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Guideline

THE LABORATORY DIAGNOSIS OF HAEMOGLOBINOPATHIES*,†

SUMMARY

The laboratory diagnosis of haemoglobinopathies, including the thalassaemias, is of growing importance, particularly because of an increasing requirement for antenatal diagnosis of significant disorders of globin chain synthesis. This guideline discusses the laboratory tests which are most useful in the diagnosis of haemoglobinopathies and describes their role in specific clinical circumstances. Of the newer technical methods, high-performance liquid chromatography (HPLC) is of considerable importance whereas isoelectric focusing (IEF) and immunoassay for variant haemoglobins have a more minor role.

Specific recommendations have been formulated for testing in relation to genetic counselling and for neonatal diagnosis. Methods used in specialized laboratories for fetal diagnosis have been tabulated. Genetic counselling requires: (i) identification of haemoglobins S, C, D-Punjab, O-Arab, E, Lepore and H, and (ii) the detection of carriers of α° and β thalassaemia. It is recommended that subjects of all ethnic groups be screened for β-thalassaemia trait, all except Northern European Caucasians for variant haemoglobins, and selected ethnic groups for α°-thalassaemia trait. Testing for β-thalassaemia trait should be carried out when the mean cellular haemoglobin (MCH) is < 27 pg and testing for α°-thalassaemia trait should be considered when the MCH is < 25 pg. Appropriate methods include HPLC or haemoglobin electrophoresis for identification of variant haemoglobins and HPLC or microcolumn chromatography for quantification of haemoglobin A2.

INTRODUCTION

Disorders of globin chain synthesis, both thalassaemias and structurally abnormal haemoglobins, are common in the U.K. and constitute a significant public health problem. Diagnosis may be required: (i) to confirm a provisional diagnosis such as sickle cell disease or β-thalassaemia major; (ii) to explain a haematological abnormality such as anaemia or microcytosis; (iii) to identify an abnormality in the presymptomatic phase, as in neonatal screening; (iv) to predict serious disorders of globin chain synthesis in the fetus and offer the option of termination of pregnancy; (v) to permit genetic counselling of prospective parents; (vi) as preoperative screening for the presence of sickle cell haemoglobin. Improved fully automated systems and reagents for techniques such as high-performance liquid chromatography (HPLC) and isoelectric focusing (IEF) have led to their introduction in many laboratories. Immunological methods for the identification of variant haemoglobins have also become available. There is therefore a need for an updated guideline defining the role of new techniques in relation to traditional techniques. To save repetition, previous guidelines (Globin Gene Disorder Working Party of the BCSH General Haematology Task Force, 1994; The Thalassaemia Working Party of the BCSH General Haematology Task Force, 1994) should be consulted. This guideline discusses techniques and defines their place in specific diagnostic settings. The detection of unstable haemoglobins, methaemoglobins and high oxygen affinity haemoglobins is not discussed but laboratories should either have methods for detecting these variant haemoglobins or should refer such samples to a reference laboratory.

It should be noted that the identification of haemoglobins is often presumptive, based on a characteristic electrophoretic mobility or other characteristics in an individual of appropriate ethnic origin. Definite identification usually requires DNA analysis or amino acid sequencing. Family studies are also of considerable importance in elucidating the nature of disorders of haemoglobin synthesis.

TECHNIQUES

Full blood count

A full blood count (FBC) is usually indicated in all individuals being investigated for a suspected disorder of globin chain synthesis. The exception is in neonatal screening. Red cell indices are of particular importance in screening for β- and α°-thalassaemia trait and in distinguishing between $\delta\beta$-thalassaemia and hereditary persistence of fetal haemoglobin.

When are further tests indicated? A blood film is useful, in addition to a blood count, when an unstable haemoglobin, sickle cell disease or thalassaemia is suspected. A reticulocyte count is indicated if haemolysis is likely, e.g. if an unstable haemoglobin, haemoglobin H disease or sickle cell disease is suspected. In addition, definitive tests to identify normal and variant haemoglobins are indicated in suspected

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haemoglobinopathies, regardless of the results of the full blood count and film.

Haemoglobin electrophoresis on cellulose acetate at alkaline pH (pH 8·2–8·6)

Cellulose acetate electrophoresis enables the provisional identification of haemoglobins A, F, S/G/D, C/E/O-Arab, H and a number of less common variant haemoglobins. With good electrophoretic techniques, haemoglobin F levels >2% can be recognized visually; when an increased level is detected, quantification is required. Good techniques also enable a split A2 band to be recognized. This is useful in helping to distinguish α-chain variants, e.g. haemoglobin G Philadelphia, from β-chain variants, e.g. haemoglobin D Punjab. It is also essential if β-thalassaemia trait is to be diagnosed in individuals who also have a δ-chain variant (see below).

Haemoglobin A2 can be quantified by cellulose acetate electrophoresis followed by elution and spectrometry, but this is a labour-intensive technique if large numbers of samples require testing. Quantification of haemoglobin A2 by scanning densitometry is not recommended as the precision is not good enough for the diagnosis of β-thalassaemia trait (ICSH, 1978).

