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ORIGINAL ARTICLE

Screening and genetic diagnosis of haemoglobinopathies

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Abstract

The haemoglobin disorders are a group of autosomal recessive disorders characterized by either the reduced synthesis of one or more normal globin chains (the thalassaemias), the synthesis of a structurally abnormal globin chain (the haemoglobin variants) or in a few cases by both phenotypes (the reduced synthesis of a Hb variant, e.g. Hb E). They are the commonest single-gene disorders known and approximately 1000 different mutant alleles have now been characterized at the molecular level. The mutations are regionally specific, with each country having its own unique spectrum of abnormal haemoglobins and thalassaemia mutations, and can occur at high gene frequencies in some ethnic groups [1]. Although haemoglobinopathy mutations are rarely found in individuals of North European origin, the number of immigrants in the North European countries is steadily increasing and the variety of their ethnic origins poses a problem for screening and accurate diagnosis.

Key Words: ??????????????????

Introduction

In England, the Department of Health has recently implemented the NHS Sickle Cell and Thalassaemia Screening Programme in order to improve the provision of screening services. This consists of two linked screening programmes for haemoglobinopathies: sickle cell screening of all newborns and sickle cell and thalassaemia screening during pregnancy. The antenatal screening programme has two approaches depending on whether the local population to be covered by the screening laboratory is defined as high prevalence or low prevalence. The Nordic countries are probably similar to the UK in that most areas are essentially low prevalence for the haemoglobinopathies, with just a few local areas of high prevalence for specific ethnic groups, and thus a similar screening protocol could be adopted.

The aim of this article is to outline the approaches to screening and the genetic diagnosis of all the haemoglobin disorders based on carrier screening and mutation analysis that is being implemented in England. The screening programmes are designed to obtain a

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reliable but essentially a presumptive diagnosis of a haemoglobinopathy phenotype in haematology laboratories, with referral to centralized DNA laboratory to obtain a definitive diagnosis in appropriate cases.

Newborn screening policy for England

The objective of the newborn screening programme is to detect all infants affected with a sickle cell disorder within the neonatal period, in order to improve outcomes through early treatment and care. Approximately 300 babies are born in England with sickle cell disease each year, and these will now be identified by a bloodspot-screening programme so that treatment can be started before 3 months of age in order to lower the risk of death or complications arising from treatable infections.

The screening programme is now part of the newborn dried blood spot programme for other conditions, including phenylketonuria and congenital hypothyroidism. The service is provided by a small number of centralized laboratories handling between 25,000 and 50,000 specimens per year. The laboratories employ two analytical techniques for screening for sickle cell disorders from dried blood spot samples: high performance liquid chromatography (HPLC) and iso-electric focusing (IEF). Either is suitable for first-line screening, the alternative procedure being used for second-line testing for validation of the presumed variant. Both procedures are capable of detecting all the common clinically significant haemoglobin variants, i.e. Hb S, C, D-Punjab, E, O-Arab, in addition to Hb F and Hb A. The sickle cell genotypes that are detected by the screening programme are Hb SS, Hb SC, Hb S/β-thalassaemia, Hb S/D-Punjab, Hb S/Lepore and Hb S/O-Arab. Hb S in combination with the deletional form of Hereditary Persistence of Fetal Haemoglobin (HPFH) will also be identified by the procedure. Characterization of Hb S/HPFH from Hb SS and Hb S/β^0-thalassaemia is achieved by family studies and further study by DNA analysis. Similarly, the identification of Hb D-Punjab, the only clinically significant D variant, can only be done by DNA analysis.

In addition to the sickle cell disorders, the newborn screening programme will detect other clinically significant haemoglobinopathies for which the patient will benefit from early follow-up. These include β-thalassaemia major and intermedia, Hb E/ β-thalassaemia and Hb H disease. The programme will also detect clinically benign haemoglobinopathies such as Hb CC, EE and carrier states for variants. These can be reported and follow-up counselling offered.

Antenatal screening policy for England

In England, screening in the antenatal period for sickle cell and thalassaemia will become an integral part of the screening package offered to all eligible pregnant women by March 2006. The programme will facilitate informed choices in screening, identify early couples at risk of a pregnancy with a sickle cell or thalassaemia disorder, and provide appropriate referral and care for prenatal diagnosis with continuation of pregnancy or termination according to women's choices.

There are two strategies for antenatal screening, one for high prevalence areas and one for low prevalence areas. High prevalence has been defined as where the prevalence of sickle cell births is greater than 1.5 per 10,000 pregnancies. In these areas, screening for sickle cell and thalassaemia will be offered to all women, by the measurement of red blood cell indices and HPLC analysis for the estimation of Hb A_2 and the detection of any

clinically significant variant. Low prevalence areas are where the prevalence of sickle cell births is lower than 1.5 per 10,000. In these areas, antenatal screening can follow a different algorithm describing a minimum screening strategy. As a minimum, all women will be screened by measurement of red blood indices and those with a MCH below 27 g dl^{-1} will have their Hb A_2 measured by HPLC for thalassaemia diagnosis. Testing for Hb S and the other variants will be based on assessment of individual risk, determined by the ethnic origin of the woman and her partner. The testing algorithms for both low and high prevalence areas can be found on the NHS Sickle Cell and Thalassaemia Screening Programme website: http://www.kcl-phs.org.uk/haemscreening.

Antenatal screening for haemoglobinopathies in England is provided by a locally based service with adequate haematology expertise as part of routine antenatal care. No minimum throughput sample number has been specified, although where small numbers may effect the timeliness of reporting, some centralization may be required. The screening programme is designed to detect the main haemoglobinopathy carrier states that can combine to give rise to the risk of an affected fetus: α^0-thalassaemia, β-thalassaemia, $\delta\beta$-thalassaemia, and Hb's C, D-Punjab, E, Lepore and S. The main carrier state combinations that can result in an affected pregnancy are summarized in Figure 1. These carrier states require partner testing and then referral of blood samples if appropriate to a DNA analysis laboratory for genotype analysis. The DNA referral guidelines and the antenatal screening algorithms for the UK antenatal screening programme have been designed to identify most but not all couples at risk for sickle cell disease or thalassaemia. They will not identify some couples at risk for Hb H disease, unusual cases of α^0-thalassaemia and silent β-thalassaemia alleles with a Hb A_2 level below 3.5 %. However, the antenatal screening laboratories can upgrade the guidelines for partner testing to take into account any specific rare haemoglobin disorders or mutations that might occur in their local population.

