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Best Practice Guidelines for carrier identification and prenatal diagnosis of haemoglobinopathies

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Guidelines prepared following an EMQN Best practice workshop held on the 2nd March 2002 in Manchester, United Kingdom, with thanks to all participants and especially Professor S.L. Thein, Dr. B. Wild, Dr A. Stephens and Dr. I. Papassotiriou for helpful comments on haematology screening.

DISCLAIMER

These Guidelines are based, in most cases, on the reports drawn up by the chairs of the disease-based workshops run by EMQN and the CMGS. These workshops are generally convened to address specific technical or interpretative problems identified by the QA scheme. In many cases, the authors have gone to considerable trouble to collate useful data and references to supplement their reports. However, the Guidelines are not, and were never intended to be, a complete primer or "how-to" guide for molecular genetic diagnosis of these disorders. The information provided on these pages is intended for chapter authors, QA committee members and other interested persons. All the guidelines are at a draft stage, and must not be used until formally published. Neither the Editor, the European Molecular Genetics Quality Network, the Clinical Molecular Genetics Society, the UK Molecular Genetics EQA Steering Committee nor the British Society for Human Genetics assumes any responsibility for the accuracy of, or for errors or omissions in, these Guidelines.

1. GENERAL BACKGROUND

1.1 Description of the disease group

Haemoglobinopathies constitute the commonest monogenic disorders worldwide (1). They are caused by mutations which affect the genes that direct synthesis of the globin chains of haemoglobin, and may result in reduced synthesis (thalassaemia syndromes) or structural changes (haemolytic anaemia, polycythemia or more rarely cyanosis).

Thalassaemia mutations and various abnormal haemoglobins interact to produce a wide range of disorders of varying degrees of severity. There are four main categories of interactions associated with severe disease states, for which genetic counselling and prenatal diagnosis is indicated (2):

Thalassaemia major (co-inheritance of β- and/or δβ-thalassaemia mutations),

a) Sickle cell disease (and analogous interactions e.g. Hb S/C, Hb S/β-thalassaemia, Hb S/D Punjab, Hb S/O Arab, Hb S/Lepore)
b) Hb E thalassaemia (co-inheritance of β-thalassaemia mutations with Hb E)
Hb Bart's Hydrops Fetalis syndrome (homozygous α^0-thalassaemia), and (rarely) Hb H Hydrops Fetalis syndrome ($\alpha^0/\alpha^T\alpha$).

In most populations β-thalassaemia syndromes (and related haemoglobinopathies) are clinically more relevant than the α-thalassaemias, since the severe forms are more common and require life-long treatment and clinical management. In contrast the severest form of α-thalassaemia, Hb Bart's Hydrops Fetalis, is incompatible with post-natal life, although prenatal diagnosis is always indicated to avoid severe toxaemic complications that occur frequently in pregnancies with hydropic fetuses.

1.2 The genes and disease-causing mutations

The major haemoglobin in adult life is HbA, a tetramer composed of two alpha and two beta globin chains ($\alpha_2\beta_2$). The gene encoding β-globin chains is located on the short arm of chromosome 11 (11p15.15), within the so-called β-gene cluster, and that encoding α-globin chains is located on the short arm of chromosome 16 (16p13.3), within the so-called α-gene cluster (1).

More than 180 mutations causing β-thalassaemia have been described, the majority of which are point mutations, and more than 80 α-thalassaemia mutations have been reported, most

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of which involve deletions from within the α-gene cluster (3). In addition more than 800 mutations causing structural variants have been characterized (3).

1.3 Approaches for carrier detection

Haemoglobinopathies are possibly unique amongst all genetic diseases in that identification of carriers is possible (and preferable) by haematological (biochemical) tests rather than DNA analysis. Any at-risk couples can then be offered reproductive choice and avoid the birth of an affected child by undergoing prenatal diagnosis, which involves mutation characterization in the parents and subsequent fetal DNA analysis. Thus genetic services for haemoglobinopathies require close collaboration between several specialities, most notably haematology and molecular genetics.

These guidelines will focus on best practice in laboratory methods and interpretation of results, but before proceeding to the methods, we wish to note some factors that should be taken into account when deciding the best strategy for a population screening programme aimed at detecting at-risk couples:

a) Frequency of the disease
b) Heterogeneity of the genetic defects
c) Knowledge of genotype-phenotype correlation
d) Resources available
e) Social, cultural and religious factors

In addition the target group for screening may include newborn, adolescent, premarital, preconceptional or antenatal, although for haemoglobinopathies, preconceptional or antenatal screening is most widely applied in most populations.