Variant haemoglobins, including haemoglobin S, can be quantitated by scanning densitometry (Legg *et al*, 1995). However, it is necessary to have good separation of bands and it should be noted that this method is imprecise if the concentration is low, e.g. haemoglobin F can be quantified with reasonable precision only when it is appreciably elevated. Quantification of variant haemoglobins can be diagnostically useful, e.g. in helping to distinguish haemoglobin Lepore from heterozygosity for haemoglobins S/D/G and in the differential diagnosis of sickle cell/β^+ thalassaemia (Sβ^+ thal) and sickle cell anaemia (SS).

When are further tests needed? Patients who show a band with the mobility of haemoglobin S require a sickle solubility test (or alternative) to confirm the presence of haemoglobin S; if this is negative an alternative technique to identify haemoglobins D and G is needed. Those with a single band with the mobility of haemoglobin S require not only a sickle solubility test but an alternative technique to distinguish sickle cell anaemia (SS) from compound heterozygosity for haemoglobin S and other haemoglobins, e.g. D and G, with the same mobility as haemoglobin S. Samples which show a band with the mobility of C/E/O-Arab require electrophoresis at acid pH (using citrate agar or a commercial or other agarose gel which has been shown to give similar separation of variant haemoglobins) or investigation by an appropriate alternative technique (see below), to distinguish between these haemoglobins and, when there is a single band with this mobility, to distinguish homozygotes from compound heterozygotes; the alternative technique should also distinguish haemoglobin C from haemoglobin C-Harlem. It should be noted that DNA analysis can be used to identify D-Punjab and O-Arab. Elevated haemoglobin F should be quantitated and a Kleihauer test should be performed when $\delta\beta$-thalassaemia is suspected (i.e. when red cell indices suggest thalassaemia, an F band is visible on electrophoresis and haemoglobin A2 is not elevated) and also when hereditary persistence of fetal haemoglobin (HPFH) is suspected.

Haemoglobin electrophoresis on citrate agar or agarose gel at pH 6·0–6·2

Electrophoresis at acid pH on citrate agar or appropriate agarose gel is usually used as a supplement to cellulose acetate electrophoresis at alkaline pH. There are differences in the relative mobilities of variant haemoglobins between citrate agar and agarose gel. Both techniques distinguish haemoglobin S from D/G but do not distinguish between most types of D and G. They will distinguish haemoglobin C from E, C-Harlem and O-Arab.

Electrophoresis at acid pH is indicated in the investigation of suspected high-affinity haemoglobins even when electrophoresis at alkaline pH is normal, since some high-affinity haemoglobins have abnormal mobility at acid pH but normal mobility at alkaline pH.

When are further tests indicated? Further tests are needed to distinguish haemoglobins D and G from each other (see above). A sickle solubility test is indicated when mobility at acid pH suggests haemoglobin C-Harlem, a variant haemoglobin in which the sickle mutation is one of two mutations, rather than haemoglobin C. A sickle cell solubility test is similarly indicated if electrophoretic mobility suggests the presence of one of the other variant haemoglobins in which the sickle mutation is one of two mutations. Performing a sickle solubility test when any variant haemoglobin is detected (World Health Organization, 1994) will avoid missing any such cases.

Quantification of haemoglobin A2 by microcolumn chromatography

This is a satisfactory method for quantification of haemoglobin A2 for the diagnosis of β-thalassaemia trait. Special columns are required for haemoglobin A2 quantification in the presence of haemoglobin S, but it should be noted that this test is not essential for diagnosis since Sβ^+ thal can be distinguished from sickle cell trait (AS) on the basis of haemoglobin S comprising a higher proportion of total haemoglobin than haemoglobin A in Sβ^+-thalassaemia (>50%) and a lower proportion (<50%) in sickle cell trait. This technique is of some value in distinguishing between SS (haemoglobin A2 <4%) and Sβ° thalassaemia (haemoglobin A2 >4%) (Dacie, 1988; Serjeant, 1992). However, it should be noted that coexisting α-thalassaemia trait will influence these values and interpretation should be undertaken with considerable caution.

When are further tests indicated? Microcolumn chromatography should be combined with haemoglobin electrophoresis at alkaline pH in order to detect any variant haemoglobins. High and low haemoglobin A2 percentages can be distinguished visually on an electrophoretic strip, and this can sometimes be a useful check on microcolumn chromatography results. It should be noted that unstable haemoglobins can be associated with an increased haemoglobin A2 percentage, and if the red cell indices do not suggest a straightforward β-thalassaemia trait then a test for an unstable haemoglobin is indicated.

Table I. Variant haemoglobins which can be distinguished from each other by isoelectric focusing.*

Instrument/reagent system	Distinguished from each other
Isolab	A, F, S, C, D-Punjab, G-Philadelphia/Lepore, E/A2/O-Arab
Helena Rapid Electrophoresis	A, F, S. C, D-Punjab, G-Philadelphia, E/A2†, O-Arab
Pharmacia Phast	Information not available

*Haemoglobins which can be distinguished from each other are separated by a comma; those which cannot be distinguished from each other are separated by a forward slash.
†Haemoglobin C may also show some overlap with haemoglobins E/A2.