DNA diagnosis policy

In England, there are three DNA analysis laboratories that undertake genotype analysis and prenatal diagnosis of the haemoglobinopathies for the national screening programme. They form a subgroup of the UK Genetic Testing Network and process approximately 300 prenatal diagnoses per year. They have produced guidelines for the referral of antenatal patient samples for DNA analysis which is published on the NHS Sickle Cell and Thalassaemia Screening Programme website as detailed above. The more specific DNA based methods should only be performed in a few centres in order to ensure stability of the assays.

The main classes of haemoglobinopathy genotypes and the current approaches to their diagnosis by DNA analysis are summarized in Table I. The key to identifying the globin gene mutation/s in a carrier or affected patient is an expert knowledge of the possible mutations and their interaction to produce a complex haematological phenotype. A patient's α- and β-genotype may be complicated, e.g. a patient with Hb S/β-thalassaemia may also be homozygous for α^+-thalassaemia and homozygous Hb G-Philadelphia. Such a patient has no Hb S or G-Philadelphia, only the hybrid variant (α^G/β^S) is visible by HPLC or electrophoresis.

Determination of carrier phenotype

The basic haematological tests required are the measurement of the mean corpuscular volume (MCV), the mean corpuscular haemoglobin (MCH) value and the quantity of Hb