2. HAEMATOLOGICAL METHODS FOR CARRIER DETECTION

"Screening" is distinct from "definitive" diagnosis in that the purpose of screening is to test for a defined set of conditions using simple biochemical tests. Screening programmes are designed using a protocol of first and second line methods in order to obtain a reliable diagnosis, which is essentially a presumptive diagnosis. If an unequivocal, definitive diagnosis is required, characterisation methods based on either protein or DNA analysis must be utilized.

With the thalassaemias, screening will detect most cases of beta thalassaemia trait. There is however no specific screening test for alpha thalassaemia trait which often remains a diagnosis made by exclusion. If an abnormal haemoglobin is found, the results obtained constitute a presumptive identification of the haemoglobin. It is important to remember that with phenotypic screening it is possible that some rarer conditions will not be detected and this has to be taken into account in the interpretation and reporting of data. For all samples, screening using haematological methods is the first step in genetic diagnosis (4).

Good laboratory practice also includes the minimization of clerical errors, particularly crucial in haematology laboratories undertaking large numbers of samples for carrier screening, sometimes numbering>1000 blood counts each day. Careful sample identification is essential (including: Full Name, Date of Birth, Sample date, if transfused in last 4 months). Bar coding is recommended. Laboratory error rates for methods utilized (if known) should be available to patients.

2.1 Basic haematology methods

2.1.1.Complete Blood Count

Recommended method: electronic measurement.

Interpretation of results: All red cell indices (and other parameters) are important in evaluation, including Hb, RBC, MCH, MCV and RDW. Important cut-off values indicating possible heterozygosity for thalassaemia include MCV <78fl and MCH <27pg. Note: Evaluation of blood count in samples >24hours old should be made with caution, as the red cells increase in size, leading to falsely raised MCV (although different analysers have variable sensitivity to this problem). .

2.1.2. Haemoglobin (Hb) pattern analysis

For a presumptive identification of an abnormal haemoglobin **methods include**:

a) *Haemoglobin electrophoresis at pH 8.6 using cellulose acetate membrane* - This method will reliably detect the common haemoglobin variants, i.e. Hb's S, C, D^{Punjab}, E, O^{Arab} and the Lepore Hbs. Hb H and Hb Barts may also be detected if suitable run times are used. Many other variants are also detectable, e.g. J's, N's, Q's, Hasharon.
b) *Haemoglobin electrophoresis at pH 6.0 using acid agarose or citrate agar gel* - This method is useful for distinguishing Hb's C, E, and O^{Arab} from each other, also Hb S and Hb D^{Punjab} from each other. Note that the migration patterns are different for acid agarose gels and citrate agar gel.
c) *Isoelectric focusing (IEF)* - IEF is a sensitive method, giving good separation of haemoglobin variants but requires considerably more expertise for interpretation than

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electrophoresis since adducted fractions also separate.

d) *High Performance Liquid Chromatography (HPLC)* - This method is recommended for simultaneous detection and quantitation of haemoglobin fractions. Since the systems are automated, operation of the analysers is simple, but interpretation of the chromatograms requires expertise. Also, attention must be paid to quality control, especially for measurement of Hb A_2. Although the cost per test is relatively high, the application is useful for large scale screening programmes.

Recommendations

a) In the presence of an abnormal haemoglobin, the use of a single test to establish presumptive identification is inappropriate and second or even third line testing procedures should be in place.
b) On most HPLC systems, derivatives of Hb S may co-elute with Hb Ao and Hb A_2; thus whenever Hb S is present, it is essential to run alkaline or acid electrophoresis to determine if Hb A is present.
c) To quantitate Hb A_2 in the presence of Hb S, electrophoresis and elution, or microcolumn chromatography with appropriate reagents for Hb S are recommended methods, rather than HPLC (although the presence of at least 50% Hb A should exclude co-existing β-thalassaemia).
d) Always analyse fresh blood samples if Hb H disease is suspected, as Hb H is unstable.