Isoelectric focusing (IEF)
The haemoglobins which can be distinguished from each other by isoelectric focusing differ between different instrument/reagent systems (Table I). In addition, an increased percentage of haemoglobin A2 may be observed but this technique has not been validated for haemoglobin A2 quantification.

When are further tests indicated? The presence of haemoglobin S should be confirmed by a sickle solubility test or an alternative technique. A high or borderline haemoglobin A2 on IEF should be confirmed by microcolumn chromatography or an alternative technique.

High-performance liquid chromatography (HPLC)
HPLC can be used for the quantification of haemoglobins A2 and F and the detection, provisional identification and quantification of variant haemoglobins. The haemoglobins which can be distinguished from each other vary somewhat between different instruments and reagent systems (Table II). HPLC provides precise quantification of haemoglobin A2 and is therefore suitable for the diagnosis of β-thalassaemia trait. However, haemoglobin A2 may not be accurately quantified in the presence of haemoglobin S. In addition, haemoglobin A2 cannot usually be separated from haemoglobin E, thus hindering the differential diagnosis between E/B° thalassaemia and homozygosity for haemoglobin E.

Automated HPLC systems which have been the subject of recent evaluations include the Bio-Rad Variant, Primus Variant System 99 and Kontron Haemoglobin System PV (Waters *et al*, 1996; Bain & Phelan, 1997a, b; Wild & Stephens, 1997).

When are further tests indicated? The nature of any variant haemoglobin detected by HPLC which is of potential clinical relevance (e.g. for genetic counselling) should be confirmed by an alternative technique.

Sickle solubility tests
The kits for sickle cell solubility tests which are predominantly used in the U.K. will detect haemoglobin S down to a concentration of 20% (and sometimes below, in some cases as low as 8%) (Bain & Phelan, 1997a). This method is

Table II. Variant haemoglobins which can be distinguished from each other by high-performance liquid chromatography.*

Instrument/reagent system	Distinguished from each other
Primus Variant System 99	A, F, S, C, E/A2, D-Punjab, G-Philadelphia, O-Arab
Kontron Instruments Haemoglobin System PV	A, F, S, C, E/A2†, D-Punjab, G-Philadelphia
BioRad Variant ('β thal short program')	A, F, S, C, E/A2, D-Punjab, G-Philadelphia, O-Arab
Glycomat 765 'Green' Kit	
In Hb A2 mode	A, F‡, S, C, D-Punjab/G-Philadelphia/E/A2
In variant mode	A, F, S, C, D-Punjab, G-Philadelphia, E/A2
Glycomat 'Gold' Kit (also Biomen Gold Kit)	
In HbA2 mode	A, F, S, C, D-Punjab/G-Philadelphia, E/A2
In variant mode	A, F, S, C, D-Punjab, G-Philadelphia, E/A2
Shimadzu Industry Standard HPLC	S, D-Punjab, G-Philadelphia, C, E/A2, O-Arab
Protech Scientific Ltd, HaemaChrom	Information not available

*This table is based on published information and on the experience of the Working Party. Haemoglobins which can be distinguished from each other are separated by a comma; those which cannot be distinguished from each other are separated by a forward slash.
†Haemoglobins E and A2 have fairly similar retention times and some overlap might occur.
‡Haemoglobin F and glycosylated haemoglobin A (haemoglobin A1) are not distinguished.

therefore capable of detecting all cases of sickle cell trait beyond the period of early infancy, even when there is coexisting α-thalassaemia trait. Kits which have been the subject of an evaluation for the Medical Devices Agency of the Department of Health and which have been found to be satisfactory are Sickledex (Ortho Diagnostics), Sickle-SOL (Baxter Diagnostics Inc.), Microgen Bioproducts S-TEST (Microgen Bioproducts Ltd) and Sickle-Check (Lorne Laboratories Ltd) (Bain & Phelan, 1997a). The method of Dacie & Lewis (1995), although less sensitive than commercial kits, also detects haemoglobin S down to a concentration of 20%. Most methods require that all negative or equivocal sickle solubility tests be centrifuged before reading to increase sensitivity and reliability.

When are further tests indicated? All positive and equivocal sickle solubility tests should be confirmed by haemoglobin electrophoresis or an alternative technique both for confirmation and to distinguish AS from SS and from compound heterozygous states. In an emergency, e.g. pre-anaesthetic, this distinction can be made with reasonable accuracy with a sickle solubility test combined with a blood film and a blood count. It is also recommended that all negative sickle solubility tests be confirmed by haemoglobin electrophoresis or an alternative technique.

In general a sickle solubility test is not indicated in an infant before the age of 6 months since a negative result may be misleading. However, a sickle solubility test can sensibly be performed in an emergency, prior to an anaesthetic, since if it is negative then it is unlikely that anaesthesia will cause any clinical problems. The wording of the report on such a test must state that a negative test does not exclude the presence of a low percentage of haemoglobin S and that further testing is necessary and will follow.