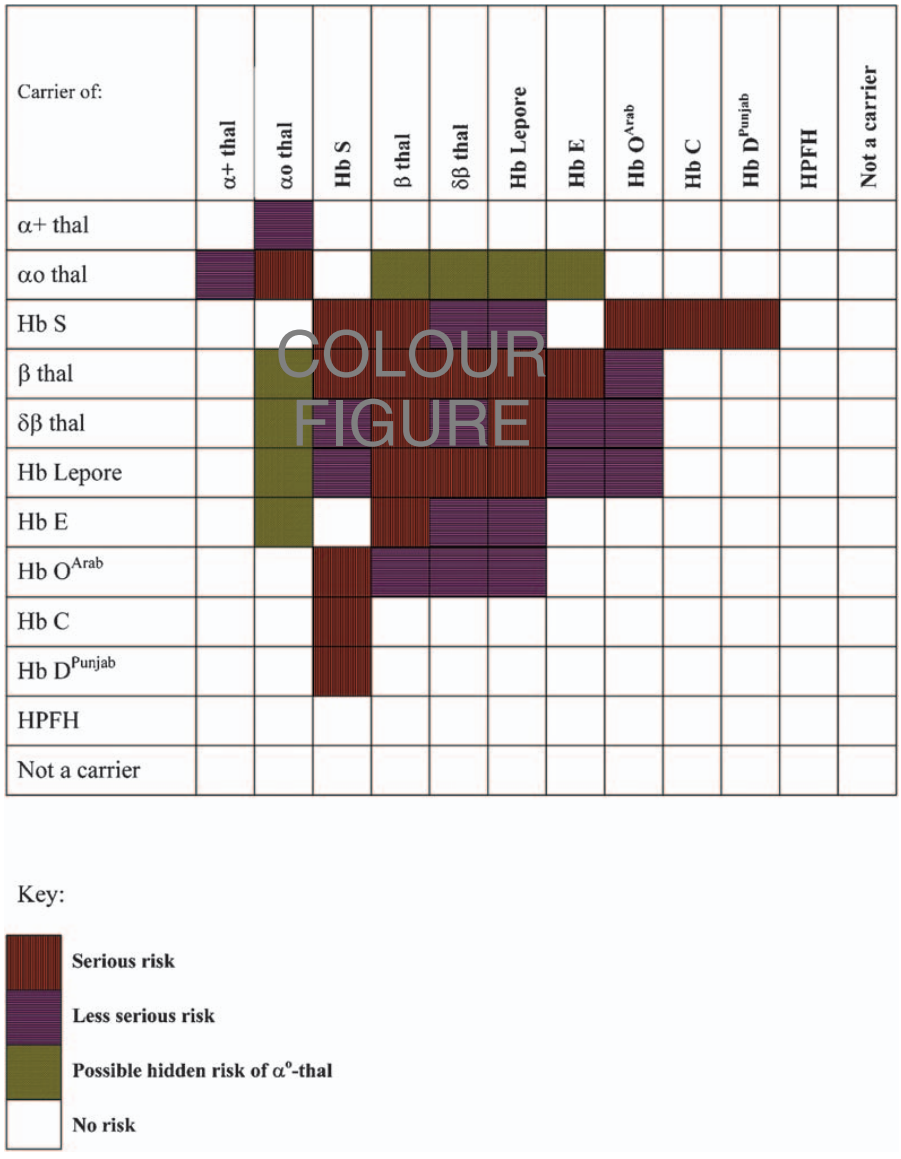


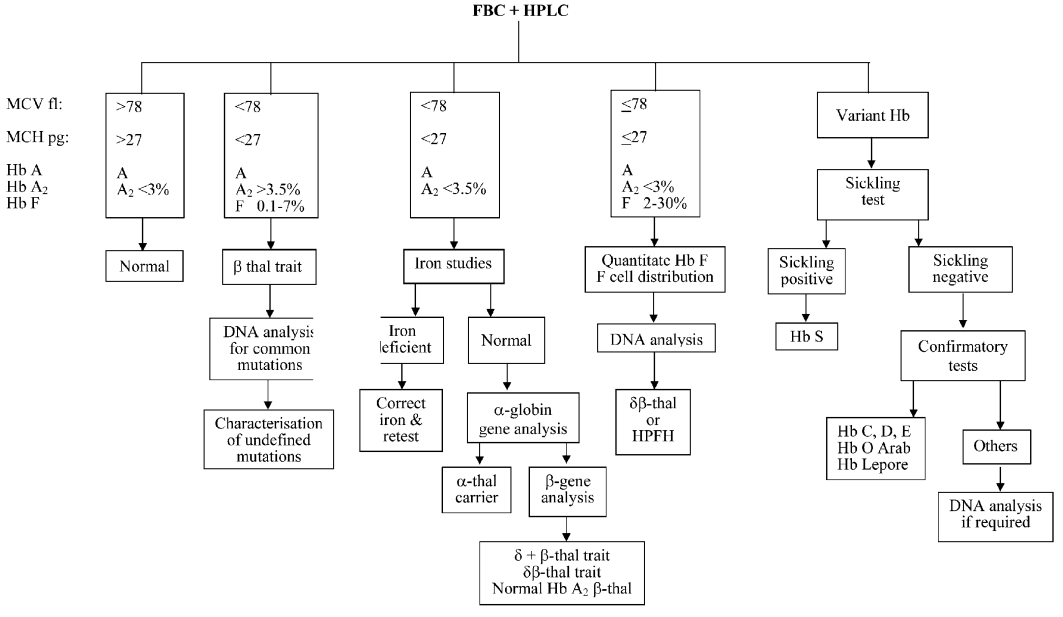
Figure 1. Summary of the main haemoglobinopathy carrier state combinations that can result in an affected pregnancy.

A_2 and Hb F [2,3]. In addition, the haemoglobin pattern needs to be examined and, traditionally, electrophoresis methods have been used for this purpose. However, if high performance liquid chromatography (HPLC) is used to quantitate the Hb A_2 and Hb F level, it will also detect most of the common, clinically relevant haemoglobin variants, such as Hb S, Hb C, Hb D-Punjab, Hb O-Arab and Hb E at the same time. A simplified flow chart based on the MCH, Hb A_2 and Hb F values that can be used for carrier screening and mutation identification is described Figure 2. This is a more comprehensive algorithm than those published by the UK NHS Sickle Cell and Thalassaemia Screening Programme, which only reaches the endpoints of partner testing or no further action required.

Table I. Haemoglobinopathies: genotypes, phenotypes and DNA diagnostic approaches.

Haemoglobinopathy	Phenotype	DNA diagnosis methods
1. Homozygous state		
α^0-thalassaemia (–/–)	Hb Bart's hydrops fetalis	Gap-PCR or S. Blot
α^+-thalasasemia (-α/-α)	No clinical problems, (same as –/αα)	Gap-PCR or S. Blot
(-α/$\alpha^T\alpha$)	Mild Hb H disease	Gap-PCR , Sequencing
($\alpha^T\alpha/\alpha^T\alpha$)	Hb H disease	Sequencing
β-thalassaemia (β^0/β^0)	Thalassaemia major	PCR: ASO or ARMS PCR:
(β^0/β^+)	Thalassaemia major	PCR: ASO or ARMS PCR
(β^+/β^+)	Thalassaemia major	PCR: ASO or ARMS PCR
(mild β^+/β^0 or β^+)	Variable: intermedia to major	PCR: ASO or ARMS PCR
(mild β^+/mild β^+)	Thalassaemia intermedia	PCR: ASO or ARMS PCR
$\delta\beta^0$-thalassaemia	Thalassaemia intermedia	Gap-PCR or S. Blot
HPFH	No clinical problems	Gap-PCR or S. Blot
Hb Lepore	Variable: intermedia to major	Gap-PCR
Hb S	Sickle cell disease	PCR: RE, ASO or ARMS
Hb C	No clinical problems	PCR: ASO or ARMS
Hb D	No clinical problems	PCR: RE, ASO or ARMS
Hb E	No clinical problems	PCR: RE, ASO or ARMS
2. Compound-heterozygous state		
α^0-thal/α^+-thal (–/-α)	Hb H disease	Gap-PCR or S. Blot
α^0-thal/α^+-thal (–/$\alpha^T\alpha$)	Severe Hb H disease	Gap-PCR or S. Blot
ααα/β^0 or β^+ thal	Mild thalassaemia intermedia	PCR: Gap, ASO or ARMS
$\delta\beta^0$ thal/β^0 or β^+ thal	Variable: intermedia to major	PCR: Gap, ASO or ARMS
$\delta\beta^0$ thal/mild β^+ thal	Mild thalassaemia intermedia	PCR: Gap, ASO or ARMS
$\delta\beta^0$ thal/Hb Lepore	Mild thalassaemia intermedia	Gap-PCR
Hb Lepore/β^0 or β^+ thal	Thalassaemia major	PCR: Gap, ASO or ARMS
Hb C/β^0 or β^+ thal	Mild thalassaemia intermedia	PCR: ASO or ARMS
Hb C/mild β^+ thal	No clinical problems	PCR: ASO or ARMS
Hb C/$\delta\beta^0$ thal	No clinical problems	PCR: Gap, ASO or ARMS
Hb D/β^0 or β^+ thal	No clinical problems	PCR: RE, ASO or ARMS
Hb E/β^0 or β^+ thal	Variable: intermedia to major	PCR: RE, ASO or ARMS
Hb E/mild β^+ thal	Mild thalassaemia intermedia	PCR: RE, ASO or ARMS
Hb O Arab/β^0 thal	Severe thalassaemia intermedia	PCR: RE, ASO or ARMS
Hb S/β^0 or β^+ thal	Sickle cell disease	PCR: RE, ASO or ARMS
Hb S/mild β^+ thal	Mild sickle cell disease	PCR: RE, ASO or ARMS
Hb S/$\delta\beta^0$ thal	Mild sickle cell disease	PCR: RE, Gap or S. Blot
Hb S/Hb C	Mild sickle cell disease	PCR: RE, ASO or ARMS
Hb S/Hb D Punjab	Sickle cell disease	PCR: RE, ASO or ARMS
Hb S/Hb O Arab	Sickle cell disease	PCR: RE, ASO or ARMS
Hb S/HPFH	No clinical problems	PCR: RE, Gap or S. Blot
3. *Hb E disorders*		
Hb E+α^0-thal/α^+-thal (–/-α)	Similar to Hb H disease	PCR: Gap, ASO or ARMS
Hb EE+α^+-thal/α^+-thal ($\alpha^T\alpha/\alpha^T\alpha$)	Mild thalassaemia intermedia	PCR: Gap, ASO or ARMS
Hb EE+α^0-tha l/α^+-thal (–/-α)	Severe thalassaemia intermedia	PCR: Gap, ASO or ARMS

