATAAA to AATAAG.[38] We attempted to detect mutations from those individual who found as unknown in the previous steps. In our study out of nine RFLP systems (ε/ *HincII, Gγ/HindIII, 3'*-ψβ*/HincII, 5'*-α*/HincII,* β*/RasI,* β*/AvaII,* β*/HinfI, Gγ/XmnI, Aγ/HindIII)* different enzymes were informative for different cases respectively. We found that offspring with homozygous patterns of either (-/-) or (+/+) would be just carriers of the thalassemia traits and they were not going to be affected. Therefore those fetuses were either just a carrier of thalassemia or normal.

The α/β globin protein ratio

Another useful specialized test helpful in confirming a thalassemia and whether it is due to α or β globin gene problem is the α/β globin protein ratio. This requires the incubation of red blood cells with a radioactive tracer such as H^3-leucine.[39] The peaks representing α and β globin proteins are then quantitated to provide α/β ratio which should equal 1.0. A ratio > 1.0 indicates β-thalassemia while a ratio <1.0 is caused by α-thalassemia. Although a useful test in some cases, the α/β ratio is no longer routinely available. This has occurred because DNA testing has essentially become the strategy of choice for testing and so few α/β ratios are now requested that laboratories do not have sufficient experience. The requirement for fresh radioactive material is another disincentive for setting up this assay. It should also be noted that the α/β ratio may not be particularly helpful if gene-gene interactions are occurring, for example, both α and β thalassemia are co-inherited. We used this method to identify mutation in some individuals who couldn't be detected by previous defined methods. We detected six individuals, with three of them showing values less than 1.0 and further investigation of these cases by using molecular techniques confirmed them as β-thalassemia heterozygous . The remaining three cases showed values more than 1.0 and were considered as α-thalassemia cases which was subsequently confirmed as α-thalassemia heterozygous by molecular techniques [Table 6].

Foetal DNA analysis

Many laboratories offering DNA diagnostics of the Hb disorders are also involved in analysis of fetal DNA for the prenatal diagnosis of these disorders. Fetal DNA is usually and preferably, obtained through chorionic villus sampling in the first trimester of pregnancy (10-

12 weeks).[40] Chorionic villus sampling provides a good yield of DNA which is isolated using conventional methods of phenol chloroform extraction after careful microscopic dissection to remove any contaminating maternal deciduas. Occasionally, if the sample is too small, it may need to be cultured increasing the risk of maternal contamination. Amniocytes obtained in the second trimester can also be used as a source of fetal DNA but the fetal cells are often contaminated with maternal cells and the results have to be interpreted with caution after analysis for maternal contamination.

Every prenatal diagnosis should be accompanied by copies of hematology results of the parents and prior confirmation of the parental phenotypes and genotypes. The PCR-based techniques that best suit the expertise of the laboratory are then used to screen for the presence of the parental mutations in the fetal DNA. Parental, appropriate positive and negative controls must always be included in the investigations. A limited number of PCR cycles (25-28) should be performed to avoid amplification of any minor DNA species and to minimize amplification of any contaminating maternal DNA. As an added precaution, maternal DNA contamination should be checked for by using polymorphic DNA markers including the variable tandem repeats (VNTRs) such as ApoB and the short tandem repeats or micro satellites.[41] This is particularly important when the fetal genotype is the same as the maternal genotype. Fetal DNA analysis should also be performed in duplicate and confirmed by an independent PCR-based technique. We did PND for 176 couples which had background of thalassemia in first pregnancy. Results of PND diagnosis in the first trimester showed 62 affected fetuses with β-thalassemia major and sickle cell disease which led to termination of the pregnancy. In order to confirm maternal DNA contamination we used polymorphic DNA markers including the variable tandem repeats (VNTRs) in all cases [Table 7]. In almost all of the cases they showed heterozygous patterns.

Table 6: Summary of the hematological data and α/β ratio in the study group

Case	Age	RBC	MCV	MCH	Hb	α/β ratio	Diagnosis
1	29	6.77	72	21.4	14.3	0.51	α-tha
2	25	6.07	69.9	21.3	12.9	0.55	α-tha
3	29	5.86	79	23.3	11.9	1.08	β-thal
4	22	5.07	68.5	22.6	10.4	1.1	β-thal
5	28	5.79	74	24	13.9	0.83	α-tha
6	28	5.53	84.7	28.5	15.7	1.06	β-thal

Conclusion

The compactness of the globin genes means that hemoglobinopathy detection is largely a PCR-based approach that can utilize direct sequencing analysis. Almost all hemoglobinopathies can be detected with the current PCR-based assays with the exception of a few rare deletions. However, the molecular diagnostic service is still under development to try and meet the demands of the population it serves. A higher throughput approach will be required to meet practice pressures and the increasing needs set by the antenatal screening program. This increasing workload dictates increasing automation, which may necessitate the use of automated robotic platforms to prepare samples and reactions and the use of automated platforms to perform the actual detection. In most populations the β-thalassemias (and related hemoglobinopathies) are clinically more relevant than the β-thalassemias. The complex mutational spectrum of the hemoglobinopathies, especially relevant in a multi-ethnic community, requires a method with the capacity to scan the β (and/or α) globin genes rapidly and accurately for all mutations. This aspect is being addressed by the development of arrays. Although in their infancy, the arrays hold great promise and are amenable to scaling up and automation. In the short term, the current generation of instruments such as the capillary electrophoresis systems, has greatly simplified DNA sequence analysis. The capillary electrophoresis system also lends itself to the multiplexed mini-sequencing methodology which is highly suitable for screening for the common globin gene mutations.

Table 7: Results of 176 couples PND tests

Case	No	Decision
β-thalassemia major	52	Termination of pregnancy
Carriers	91	Continuing the pregnancy
Sickle cell	10	Termination of pregnancy
α or β-thalassemia/Hb variant	4	Continuing the pregnancy
Normal	19	Continuing the pregnancy
Total	176	

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