Immunoassay for variant haemoglobins

Kits are currently available for the immunoassays of haemoglobins S, C, E and A, known respectively as HemoCard Hemoglobin S, HemoCard Hemoglobin C, HemoCard Hemoglobin E and HemoCard Hemoglobin A (Isolab Inc.). This technique is potentially useful and, when functioning properly, all HemoCards detect the relevant variant haemoglobins at least down to 10% and sometimes down to 5% (Bain & Phelan, 1997a, b; Chapman *et al*, 1997). There have, however, been problems with intermittent failure of the method.

Quantification of haemoglobin F and Kleihauer test

Quantification of haemoglobin F is indicated if raised haemoglobin F is detected beyond early infancy, e.g. in sickle cell disease, thalassaemia major or intermedia, suspected hereditary persistence of fetal haemoglobin (HPFH) and suspected $\delta\beta$-thalassaemia. A Kleihauer test should be performed whenever an increased percentage of haemoglobin F is detected and the differential diagnosis is between $\delta\beta$-thalassaemia trait, when the distribution of haemoglobin F is usually heterocellular, and HPFH, in which the distribution of haemoglobin F is usually pancellular. A 2-minute alkali denaturation test is recommended for the quantification of haemoglobin F. Techniques for the Kleihauer test will be the subject of a separate BCSH guideline. It should be noted that in the differential diagnosis of HPFH and $\delta\beta$-thalassaemia, quantification of haemoglobin F and a Kleihauer test are supplementary to the assessment of red cell indices.

Haemoglobin H inclusions

A haemoglobin H preparation is indicated to confirm the presence of haemoglobin H in suspected inherited or acquired haemoglobin H disease.

CLINICAL SETTINGS IN WHICH TESTS ARE REQUIRED

For genetic counselling of prospective parents either before or during pregnancy

The disorders of globin chain synthesis which should be predicted in a fetus and the abnormalities which should therefore be detected in prospective parents are given in Table III. A flow chart illustrating the procedures for their detection is given in Fig 1. It should be noted that it may not always be possible to predict whether a fetus will have β-thalassaemia major or β-thalassaemia intermedia. Selective testing may be considered if the percentage of patients from ethnic minorities is low (Working Party of the Standing Committee on Sickle Cell, Thalassaemia and other Haemoglobinopathies, 1993), but this policy is dependent on reliable information on ethnic origin being available. It should be noted that screening for haemoglobinopathies and thalassaemias is just as important when pregnancy may result from artificial insemination from a donor or from *in vitro* fertilization as when conception occurs naturally.

β-Thalassaemia trait

The detection of the great majority of cases of β-thalassaemia trait requires either (a) that all women are screened for β-thalassaemia trait by scrutiny of the red cell indices, or (b) that all women are tested for β-thalassaemia trait, regardless of red cell indices, by universal measurement of haemoglobin A2 percentage. Selection by ethnic origin is undesirable since β-thalassaemia trait and haemoglobin Lepore (which has the same significance) can occur in a very wide range of ethnic groups including Northern Europeans. If a woman is already pregnant, testing should be done regardless of apparent iron deficiency since a diagnosis of iron deficiency does not exclude coexisting β-thalassaemia. When screening is based on red cell indices, all women with an MCH <27 pg (Rogers *et al*, 1995) should have further tests performed. The screening of all women necessitates the use of a less labour-intensive technique such as HPLC whereas selective screening can be carried out by either cellulose acetate electrophoresis and microcolumn chromatography or by HPLC. If numbers are relatively small, electrophoresis followed by elution can be used. Important technical points which should be considered in quantifying haemoglobin A2 have been discussed in detail in a previous BCSH guideline (The Thalassaemia Working Party of the BCSH General Haematology Task Force, 1994). Both $\delta\beta$-thalassaemia trait and the presence of haemoglobin Lepore have the same

Table III. Disorders of globin chain synthesis which should be predicted in a fetus and prospective parents.

Disorders of globin chain synthesis which should be predicted in a fetus
β-Thalassaemia major (including haemoglobin E/β°-thalassaemia)
β-Thalassaemia intermedia (including haemoglobin E/β°-thalassaemia)
Haemoglobin Barts hydrops fetalis
Sickle cell anaemia
Compound heterozygous states causing other forms of sickle cell disease (sickle cell/β-thalassaemia, sickle cell/haemoglobin C disease, sickle cell/haemoglobin D-Punjab, sickle cell/haemoglobin O-Arab)
Disorders of globin chain synthesis which should be detected in prospective parents
β-Thalassaemia* heterozygosity or compound heterozygosity
Haemoglobin E heterozygosity, homozygosity or compound heterozygosity
α°-Thalassaemia heterozygosity or haemoglobin H disease
Sickle cell heterozygosity, homozygosity, or compound heterozygosity
Haemoglobins C, D-Punjab and O-Arab

* The presence of haemoglobin Lepore or of $\delta\beta$-thalassaemia trait has exactly the same significance as β-thalassaemia trait and diagnosis of these traits is required whenever diagnosis of β-thalassaemia trait is required.

significance as β-thalassaemia; haemoglobin electrophoresis at alkaline pH and quantification of haemoglobin F, when elevated, by an alkali denaturation test will permit their diagnosis. Both can also be diagnosed by means of IEF or HPLC. It should be noted that quantification of haemoglobin F is not essential in straightforward β-thalassaemia trait since, although it is elevated in a significant proportion of patients, the diagnosis can be made from consideration of the red cell indices and the proportion of haemoglobin A2. However, an elevated level should be noted because of its relevance to a Kleihauer test performed for quantification of feto-maternal haemorrhage. Each laboratory should have procedures in place to ensure that the presence of an elevated proportion of haemoglobin F in a pregnant woman does not lead to misdiagnosis of feto-maternal haemorrhage and that an alternative technique is available for ensuring that the dose of anti-D given to Rhesus negative women is adequate.