2.1.3. Quantitation of Hb A_2

Methods include:

a) *Hb electrophoresis with automatic densitometry* – not recommended.
b) *Electrophoresis and elution* – accurate but time-consuming.
c) *Microchromatography* - accurate but time-consuming.
d) *HPLC* – accurate in the absence of variants (see above) and high-throughput

Interpretation of results: Important cut-off value indicating heterozygosity for β-thalassaemia: Hb A_2 >3.5%. Borderline levels of 3.1-3.5% (depending upon laboratory) indicate further investigation required (**see Tables 2.3.1 and 2.3.2**).

2.1.4. Quantitation of Hb F

Methods include:

a) *Alkali denaturation* - The modification by Pembrey et al (5) has excellent reproducibility, in most ranges of Hb F, giving worthwhile results in virtually all clinical situations (if used carefully).
b) *HPLC* – on some systems may be inaccurate for Hb F values <1%, although for the Biorad HPLC system, accurate quantitation of Hb F can be achieved using the lytic solution for Hb A_{IC}.
c) Interpretation of results: Important cut-off value indicating heterozygosity for δβ-thalassaemia are Hb F>5% in the presence of low red cell indices and a normal Hb A_2 level. However, Hb F may increase up to 3% in pregnancy, making values in the range of 3-5% difficult to interpret. Values above 5% may indicate the presence of heterocellular HPFH. Follow-up at 6 months post natal would clarify the individual's normal level.

Note: WHO International Reference Reagents are available for Hb A_2 quantitation by electrophoresis & elution, microcolumn chromatography, and by HPLC. Also for Hb F quantitation using the 2 minute alkali denaturation method by Pembrey et al (5).

2.2 Supplementary haematological methods

2.2.1.Iron (Fe) status

a) *Zinc protoporphyrin (ZnPP)* – sample can be analysed from same tube as blood count, and sample is stable for long time period. Analysis is fast, simple and cheap, although it requires specific instrument. ZnPP is elevated in iron deficiency, but may be falsely high in lead intoxication or if the bilirubin levels are raised.
b) *Ferritin* – most popular test for indicating iron deficiency, but it is expensive and may be falsely high during infection, liver disease or neoplasia.
c) *Transferrin saturation (Iron/Total Iron Binding Capacity)*– more accurate than ferritin but there is no internationally recognized standard protocol.

Interpretation of results: Measurement of iron status in samples with hypochromic, microcytic indices but with normal Hb A_2 and F is useful to distinguish between cases of uncomplicated iron deficiency and those with possible alpha thalassaemia trait or silent beta thalassaemia trait in whom the iron status is normal. This is a useful approach not only to prevent unnecessary further investigation but in some cases inappropriate iron therapy. However, it is important to note that iron deficiency can co-exist with the thalassaemias, and such cases could be misinterpreted. It is sometimes necessary to recommend repeating the

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haematology screen after correction of iron deficiency (assuming that there is no time limit with an on-going pregnancy).

2.2.2. Globin chain synthesis

May provide useful information for diagnosing atypical cases.

a) *CMC chromatography method*. - Very accurate but time consuming method for evaluating relative rate of globin chains synthesised in reticulocytes.
b) *HPLC* – Potentially a less time consuming method, but it needs careful standardisation to be accurate and reliable.
c) *IEF*- rapid and convenient, with potential to process multiple samples simultaneously (6).

2.2.3. Globin chain separation

Can be undertaken either by *HPLC or IEF*, and is useful for indicating which globin chain is affected, thus giving evidence for the nature and potential significance of the variant.

2.2.4. Functional tests for Hb variants

a) *Sickle tests* - if there is an abnormal fraction that runs in the position of Hb S, then the sickle solubility test should be undertaken. **Note**: Some other (rare) haemoglobins also have reduced solubility and thus have a positive solubility test but do not migrate to the same position as Hb S.
b) *Heinz body formation* – not very specific, but useful for detecting presence of unstable variants
c) *Oxygen dissociation curve* – maybe useful for implicating presence of Hb variants with altered oxygen affinity.

2.2.5 Mass spectrometry

Specialized method based on analysing tryptic digests of whole blood. Although there may be only a small shift in mass for some common variants, it is very effective for the characterisation of Hb variants especially if used in conjunction with other methods (7).

2.2.6. Immunological measurement of F-cells

Specialized method which uses monoclonal antibodies against γ-globin chains to label Hb F containing red cells. F–cells can be counted using fluorescently-activated cell sorting (FACS) or on a slide as a smear. Useful for distinguishing between heterocellular and pancellular conditions of HPFH (amongst more specialized applications).