Testing for thalassaemia is usually carried out when the MCH is <27 pg, although in very rare instances individuals may have thalassaemia trait and a normal MCH, either due to a silent β-thalassaemia mutation or the co-inheritance of α- and β-thalassaemia. The MCH value is more reliable for thalassaemia diagnosis than the MCV. In samples greater than 24 h old, the red cell indices can be misleading, as the MCV increases by up to 5 fl. Thus, evaluation of blood count in old samples should be made with caution. The phenotype of a carrier usually fits one of the following categories for further molecular investigations:



(a) *Raised Hb A_2, reduced MCH*

When the MCH is below 27 pg and the Hb A_2 is above 3.5 %, a diagnosis of heterozygous β-thalassaemia is made. The majority of β-thalassaemia carriers, either with a β^0 or severe β^+-type mutation, are characterized by a markedly low MCH (19–23 pg) and an elevated Hb A_2 level in the range 4.0–6.0 %. The latter is the most phenotypic feature of β-thalassaemia trait, although there is also a slightly raised Hb F level (1–3 %) in about 30 % of cases. However, some β-thalassaemia carriers have atypical Hb A_2 levels. Some show unusually high Hb A_2 levels of 6.5–9.0 % and a variable elevated Hb level of 3–15 %. The molecular lesions in such cases are large deletions that remove the 5′ promoter region of the β-globin gene. If the Hb A_2 level lies between 3.5 % and 4.0 %, it usually indicates the carrier has a mild β^+-thalassaemia mutation. These mutations can also give rise to a Hb A_2 slightly below 3.5 %, and thus also feature in category (b) below.

Some rare molecular lesions of the β-globin gene produce highly unstable β-chains that precipitate in bone marrow precursors. This results in ineffective erythropoiesis and a clinical phenotype of thalassaemia intermedia with a raised Hb A_2. Because of the precipitation in early red cell precursors, the β-chain variant is usually undetectable in peripheral blood. These mutations, known as dominant β-thalassaemia alleles, are usually located in exon 3 [4].

Both the MCH and the Hb A_2 level of β-thalassaemia carriers may be influenced by other factors. The co-inheritance of α-thalassaemia may result in a carrier having a nearly normal MCH value with a clearly raised HbA_2 level. A high frequency of both α- and β-thalassaemia is found in some countries in Southeast Asia, the Mediterranean area, and possibly the Middle East, and therefore the relevant α^0-thalassaemia mutations should always be screened by DNA analysis in β-thalassaemia and Hb E carriers from these regions. The presence of a δ-chain variant will split the Hb A_2 into two equal peaks on HPLC, while the presence of an α-chain variant will split the Hb A_2 into two unequal peaks, the abnormal one being 25 % of the normal one.

Iron deficiency may reduce the Hb A_2 level, although the typically raised values of heterozygous β-thalassaemia are not usually lowered below 3.5 % unless a very severe anaemia is present [5]. However if the mutation is a mild β^+-thalassaemia type that normally gives a Hb A_2 between 3.3 % and 4.0 % (as described above), iron deficiency may produce a falsely low level below 3.5 %. Iron deficiency is excluded by the measurement of erythrocyte zinc protoporphyrin or the evaluation of transferrin saturation.

(b) *Normal Hb A_2, reduced MCH*

When the MCH is below 27 pg, the Hb A_2 is below 3.5 % and the Hb F level normal, the diagnosis may be iron deficiency, α-thalassaemia, $(\varepsilon\gamma\delta\beta)^0$-thalassaemia, β- and δ-thalassaemia or mild β-thalassaemia trait with a normal Hb A_2. After iron deficiency is excluded, the different thalassaemia determinants leading to this phenotype are identified by α-, δ- and β-globin gene mutation analysis.

Carriers of α^0-thalassaemia usually have a MCH below 25 pg unless they also have β-thalassaemia trait. However, individuals with homozygous α^+-thalassaemia also have a MCH below 25 pg and the different forms of α-thalassaemia can only be differentiated reliably by gene analysis. Individuals with α^0-thalassaemia trait may have a few red cells with Hb H inclusions, but their absence does not exclude this genotype. The detection of Hb H by electrophoresis or HPLC indicates Hb H disease, usually resulting from the combination of α^0-thalassaemia trait and α^+-thalassaemia trait.

The UK antenatal screening programme has tried to simplify the diagnosis of α-thalassaemia by limiting its approach to just the diagnosis of carriers of α^0-thalassaemia. By screening out the carriers of α^+-thalassaemia, the workload for midwives and others responsible for requesting partner tests and for the DNA analysis laboratories is considerably reduced and there is less anxiety for women and their families who are mostly carriers of α^+-thalassaemia. The screen consists, first, of only considering women who have a MCH below 25 pg, and then of these, only requesting partner testing if the woman's family originally came from China, Southeast Asia, Greece, Turkey, Cyprus or is of unknown origin.

$(\varepsilon\gamma\delta\beta)^0$-Thalassaemia trait is a very rare condition described in just a few families. Newborns heterozygous for this condition are severely anaemic but improve after 3 months and adults a mild anaemia with reduced MCH and MCV levels, a normal Hb F and a normal Hb A_2 value. δ-Thalassaemia has mainly been described in individuals from Mediterranean countries and its co-inheritance with β-thalassaemia trait reduces the Hb A_2 value to below 3.5 %. There is no PCR test currently available for any of the $(\varepsilon\gamma\delta\beta)^0$-thalassaemia deletion mutations.