It should be noted that there are some problem areas in the diagnosis of β-thalassaemia trait. One is the existence of β-thalassaemia trait in which the haemoglobin A2 level is borderline or even normal. These cases can be divided into (i) those with both normal red cell indices and normal haemoglobin A2, and (ii) those with abnormal red cell indices but a normal haemoglobin A2. The majority of heterozygotes of the former group will be missed in the routine diagnostic laboratory. The commonest mutations responsible are -101 (C→T) and -92 (C→T). Mean values reported for individuals carrying the latter mutation are MCV 83·9 fl, MCH 28·6 pg and Hb A2 3·4% (Pagano *et al*, 1995). Patients in the second group, i.e. those with abnormal red cell indices and a normal haemoglobin A2 concentration, have an α-thalassaemia trait-like phenotype. This abnormality results from a small group of mild β-thalassaemia mutations, such as CAP+1 (A→C) in South Asians (Indians) and, occasionally from IVI-6 (T→C) in Mediterranean individuals. Heterozygotes for the CAP+1 mutation have, for example, been observed to have the following mean values: MCV 79 fl, MCH 24·7 pg, Hb A2 3·4% (personal observations, Dr J. Old). The phenotype of abnormal red cell indices with a normal haemoglobin A2 concentration can also result from the co-inheritance of δ-thalassaemia (in *cis* or *trans*) and a 'high haemoglobin A2' β^{+}- or β°-thalassaemia mutation. Such co-inheritance of β- and δ-thalassaemia trait occurs in Sardinians and in Cypriots. The majority of such cases will be detected if the procedures we recommend for the detection of α°-thalassaemia trait are followed (see Fig 1).

It should also be noted that co-inheritance of either α°-thalassaemia trait or homozygous α^{+}-thalassaemia trait and mild β-thalassaemia trait makes it more likely that the diagnosis of β-thalassaemia trait will be missed. In a small proportion of such individuals the MCV and MCH will be raised to normal values. This modifying effect means that the diagnosis of β-thalassaemia trait may be missed in individuals with mild β^{+}-thalassaemia mutations such as CAP+1 (A→C) and coexisting α-thalassaemia trait. The co-inheritance of α-thalassaemia trait and a β-thalassaemia mutation, which would usually lead to abnormal red cell indices but a normal haemoglobin A2 concentration, may result in the patient having normal red cell indices as well as a normal haemoglobin A2 concentration.

A further problem occurs if an A2 variant is present. Failure to detect a split A2 band may cause the diagnosis of β-thalassaemia trait to be missed as a result of incorrect haemoglobin A2 quantification.

α°-Thalassaemia trait

Screening for α°-thalassaemia trait (genotype $--/\alpha\alpha$) by scrutiny of the red cell indices (supplemented by measurement of haemoglobin A2 to exclude β-thalassaemia trait