3. MOLECULAR DIAGNOSIS

Almost all methods for DNA analysis of haemoglobinopathies currently in use are based on the polymerase chain reaction. There are now many different PCR-based techniques that can be used to detect the globin gene mutations, including 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. All are recommended for use as best practice, each method having its own advantages and disadvantages. (see Table 3.2.1). The particular methods chosen by a laboratory for the diagnosis of the globin gene point mutations or deletions 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 (population groups) being tested. It is best practice for any DNA diagnostic laboratory to have at least two alternative methods for detecting each mutation.

3.1.1 Diagnostic strategy

The haemoglobinopathies are regionally specific, with each population having a unique combination of abnormal haemoglobins and thalassaemia disorders. The spectrum of mutations and the mutation frequencies have been published for most populations, usually consisting of a limited number of common mutations and a slightly larger number of rare mutations (8). Therefore knowledge of the ethnic origin of a patient simplifies the diagnostic strategy, enabling a quick identification of the underlying defects in most cases.

3.1.2 α-Thalassaemia

Gap-PCR (amplification across the breakpoints of a deletion) provides a quick diagnostic test for α^+-thalassaemia and α^o-thalassaemia deletion mutations but requires careful application for prenatal diagnosis, since the method may be susceptible to false negative results caused by allele drop out (ADO). The first gap-PCR assays were subject to technical failure through allele drop out but more recent published primers and conditions result in more robust assays (9,10).

Most of the common α^0-thalassaemia deletions can be diagnosed by gap-PCR: the $--^{SEA}$ allele, found in Southeast Asian individuals; the $--^{MED}$ and $-(\alpha)^{20.5}$ alleles found in Mediterranean individuals; the $--^{FIL}$ allele, found in Fillipino individuals and finally the $--^{THAI}$ allele, found in Thai individuals. Two α^+-thalassaemia deletions can be diagnosed by gap-PCR: the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ alleles. The former is found in African, Mediterranean, Asian and Southeast

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Asian populations, while the latter is found in Southeast Asia and the Pacific populations. However it is good practice to screen for both deletions in any individual suspected of having α^+-thalassaemia.

Southern blotting using ζ-gene and α-gene probes must be used to diagnose all the other α^o and α^+-thalassaemia deletion mutations. This approach also detects α-gene rearrangements (the triple and quadruple α-gene alleles).

α^+-Thalassaemia may also be caused by point mutations in one of the two α-globin genes. These non deletion alleles can be detected by PCR using a technique of selective amplification of each α-globin gene followed by a general method of mutation analysis such as SSCP or DNA sequence analysis (11). Several of the non deletion mutations alter a restriction enzyme site and may be diagnosed by selective amplification and restriction endonuclease analysis, e.g. the mutation for Hb Constant Spring in Asians, or the ATG→ACG α2 gene mutation and the IVS1 donor site -GAG↑GT-deletion in Mediterraneans (12). If the common non deletion mutations in the local population are known, the use of mutation-specific tests is recommended.

3.1.3 β-Thalassaemia

A limited number of β-thalassaemia mutations are prevalent in most of the populations at risk for severe thalassaemia and in practice this permits the most appropriate probes or primers to be selected according to the carrier's ethnic origin. The most commonly used screening procedures for known mutations are the reverse dot blot analysis with allele specific oligonucleotide probes (13), and primer specific amplification (ARMS) (14). Restriction enzyme analysis of amplified β gene product is useful for a limited number of mutations (15).

When a β thalassaemia mutation can not be defined by one of the direct mutation detection methods, characterization of the mutation may be done by using denaturing gradient gel electrophoresis (DGGE) (16) or single-strand conformation polymorphism (SSCP) analysis (17) to localize possible mutations within the β-globin gene, followed by direct sequencing on amplified single-strand DNA either manually or automatically (18). Alternatively, for those laboratories which have access to automated DNA sequencing facilities it may be more efficient to proceed directly to DNA sequence analysis if a mutation has not been identified by the techniques for known mutations. DGGE is also useful for directing mutation identification when using mutation specific assays such as ARMS or RE-PCR (see Table 3.2.1), since most mutations have characteristic heteroduplex patterns with DGGE analysis.

Small deletions are detected by polyacrylamide gel electrophoresis of the amplified β gene product. Some of the larger deletions that remove the β globin gene may be identified by gap-PCR (including Hb Lepore, some δβ-thalassaemia deletions and the HPFH1/2/3 deletion mutations) (19), or by Southern blot analysis.