β-Thalassaemia trait with a normal Hb A2 and reduced MCH is also a very rare condition. Carriers of a mild β^+-thalassaemia mutation may have reduced MCV and MCH values with a normal or slightly raised HbA_2 level ranging from 3.4 % to 3.8 % The most common such mutations are the Asian Indian mutation CAP+1 (A→C), Mediterranean mutation IVS1-6 (T→C) and the African mutation Poly A T→C.

(c) *Normal HbA_2, reduced MCH and raised Hb F*

In rare instances where a low MCH and normal (or reduced) HbA_2 level is observed in combination with a raised Hb F level of 2–30 %, $\delta\beta$-thalassaemia trait or HPFH should be suspected. It is important to differentiate $\delta\beta$-thalassaemia from hereditary persistence of fetal haemoglobin (HPFH) for genetic counselling because compound heterozygotes for

HPFH and β-thalassaemia have a silent or very mild phenotype, in contrast to the combination of $\delta\beta$-thalassaemia and β-thalassaemia which may result in β-thalassaemia major. Although HPFH is associated with normal red cell indices, individuals with HPFH trait may also have α-thalassaemia and thus the MCH is not a reliable parameter for the differentiation between HPFH and $\delta\beta$-thalassaemia. The distinction may be made haematologically by analysing the red blood cell distribution of Hb F. Hb F is usually heterogeneously distributed in $\delta\beta$-thalassaemia trait in contrast to HPFH trait in which it is homogenously distributed. However, a definitive diagnosis can only be made by identification of the deletion mutation by DNA analysis.

(d) *Normal Hb A_2, normal MCH*

This is the phenotype of a silent β-thalassaemia allele, such as the Mediterranean mutation -101 (C→T) and also of the triplicated α-gene allele. Silent β-thalassaemia mutations are associated with Hb A_2 levels below 3.5 % and a minimal deficiency of β-globin production, resulting in an extremely mild β-thalassaemia phenotype. They are extremely uncommon and result in the condition of mild thalassaemia intermedia in the homozygous state or in combination with a severe β^+- or β^0-thalassaemia mutation. Carriers may escape identification in population screening, and identification is usually retrospective, by analysis of parent(s) of patients with mild thalassaemia intermedia. Co-inheritance of the triplicated α-gene allele with β-thalassaemia trait may result in some patients in the phenotype of very mild thalassaemia intermedia, but simple carriers and homozygotes of the allele are completely normal.

(e) *Abnormal haemoglobins*

The most common clinically relevant abnormal haemoglobins (Hb S, C, D-Punjab, O-Arab, and E) can all be characterized by haemoglobin electrophoresis or HPLC. The electrophoresis method usually performed is cellulose acetate electrophoresis at alkaline pH. However, alternative methods may be used, such as isoelectric focusing (IEF). Although not the cheapest method, IEF offers the best resolution identification of abnormal haemoglobins by haemoglobin electrophoresis. HPLC is accurate, fast and quantitates the variant, providing an elution time for variant identification. This is usually the first-line screening method for the common variants, and then electrophoresis is used for confirmation.

In this way, the common clinically important ones can be characterized, apart from Hb D-Punjab, which requires DNA analysis to distinguish it from the other D variants. If IEF is used for the electrophoretic method, the resolution is so good that other common variants with no clinical significance may be characterized by the combination of HPLC retention time and the IEF position. Although a specific elution time at HPLC and a specific band position at electrophoresis does not give a precise identification of the variant, some of the commoner ones may be characterized. For example, Hb G-Philadelphia and Hb J-Meerut are fairly common in patients of African and Indian origin and can be putatively identified in this way. However, screening programmes are bound to reveal some rare variants that cannot be putatively identified from past experience of the chromatograms, etc. Most of these will have no clinical significance, but just a few, particularly the unstable haemoglobins or those with altered oxygen affinity, can produce clinical manifestations, especially in combination with β-thalassaemia trait [1]. It is

recommended that rare variants are referred to a specialized laboratory for a precise identification.

A precise identification is achieved by mutation analysis methods such as DNA sequencing, restriction enzyme analysis, dot blotting or ARMS-PCR, or by amino acid analysis using mass spectrometry. The only exception is Hb S and its analogous variants (Hb C-Harlem, S-Antilles, S-Oman, S-Providence, S-Travis and C-Ziquinchor), identified more simply by the sickling test.

Note that the Hb A_2 level in Hb AS individuals is often slightly raised above the normal level (3.5–4.0 %), but this never signifies the presence of a co-inherited β-thalassaemia gene unless the amount of Hb S is greater than 50 %. When this is the case, the genotype may be Hb S/β-thalassaemia, Hb S/$\delta\beta$-thalassaemia, Hb S/HPFH or Hb SS. DNA studies and/or haematological analysis of the patient's parents are required to identify the correct genotype. Some β-chain variants, e.g. Hb S and Hb E, are subject to a reduced expression in heterozygotes with co-inherited α-thalassaemia. For sickle cell trait, normal individuals have 35–40 % Hb S, those with α^+-thalassaemia trait have 29–34 %, while those with homozygous α^+-thalassaemia have 24–28 % [1].

Determination of carrier genotype

A variety of techniques based on the amplification of DNA by the polymerase chain reaction (PCR) have been developed to identify the globin gene mutations. These techniques include dot blot analysis, reverse dot blot analysis, the amplification refractory mutation system (ARMS), denaturing gradient gel electrophoresis, mutagenically separated PCR, gap PCR and restriction endonuclease analysis. Each method has its own advantages and disadvantages and all are recommended for use in best practice guidelines [6]. The particular ones chosen by a laboratory depends not only on the technical expertise available in the diagnostic laboratory but also on the type and variety of the mutations likely to be encountered in the individuals being screened.