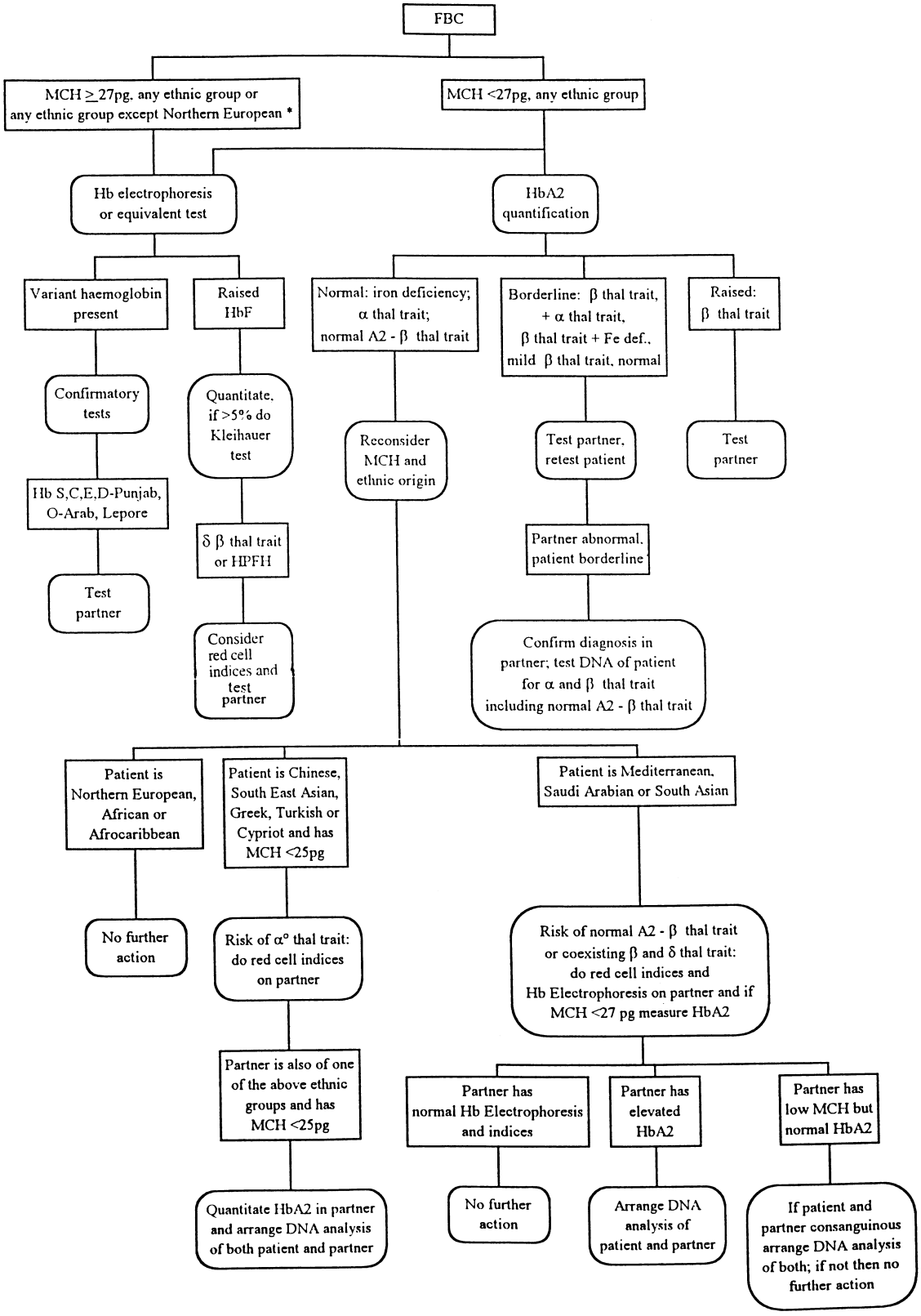

Fig 1. Flow chart demonstrating procedure for diagnosis of α°-, β- and $\delta\beta$-thalassaemia trait and clinically significant haemoglobin variants in pregnant women ('patients') and their partners. * Selective screening is acceptable in low incidence areas but only if accurate information on ethnic origin is available.

when the MCH is appropriately reduced) should be carried out in all women of the following ethnic origins: Chinese, South-East Asian, Greek, Turkish, Cypriot. The need to screen other Mediterranean populations is less well established. If the woman and her partner are consanguineous the indications for screening are stronger. It is not necessary to screen South-Asian (Indian subcontinent), African and Afro-Caribbean women for α° thalassaemia since the α-thalassaemia trait phenotype is likely to arise from the $-\alpha/-\alpha$ genotype and not the $--/\alpha\alpha$ genotype. However, it should be noted that a borderline haemoglobin A2 in a patient with thalassaemic indices may be consequent on the coexistence of α- and β-thalassaemia (with or without iron deficiency); further investigation is necessitated by the possibility of β-thalassaemia trait, not the possibility of α-thalassaemia trait. It is considered reasonable not to test Northern European women since although α°-thalassaemia trait does occur in white British individuals it is very uncommon and unless the partner was from a high incidence ethnic group an adverse fetal outcome would be extremely unlikely. The likelihood of detecting a significant abnormality must be set against the expense of testing and the anxiety generated.

Initial screening for α°-thalassaemia trait is by the red cell indices (Higgs, 1993). Further testing is indicated if the MCH is <25 pg and β- and $\delta\beta$-thalassaemia trait and haemoglobin Lepore have been excluded. Occasional cases of α°-thalassaemia trait have an MCH between 25 and 26 pg, but in large series such cases constitute no more than 1%. Of a total of more than 270 cases of α°-thalassaemia trait diagnosed in four British laboratories in recent years, only two patients had an MCH between 25 and 26 pg and one of these had active liver disease. Another had such a high MCH that the result was probably erroneous.

To avoid unnecessary expensive investigations and the engendering of anxiety in suspected α°-thalassaemia trait it is useful to determine the red cell indices of the partner and proceed to DNA analysis only if both partners have an MCH <25 pg.

It should be noted that the presence of β-thalassaemia trait does not exclude the simultaneous presence of α°-thalassaemia trait. Failure to detect both abnormalities may lead to a failure to predict haemoglobin Barts hydrops fetalis when one partner has α°-thalassaemia trait and the other has both α°- and β-thalassaemia trait. In appropriate ethnic groups (i.e. those listed above) further detailed investigation is therefore indicated if one partner has β-thalassaemia trait and the other has probable α°-thalassaemia trait (Lam *et al*, 1997).