3.1.4 Common Hb Variants

The clinically important variants, Hb S, Hb C, Hb E, Hb D^{Punjab} and Hb O^{Arab}, can be diagnosed by dot blot hybridisation, the ARMS technique or direct sequencing. All except Hb C can also be diagnosed by restriction endonuclease digestion of amplified β gene product (RE-PCR). For the many other haemoglobin variants, positive identification at the DNA level is achieved by selective globin gene amplification and DNA sequence analysis.

3.2 ADVANTAGES AND DISADVANTAGES OF MUTATION DETECTION METHODS

3.2.1 Detection of known mutations (see table 3.2.1)

3.2.2 Indirect mutation detection or detection of unknown mutations (see table 3.2.2)

4. FETAL DNA ANALYSIS

It is best practice for all couples undergoing prenatal diagnosis to be counselled by a qualified health professional well versed in the molecular diversity of the haemoglobinopathies. No woman should undergo prenatal diagnosis unless she has been counselled by a qualified health professional, and preferably been provided with appropriate information leaflets. A good selection of these are available on the web-page www.chime.ucl.ac.uk/APoG1.

Problems related to PCR-based prenatal diagnosis include the high sensitivity to maternal DNA contamination and the complex battery of probes and primers necessary to detect a wide range of thalassaemia mutations. The following procedures are intended to minimise the diagnostic error rate.

4.1.1 Parental Blood Samples

1. Copies of haematology results should be sent to molecular diagnostic laboratory.

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2. Blood samples should be obtained from both parents to confirm phenotype of parents by full blood count and haemoglobinopathy screen such as electrophoresis and as source of control DNA for the molecular analysis.

This should be repeated with every prenatal diagnosis that a couple undergoes.

4.1.2 Partner not available for testing

There are cases where a carrier woman requests prenatal diagnosis although her partner is unavailable for testing. In such situations it is important to evaluate risk of a major haemoglobinopathy in the fetus.

1. For a sickle cell trait mother and untested partner
2. If an AS genotype is diagnosed in the fetus then test for common beta thalassaemia mutations and any other haemoglobinopathy genes (especially β^C or β^D) known to exist in the partner's ethnic group.
3. For a beta thalassaemia trait mother and untested partner
4. If the mother's β thalassaemia mutation is diagnosed in the fetus, the possibility of the fetus being homozygous or compound heterozygous for beta thalassaemia should be excluded by testing for the β thalassaemia mutations and any other β haemoglobinopathy genes found in the fathers ethnic group. Alternatively the fetal DNA sample may be sequenced.

4.2 Fetal Sampling

There are three possible procedures, chorionic villus sampling, amniocentesis and fetal blood sampling. Prenatal diagnosis of haemoglobinopathies should preferably be carried out by a chorionic villus sample in the first trimester of pregnancy (10-12 weeks).

4.2.1 Chorionic Villus Sampling

1. Provides good source of DNA
2. Risk of maternal contamination is low with careful microscopic dissection to remove contaminating maternal decidua.
3. There is a risk of maternal contamination if sample is cultured, although this should not be necessary if sample is of adequate size.
4. Risk of miscarriage is low if sample taken in experienced centre
5. Result available early in pregnancy

4.2.2 Amniocentesis

1. Amniocytes can be used for molecular analysis directly spun down from the amniocentesis sample. This usually yields sufficient DNA for analysis with PCR-based methods.

NOTE: Direct analysis should be carried out with caution as the fetal cells are invariably contaminated with maternal cells.

2. For greater amounts of fetal DNA, samples have to be cultured for 10-14 days. Culture of the cells reduces risk of maternal contamination, but result is delayed.
3. Risk of miscarriage following amniocentesis is low if sample taken in experienced centre.
4. Result available later in pregnancy as amniocentesis cannot be performed earlier than about the 16th week.

4.2.3 Fetal Blood Sampling

1. 1-2 ml of fetal blood obtained, which can be used for molecular analysis or globin chain biosynthesis studies. The latter can be used when parental mutations are not known, if a couple present late, or if partner is unavailable for testing. The diagnosis is based on the relative synthesis of β-globin (representing HbA) and γ- globin (representing HbF). A β/γ chain synthesis ratio above 0.02-0.03 (slightly variable between laboratories) indicates an unaffected fetus. **Note:** When using this technique results should be interpretated with care as mild β^+ mutations can produce higher levels of β globin, leading to risk of misdiagnosis (20). Overall globin chain biosynthesis in fetal blood is no longer used by most centres
2. Higher rate of miscarriage
3. Carried out late in pregnancy (after 18-20 weeks).