(1) β-*Thalassaemia*

More than 170 different β-thalassaemia have now been characterized, the majority of which are point mutations or deletions/insertions of just one or two nucleotides [7]. An updated list can be accessed on the internet at http://www.globin.cse.psu.edu/. These are diagnosed in most laboratories by two main PCR-based approaches: allele-specific oligonucleotide hybridization or allele-specific priming. The small number of large deletion mutations may be diagnosed by gap PCR. All of the mutations are regionally specific and the spectrum of mutations has now been determined for most at-risk populations. The strategy for identifying β-thalassaemia mutations is usually based on the knowledge of the common and rare mutations in the ethnic group of the individual being screened. Most populations have been found to have just a few of the common mutations together with a larger and more variable number of rare ones. The common mutations (alleles at a relative gene frequency of greater than 1 %) for the four main ethnic groups are listed in Table II.

Usually, the common ones are analysed first using a PCR technique that allows the detection of multiple mutations simultaneously. This approach will identify the mutation in more than 90 % of cases. If the mutation remains unidentified, a further screening for the possible rare mutation will identify the defect in most cases. Mutations remaining unknown after the screen for rare mutations are characterized by direct DNA sequence analysis. Before sequencing, some laboratories localize the site of the mutation by the application of

Table II. Distribution of the common β-thalassaemia mutations expressed as percentage gene frequencies of the total number of thalassaemia chromosomes studied.

	Mediterranean			Indian		Chinese		African
Mutation	Italy	Greece	Turkey	Pakistan	India	China	Thailand	African-American
−88 (C→T)					0.8			21.4
−87 (C→G)	0.4	1.8	1.2					
−30 (T→A)			2.5					
−29 (A→G)						1.9		60.3
−28 (A→G)						11.6	4.9	
CAP+1 (A→C)					1.7			
CD5 (-CT)		1.2	0.8					
CD6 (-A)	0.4	2.9	0.6					
CD8 (-AA)		0.6	7.4					
CD8/9 (+G)				28.9	12.0			
CD15 (G→A)				3.5	0.8			0.8
CD16 (-C)				1.3	1.7			
CD17 (A→T)						10.5	24.7	
CD24 (T→A)								7.9
CD30 (G→A)				0.9				
CD30 (G→C)				3.5	0.9			
CD39 (C→T)	40.1	17.4	3.5					
CD41/42 (-TCTT)				7.9	13.7	38.6	46.4	
CD71/72 (+A)						12.4	2.3	
IVSI-1 (G→A)	4.3	13.6	2.5					
IVSI-1 (G→T)				8.2	6.6			
IVSI-5 (G→C)				26.4	48.5	2.5	4.9	
IVSI-6 (T→C)	16.3	7.4	17.4					
IVSI-110 (G→A)	29.8	43.7	41.9					
IVSII-1 (G→A)	1.1	2.1	9.7					
IVSII-654 (C→T)						15.7	8.9	
IVSII-745 (C→G)	3.5	7.1	2.7					
619 bp deletion				23.3	13.3			
Others	4.1	2.2	9.7	0.5	0.9	6.8	7.9	10.6

CD=Codon, IVS=intervening sequence, bp=base pairs.

a non-specific detection method such as denaturing gradient gel electrophoresis (DGGE). However, using the latest capillary-based sequencers, the whole β-globin gene may be sequenced directly in just three sequencing runs, eliminating the need for DGGE.

Allele-specific oligonucleotide hybridization. – Mutation detection by the hybridization of allele-specific oligonucleotide probes (ASOs) was the first PCR method to be developed. The technique has been applied in many laboratories with great success, especially for populations with just one common mutation and a small number of rare ones, such as the one in Sardinia [8]. However, when screening for a large number of different mutations the method becomes time consuming because of the need for separate hybridization and washing steps for each mutation.

The reverse dot-blotting technique was developed to allow multiple mutations to be tested for in one hybridization reaction and the method is more suitable for the use of a non-isotopic labelling. In this method, unlabelled ASO probes complementary to the mutant and normal DNA sequences are fixed to a nylon membrane strip in the form of dots

or slots, and labelled amplified genomic DNA is then hybridized to the filter using a two-step procedure with one nylon strip for the common mutations and a second one for the rare mutations. This has been described for diagnosis of Mediterranean [9], African-American [10] and Thai β-thalassaemia mutations [11].

Allele-specific priming methods. – These methods are based on the principle that a perfectly matched PCR primer is much more efficient in directing primer extension than a mismatched one. The mostly widely used technique is the amplification refractory mutation system (ARMS). As with the ASO approach, a panel of mutant-specific primers is required for mutation screening and can be made for the common mutations in each ethnic group [12]. Table III lists the ARMS primers developed in my laboratory for detection of the common mutations. For genotyping homozygous patients and for prenatal diagnosis, an ARMS primer complementary to the normal sequence at the mutation site being screened for is required in a separate reaction [13]. Variations of the ARMS technique include multiplexing of the primers to detect more than one mutation [14] and the detection of the whole genotype using both normal and mutation-specific primers in the same reaction, either using fluorescent labels [15] or primers of different lengths [16].

Restriction enzyme analysis of amplified product. – This is a useful but limited technique because very few β-thalassaemia mutations create or abolish a restriction endonuclease site and generate diagnosable products. Mutations that do not naturally create or abolish restriction sites may be diagnosed by the technique of amplification created restriction sites (ACRS). This method uses primers that are designed to insert new bases into the amplified product in order to create a restriction enzyme recognition site adjacent to the mutation sequence. The technique has been applied in the detection of Mediterranean β-thalassaemia mutations [17].

Gap-PCR. – The technique of gap PCR is used to detect β-globin gene deletion mutations. Primers complementary to the breakpoint sequences amplify a deletion-specific fragment that spans the deletion [18]. For large deletions, the distance between the two primers is too great to amplify normal DNA and the normal allele may be detected by amplifying between sequences spanning one of the breakpoints [19]. Eight β-thalassaemia deletions, ranging in size from 290 bp to 45 kb, can be diagnosed by gap-PCR, as listed in Table V.