Variant haemoglobins

Screening for variant haemoglobins should be carried out on pregnant women of all ethnic groups with the possible exception of Northern Europeans. Ideally women of all ethnic groups should be screened since clinically significant variant haemoglobins also occur, albeit at a low frequency, in Northern Europeans, and since reliable information on ethnic origin is often not available. Screening can be done by cellulose acetate electrophoresis at alkaline pH, IEF or HPLC. When electrophoresis is used as the primary method the detection of a variant haemoglobin with the mobility of haemoglobin S requires a sickle solubility test or an immunoassay for haemoglobin S for confirmation. Further testing should also be performed to identify haemoglobin D-Punjab (which is clinically significant) and to distinguish it from haemoglobin G-Philadelphia (which is not); this may be done by IEF or HPLC. Further testing should be performed to distinguish between haemoglobins C, E and O-Arab. IEF, HPLC or an immunoassay are suitable.

Testing of partners

The tests necessary in the partner when a variant haemoglobin or α- or β-thalassaemia trait is detected are given in Table IV.

Diagnosis of disorders of haemoglobin synthesis in the fetus

Diagnosis of disorders of globin chain synthesis in the fetus is indicated when a serious disorder is predicted (see Table II). Fetal DNA diagnosis should be carried out in specialized laboratories in accordance with the previous BCSH guideline (The Thalassaemia Working Party of the BCSH General Haematology Task Force, 1994). Tests most commonly used in the National Haemoglobinopathy Reference Laboratory, including tests which have been developed since the previous guideline, are given in Table V.

Submission of samples for fetal diagnosis to a reference laboratory should ideally be preceded by submission of samples from both parents, together with precise details of their respective ethnic origins and a relevant clinical history.

Neonatal screening

Neonatal screening is performed to detect the presence of clinically important haemoglobin variants and the absence of haemoglobin A.

Blood samples. Testing can be carried out on liquid blood, either cord blood or capillary specimens, or on capillary specimens blotted onto filter paper and allowed to dry. The latter are usually known as 'Guthrie spots'. Liquid blood specimens are less suitable for large-scale testing programmes. Guthrie spots have the advantage that collection of specimens for haemoglobinopathy screening can be carried out at the same time as collection of specimens for other neonatal screening programmes. When cord blood samples are used the risk of maternal contamination should be minimized by obtaining the specimen by needle aspiration from a cleaned site on the cord. Maternal contamination (estimated to occur in <0·5% of samples) should be suspected if haemoglobin A2 is present or if there is a high percentage of haemoglobin A. However, it should be noted that some neonatal samples do contain haemoglobin A2.

Techniques. Liquid blood samples can be screened using cellulose acetate electrophoresis, IEF or HPLC techniques. Eluates from Guthrie spot cards provide a more dilute sample which may not always be suitable for analysis by cellulose acetate electrophoresis. HPLC or IEF is therefore more appropriate as a first-line technique. These samples are subject to increasing degradation of haemoglobins with age. Spots should therefore ideally be tested within 7 d of sampling. Ageing of the dried blood samples leads to

Table IV. Tests to be performed in the partner of a woman with a disorder of globin chain synthesis.*

Disorder found in woman	Tests to be performed in partner to exclude clinically important interactions†
β-Thalassaemia trait‡	Red cell indices and haemoglobin A2 quantification when indicated to exclude β-thalassaemia trait‡; haemoglobin electrophoresis or alternative to exclude sickle cell trait, haemoglobin E and haemoglobin O-Arab
Haemoglobin Lepore and δβ-thalassaemia trait	As for β-thalassaemia trait
α°-Thalassaemia trait	Red cell indices, proceeding to DNA analysis in both parents if ethnic origin is appropriate and MCH is <25 pg in both
Haemoglobin S	Haemoglobin electrophoresis (or alternative technique) to exclude haemoglobins S, C, D-Punjab and O-Arab; red cell indices and, if indicated, quantification of haemoglobin A2 to exclude β-thalassaemia trait‡
Haemoglobins C and D-Punjab	Haemoglobin electrophoresis to exclude haemoglobin S
Haemoglobin O-Arab	Haemoglobin electrophoresis to exclude haemoglobin S and, if MCH is <27 pg, haemoglobin electrophoresis and quantification of haemoglobin A2 to exclude β-thalassaemia trait‡
Haemoglobin E	Red cell indices and quantification of haemoglobin A2, if indicated, to exclude β-thalassaemia trait‡

*Or tests to be performed in a woman when her partner is found to have a disorder of haemoglobin synthesis.

†In this table the term 'haemoglobin electrophoresis' should be interpreted as 'haemoglobin electrophoresis or alternative technique such as HPLC'.

‡The presence of haemoglobin Lepore or of δβ-thalassaemia trait has exactly the same significance as β-thalassaemia trait and diagnosis of these traits is required whenever diagnosis of β-thalassaemia trait is required.

Table V. Techniques for fetal DNA analysis for disorders of globin chain synthesis most often used in the National Haemoglobinopathy Reference Laboratory.