4.3.Molecular Analysis

4.3.1 Genotype Analysis

The laboratory carrying out the molecular analysis should choose the technique(s) that best suits their laboratory, expertise and population. The techniques have been discussed in section 3.

1. Always analyse parental and the appropriate control DNA's simultaneously with the fetal DNA and use a blank control sample.
2. Perform duplicate tests to minimize human errors.
3. To monitor potential laboratory errors such as partial digestion or allele drop use two independent diagnostic methods on each sample for each mutation being investigated.
4. Use a limited number of amplification cycles to minimise co-amplification of any maternal DNA.

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4.3.2 Maternal Contamination

Polymorphism analysis excludes maternal contamination (and may also identify non-paternity).

1. Check for maternal DNA contamination in every case ESPECIALLY when the fetal genotype is same as mother's genotype. The choice of polymorphic markers available is wide, including Short Tandem Repeat (STR) markers such as D21S11, D21S1414, D18S535 (21) or Variable Number Tandem Repeat (VNTR's) markers such as ApoB, IgJH and Has-ras (22).
2. When the fetal genotype is same as the mother's, and no informative marker to indicate presence/absence of maternal contamination is found, the fetal diagnosis report should state these findings and indicate greater risk of error in fetal result.

4.4 Patient consent and Reports

1. There should be a consent form signed by patient and counsellor accompanying the fetal sample.
2. The fetal DNA report should detail types of DNA analysis performed and clearly state the risk of misdiagnosis based on reported technical errors of the protocols utilized. Laboratory error rates should be documented and explained to patients for all methods.

4.5 Prenatal diagnosis follow-up

1. Ideally confirm fetal DNA diagnosis at birth through a request for cord blood sampling that can be sent out with fetal diagnosis report. Haematological, haemoglobin and DNA analysis also requested by some centres.
2. Ideally foetal material should be requested when affected pregnancies are terminated to confirm prenatal diagnosis result.

4.6 Audit

National registers should exist to audit services for prenatal diagnosis. In the UK the three diagnostic laboratories enter data for each diagnosis onto a shared register and aggregated data can be used for national audit of antenatal carrier screening and utilisation of Prenatal Diagnosis by risk, ethnic group and region (23). It can also be used to report on the accuracy of prenatal diagnosis (24). Audit should be an on-going activity that aims to identify any weaknesses in the prenatal diagnosis services, directing ways for improvement.

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Table 2.3.1 Possible interpretation of haematology not consistent with typical β-thalassaemia trait

Haematological Parameters	**Possible interpretation**
Reduced red cell indices & normal Hb electrophoresis (including Hb A_2)	Iron deficiency heterozygous α-thalassaemia heterozygosity for mild β-thalassaemia mutations (sometimes Hb A_2 is borderline raised) co-inheritance of heterozygous δ- with β-thalassaemia heterozygous γδβ-thalassaemia
Normal/borderline reduced red cell indices with raised Hb A_2	Interaction of α- with β-thalassaemia
Normal or reduced red cell indices with raised Hb F (and normal HbA_2).	Heterozygous δβ-thalassaemia or HPFH.
Normal red cell indices with normal HbA_2	Triplication of alpha genes (when implicated in family studies), or mild β-thalassaemia mutation.

Note: Some Hb variants are not detected by electrophoretic or chromatographic procedures, but may be suspected due to the presence of abnormal haematological parameters and/or clinical symptoms. In such cases it is recommended that samples are analysed using mass spectrometry or DNA methods. Occasionally hyperunstable variants are present and these may only be found by DNA methodology as the protein produced is so unstable.

Table 2.3.2 Genotypes associated with borderline/normal Hb A_2 levels – a guideline of related haematological and biosynthetic characteristics.