PCR methods for undefined mutations. – A number of techniques have been applied for the detection of undefined β-thalassaemia mutations. These include denaturing gradient gel electrophoresis (DGGE) and heteroduplex analysis using non-denaturing gel electrophoresis [20]. However, direct DNA sequencing can now be done very efficiently using an automated capillary-based machine utilizing fluorescence detection technology, and thus is the method of choice.

(2) $\delta\beta$-*Thalassaemia, Hb Lepore and HPFH*

Gap-PCR is used to diagnose the $\delta\beta$-thalassaemia, Hb Lepore and HPFH deletion mutations [19]. Note that it can only be used for deletions in which both breakpoints have been sequenced (listed in Table V). These are six $\delta\beta$-thalassaemia deletions (the Spanish, Sicilian, Vietnamese and Chinese deletions, and the Turkish and Indian inversion/deletion mutations), the Hb Lepore deletion three HPFH deletions (the African HPFH1 and 2

Table III. Primer sequences used for detection of the common β-thalassaemia mutations by the allele-specific priming technique.

Mutation	Oligonucleotide sequence	Second primer	Product size (bp)
−88 (C→T)	TCACTTAGACCTCACCCTGTGGAGCCTCAT	A	684
−87 (C→G)	CACTTAGACCTCACCCTGTGGAGCCACCCG	A	683
−30 (T→A)	GCAGGGAGGGCAGGAGCCAGGGCTGGGGAA	A	626
−29 (A→G)	CAGGGAGGGCAGGAGCCAGGGCTGGGTATG	A	625
−28 (A→G)	AGGGAGGGCAGGAGCCAGGGCTGGGCTTAG	A	624
CAP+1 (A→G)	ATAAGTCAGGGCAGAGCCATCTATTGGTTC	A	597
CD5 (-CT)	TCAAACAGACACCATGGTGCACCTGAGTCG	A	528
CD6 (-A)	CCCACAGGGCAGTAACGGCAGACTTCTGCC	B	207
CD8 (-AA)	ACACCATGGTGCACCTGACTCCTGAGCAGG	A	520
CD8/9 (+G)	CCTTGCCCCACAGGGCAGTAACGGCACACC	B	225
CD15 (G→A)	TGAGGAGAAGTCTGCCGTTACTGCCCAGTA	A	500
CD16 (-C)	TCACCACCAACTTCATCCACGTTCACGTTC	B	238
CD17 (A→T)	CTCACCACCAACTTCAGCCACGTTCAGCTA	B	239
CD24 (T→A)	CTTGATACCAACCTGCCCAGGGCCTCTCCT	B	262
CD30 (G→A)	TAAACGTGTCTTGTAACCTTGATACCTACT	B	280
CD30 (G→C)	TAAACCTGTCTTGTAACCTTGATACCTACG	B	280
CD39 (C→T)	CAGATCCCCAAAGGACTCAAAGAACCTGTA	B	436
CD41/42 (-TCTT)	GAGTGGACAGATCCCCAAAGGACTCAACCT	B	439
CD71-72 (+A)	CATGGCAAGAAAGTGCTCGGTGCCTTTAAG	C	241
IVSI-1 (G→A)	TTAAACCTGTCTTGTAACCTTGATACCGAT	B	281
IVSI-1 (G→T)	TTAAACCTGTCTTGTAACCTTGATACCGAAA	B	281
IVSI-5 (G→C)	CTCCTTAAACCTGTCTTGTAACCTTGTTAG	B	285
IVSI-6 (T→C)	TCTCCTTAAACCTGTCTTGTAACCTTCATG	B	286
IVSI-110 (G→A)	ACCAGCAGCCTAAGGGTGGGAAAATAGAGT	B	419
IVSII-1 (G→A)	AAGAAAACATCAAGGGTCCCATAGACTGAT	B	634
IVSII-654 (C→T)	GAATAACAGTGATAATTTCTGGGTTAACGT*	D	829
IVSII-745 (C→G)	TCATATTGCTAATAGCAGCTACAATCGAGG*	D	738
β^S: CD6 (A→T)	CCCACAGGGCAGTAACGGCAGACTTCTGCA	B	207
β^C: CD6 (G→A)	CCACAGGGCAGTAACGGCAGACTTCTCGTT	B	206
β^E: CD26 (G→A)	TAACCTTGATACCAACCTGCCCAGGGCGTT	B	236

The above primers are coupled as indicated with primers A, B, C or D. A: CCCCTTCCTATGACATGAACTTAA; B: ACCTCACCCTGTGGAGCCAC; C: TTCGTCTGTTTCCCATTCTAAACT; or D: GAGTCAAGGCTGAGAGATGCAGGA. The control primers used for all the above mutation-specific ARMS primers except the two marked * are primers D plus E: CAATGTATCATGCCTCTTTGCACC. For IVSII-654 (C→T) and IVSII-745 (C→G), the $^G\gamma$-*Hin*d III RFLP primers (Forward: AGTGCTGCAAGAAGAACAACTACC; Reverse: CTCTGCATCATGGGCAGTGAGCTC) are used as control primers.

deletions, and the Indian HPFH3 deletion) and the Hb Kenya deletion gene (which is classed as a HPFH allele). The other known $\delta\beta$-thalassaemia and HPFH deletions can only be diagnosed by Southern blot analysis at the moment. However, the technique of multiplex ligation-dependent probe amplification (MLPA), which has already been used successfully to detect large deletions in a number of other genes [21], is expected to replace Southern blot analysis in the near future.

(3) α-*Thalassaemia*

α-Thalassaemia alleles result from mutations affecting either one α-globin gene (α^+-thalassaemia) or both α-globin genes on the same chromosome (α^0-thalassaemia). The

majority of the mutations are gene deletions but a small number of point mutations within one of the two α-globin genes resulting in α^+-thalassaemia have been described [1]. The seven most common can be diagnosed by gap-PCR (Table IV). The remainder of the eletion alleles can only be diagnosed by Southern blot analysis, although, as for the β-gene cluster deletions, the MLPA technique is expected to replace Southern blot analysis in the near future.

α^0-Thalassaemia is found mainly in patients of Mediterranean or Southeast Asian in origin. Although one or two mutations have been described in patients of Asian Indian or African origin, it is extremely uncommon and patients with the phenotype of α^0-thalassaemia trait usually have the genotype of homozygous α^+-thalassaemia. The UK antenatal screening strategy for α-thalassaemia is based on this principle. α^+-Thalassaemia can reach high gene frequencies in parts of Africa and Asia, with the $-\alpha^{3.7}$ deletion being the predominant mutation in African, Mediterranean and Asian individuals and the $-\alpha^{4.2}$ being more common in Southeast Asian and the Pacific islands populations. The strategy for screening is based on ethnic origin of the individual, although PCR now makes it easy to screen for all the common deletion mutations in any individual.

Gap-PCR for α^+- and α^0- thalassaemia deletions. – Multiplex Gap-PCR is now used routinely for the diagnosis of seven deletional α-thalassaemia alleles. The two common α^+-thalassaemia deletion genes ($-\alpha^{3.7}$ and $-\alpha^{4.2}$) are diagnosed in one reaction, the three Southeast Asian α^0-thalassaemia deletions ($^{-SEA}$, $^{-THAI}$ and $^{-FIL}$) in a second reaction, and two Mediterranean α^0-thalassaemia deletions ($^{-MED}$ and $-(\alpha)^{20.5}$) in a third reaction

Table IV. Globin gene deletion mutations diagnosable by gap-PCR.

Disorder	Mutation	Distribution
α^0-thalassaemia	$^{-SEA}$	Southeast Asia
	$^{-MED}$	Mediterranean
	$-(\alpha)^{20.5}$	Mediterranean
	$^{-FIL}$	Philippines
	$^{-THAI}$	Thailand
α^+-thalassaemia	$-\alpha^{3.7}$	Worldwide
	$-\alpha^{4.2}$	Worldwide
β^0-thalassaemia	290 bp deletion	Turkey, Bulgaria
	532 bp deletion	African
	619 bp deletion	India, Pakistan
	1393 bp deletion	African
	1605 bp deletion	Croatia
	3.5 kb deletion	Thailand
	10.3 kb deletion	India
	45 kb deletion	Philippines, Malaysia
Hb Lepore	Hb Lepore	Mediterranean, Brazil
$(\delta\beta)^0$ thalassaemia	Spanish	Spain
	Sicilian	Mediterranean
	Vietnamese	Vietnam
	Macedonian/Turkish	Macedonia, Turkey
$(^A\gamma\delta\beta)^0$ thalassaemia	Indian	India, Bangladesh, Kuwait
	Chinese	Southern China
HPFH	HPFH1	Africa
	HPFH2	Ghana, Africa
	HPFH3	India
	Hb Kenya	Africa

[22–24]. Care has to be taken to minimize the possible problem of allele drop-out. In carriers where the ethnic group is not known or different, screening for both sets of α^0-thalassaemia mutations is recommended.

α^+-Thalassaemia point mutations. – The non-deletion α^+-thalassaemia mutations can be identified by all PCR techniques for point mutation analysis, following selective amplification of the α-globin genes [25]. Several of the non-deletion α^+-thalassaemia mutations create or destroy a restriction enzyme site and may be analysed by restriction enzyme digestion of the amplified product. For example, Hb Constant Spring mutation can be diagnosed by *Mse* I digestion [26]. For undefined mutations, DNA sequence analysis is the method of choice, although a method combining DGGE and single strand conformation analysis (SSCA) with DNA sequencing has been used [27].

(4) *Abnormal haemoglobins*

More than 700 haemoglobin variants have been described to date (an updated list can be accessed on the internet at http://www.globin.cse.psu.edu/), of which the clinically most important ones that can be diagnosed by the simple DNA analysis methods are described below. A definitive identification of the other variants requires DNA or amino acid sequence analysis, although a few have had ARMS primers developed for their quick diagnosis [28].

Hb S. – The Hb S mutation CD6 (A→T) destroys the recognition site of the restriction enzyme Dde I at codon 6. Diagnosis of the mutation by Dde I digestion of amplified globin gene fragment is the method of choice. This test also gives a positive signal for the two β-thalassaemia mutations CD6 (-A) and CD5 (-CT). Thus care must be taken when analysing the genotypes of patients with possible Hb S/β-thalassaemia. Care is also required in analysing patients with possible Hb S/HPFH or Hb S/$\delta\beta$-thalassaemia phenotypes, as these appear to have a Hb SS genotype by Dde I analysis. A family study of the parental phenotypes is often required for the investigation of genotypes of patients with sickle cell disorders. Other PCR-based techniques, such as dot blotting or ARMS, are also used for confirmation of the Hb S mutation in carriers.

Hb C. – The Hb C mutation, CD6 (G→A), unfortunately does not destroy the *Dde* I site at codon 6. Thus ARMS or ASO dot blotting must be used to detect the Hb C mutation.

Hb D-Punjab and Hb O-Arab. – The Hb D-Punjab mutation, CD121 (G→C), and the Hb O-Arab mutation, CD121 (G→A), destroy an *Eco*R I site at codon 121, and diagnosis may be carried by *Eco*R I digestion of amplified globin gene fragment. However, this assay does not distinguish between the two variants (and also the rare variant Hb D-Neath), and thus the DNA result must be combined with HPLC or electrophoresis data to identify each variant in carriers. An ARMS primer is now used in my laboratory to diagnose Hb D-Punjab.

Hb E. – The Hb E mutation, CD26 (G→A), abolishes an *Mnl* I site and may be diagnosed by PCR amplification and restriction enzyme analysis of the product. However, the digestion products are quite small in size due to nearby *Mnl* I sites and the Hb E mutation is better diagnosed by the use of ASO probes or ARMS primers.

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