GENOTYPE	**MCV fl**	**MCH pg**	**HbA_2**	**α/β ratio**
β -101 (C→T)	88.5 ± 7.8	30.1 ± 1.0	3.1 ± 1.0	1.3 ± 0.4
β -92 (C→T)	83.0 ± 6.0	28.3 ± 2.0	3.5 ± 0.4	1.3 ± 0.8
β +33 (C→G)	82.0 ± 9.2	27.1 ± 3.4	2.5 ± 1.4	1.3 ± 0.6
Cap+1 (A→C)	23-26*	75-80*	3.4-3.8*	-
β IVS1-6 (T→C)	71.0 ± 4.0	23.1 ± 2.2	3.4 ± 0.2	1.9 ± 1.0
βIVS2-844 (C→G)	96.0 ± 4.0	30.3 ± 1.8	3.2 ± 0.2	1.0 ± 0.6
β +1480 (C→G)	88.3 ± 9.5	27.9 ± 6.0	2.7 ± 0.8	1.6 ± 0.4
ααα/αα	85.5 ± 7.8	30.4 ± 5.0	2.8 ± 0.6	1.2 ± 0.4
δ + β thalassaemia	67.6 ± 7.6	21.8 ± 3.6	3.3 ± 0.4	1.7 ± 0.6

Values (mean±2SD or range*) are a **guideline** and represent those reported in various studies on carriers of these mutations (prepared by R.Galanello).

Note: It is recommended that subjects with borderline HbA_2 levels, particularly spouses of a typical β-thalassaemia carrier, should be extensively investigated (α and β gene analysis, globin biosynthesis), although the majority usually have normal β and α globin genes. Borderline-raised HbA_2 levels in normal individuals is probably explained as the extreme distribution of the normal range of the HbA_2.

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3.2.1 *Detection of known mutations*

Advantages	Disadvantages
ASO dot blot hybridisation • Widely applicable and reliable	***ASO dot blot hybridisation*** • Traditional protocols use radioactively labeled probes • Time consuming and can only screen one mutation at a time • Expensive
Reverse dot blot hybridisation (RDB) • Simultaneous screening for many mutations • Usually no radioactivity • Relatively inexpensive • Simple, rapid & reliable	***Reverse dot blot hybridisation (RDB)*** • Need sample controls to standardize new mutations • Need good technical expertise in the laboratory to set up and validate RDB • Kits not always be reliable (experience of some labs demonstrates batch to batch variation)*
ARMS-PCR • Simple, rapid & inexpensive • Suitable for technical modification • Can be multiplexed to detect >1 mutation	***ARMS-PCR*** • Need control DNA to validate test and some rare mutations unavailable in homozygous state • Primers can degrade, giving non-specific signal
GAP-PCR • Simple, rapid & inexpensive • Can be multiplexed to detect >1 mutation	***GAP-PCR*** • Need control DNA to validate test • Limited to diagnosis of deletions with known DNA breakpoint sequences • Amplification of α-genes technically difficult • Possibility of allele drop-out (and thus not recommended for prenatal diagnosis especially for homozygosity of α^{o}-thalassaemia)
Restriction enzyme (RE)-PCR • Simple & rapid • Reliable	***Restriction enzyme (RE)-PCR*** • Not all mutations are amenable • Need care to avoid partial digestion problems • 'Frequent cutter' enzymes not very useful • Some enzymes costly

*There are a limited number of commercial kits available, but as any other method they should not be used in the absence of alternative methods in the diagnostic lab.

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3.2.2 *Indirect mutation detection or detection of unknown mutations*

Advantages	Disadvantages
DGGE • Relatively cheap. • Suitable for large scale screening. • Characteristic patterns due to heteroduplexes. • Predictive computer programs make it easier to optimize.	***DGGE*** • Experience required to interpret results as DGGE detects polymorphic as well as disease-causing mutations • CG-rich regions difficult to investigate • Sometimes laborious to optimise conditions • Overall DGGE is technically demanding.
SSCP • Use of automated equipment makes it rapid, reproducible and relatively simple. • Very sensitive especially for micro-deletions/insertions. • GC-richness not the limiting factor (as for DGGE).	***SSCP*** • Manual methods may require use of radioactivity for DNA labeling. • Sensitivity decreases with fragment length. • Optimisation highly empirical. • No distinction between different substitutions at the same position. • Relatively expensive.
Direct sequencing (Automated) • Use of automated sequencers makes it more rapid and easier. • In some systems ddNTP's can be labelled, precluding use of modified primers • Mutation directly characterised.	***Direct sequencing (Automated)*** • Relatively expensive investment. • PCR-products need to be purified.

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