α°-Thalassaemia	Southern blot analysis Gap PCR
β-Thalassaemia	
Known mutations	PCR, allele-specific priming GAP PCR
Unknown mutations	DGGE or heteroduplex analysis RFLP linkage DNA sequencing
Hb Lepore	GAP PCR
δβ-Thalassaemia	GAP PCR
Hereditary persistence of fetal haemoglobin	GAP PCR
Hb S	PCR, DdeI digestion
Hb C	PCR, allele-specific priming
Hb E	PCR, allele-specific priming
Hb D-Punjab	PCR, EcoRI digestion
Hb O-Arab	PCR, EcoRI digestion

conversion to methaemoglobins. This in turn leads to variation in the baseline of chromatograms for all HPLC systems together with an increased number of peaks in the early part of the trace, interfering, in particular, with the detection and quantification of haemoglobin H and haemoglobin Barts. This may not be deemed clinically important as neonatal screening does not usually aim to detect haemoglobin H disease or α°-thalassaemia trait. Ageing of blood samples also leads to widening of the peaks of normal and variant haemoglobins, requiring careful interpretation. Both IEF and HPLC appear to detect all clinically significant variants and detect haemoglobin A down to concentrations of <5%.

Sickle solubility tests are not sensitive enough to detect the small quantities of haemoglobin S found in neonatal samples. HaemoCard kits may be sensitive down to concentrations of 10% of haemoglobins S, C and E. However, there is some variation in sensitivity between cards and there is no HemoCard available for haemoglobin D-Punjab or haemoglobin O-Arab.

A presumptive identification of any variant haemoglobin detected can be made on the same sample using a second technique. Further confirmation and clarification of the clinical significance of abnormalities detected should be carried out at a later date, ideally around 6 weeks of age.

The ICSH has published recommendations for neonatal screening for haemoglobinopathies (ICSH, 1988). Available methods have been assessed more recently for the Medical Devices Agency of the Department of Health (Chapman *et al*, 1997).

Preoperative screening

Screening for haemoglobin S is traditionally carried out before surgery so that anaesthetists can be aware of the potential clinical problems. However, it should be noted that with modern anaesthetic techniques such testing may be unnecessary. If testing is considered necessary then all patients who are not of Northern European origin should be tested.

For routine planned surgery a full blood count, sickle solubility test and haemoglobin electrophoresis are usually performed in advance. For emergency surgery, a full blood count and sickle solubility test are usually performed. If the sickle solubility test is positive but the blood count is normal then SS and Sβ°-thalassaemia can be excluded. A blood film will help to exclude Sβ^{+}-thalassaemia and sickle cell/haemoglobin C disease. Some HPLC systems can accept samples out of sequence and provide a rapid answer.

Investigation of microcytosis

If the full blood count is suggestive of iron deficiency then haemoglobin electrophoresis is not an appropriate initial investigation (except in a pregnant woman where a rapid diagnosis is required). Iron deficiency can lower the haemoglobin A2 concentration and, although most cases of β-thalassaemia trait can be diagnosed despite iron deficiency, cases which otherwise would have only a mild elevation of the haemoglobin A2 may be missed (Wasi *et al*, 1968; Alperin *et al*, 1972; Kattamis *et al*, 1972). The patient should be investigated for iron deficiency and treated if appropriate. If there is a persistent microcytosis after recovery from iron deficiency, further investigation should be undertaken.

If the red cell indices are strongly suggestive of thalassaemia trait (e.g. high RBC, normal Hb and low MCV), then investigation for β-thalassaemia trait (and in appropriate ethnic groups haemoglobin E trait) is an appropriate initial investigation. Any one of the methods recommended above for quantification of haemoglobin A2 is satisfactory (HPLC, IEF or haemoglobin electrophoresis at alkaline pH plus elution or microcolumn chromatography). If iron deficiency is excluded and the haemoglobin A2 percentage is normal, the diagnosis of α-thalassaemia trait should be considered. If the patient is in the reproductive age range, is of an appropriate ethnic group (see above) and the MCH is <25 pg, definitive testing for α°-thalassaemia trait should be considered. If the patient is not of an ethnic group in which α°-thalassaemia trait is likely or if the MCH is >25 pg, further testing is not indicated. The report can be worded 'haemoglobin electrophoresis (or HPLC) is normal; alpha thalassaemia trait not excluded'. In appropriate ethnic groups (e.g. Cypriots and Sardinians) the possibility should be borne in mind that thalassaemic indices with a normal haemoglobin A2 may be due to coexisting β- and δ-thalassaemia. The diagnosis of β-thalassaemia trait can be confirmed by globin chain synthesis or DNA studies.

In the non-urgent investigation of microcytosis the various published formulae for discriminating between iron deficiency and thalassaemia trait (summarized in Bain, 1995) can be used to indicate which diagnosis is more likely and which test should be carried out first. However, it should be noted that such formulae should not be used in decision making with reference to women who are already pregnant, since the question which is then being asked is not which diagnosis is more likely but whether β- and α°-thalassaemia trait can be excluded.

Investigation of other haematological abnormalities

Investigation to confirm or exclude the presence of a variant haemoglobin may be useful in the investigation of target cells, irregularly contracted cells and, occasionally, an unexplained high haemoglobin concentration or cyanosis, or when non-lysis of red cells in an automated haematology counter suggests the possible presence of a variant haemoglobin. Haemoglobin electrophoresis, IEF or HPLC is suitable.

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USEFUL ADDRESSES

National Haemoglobinopathy Reference Service, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DS. Telephone 01865 222449 or 01865 222388 (Lab); fax 01865 222500; e.mail: jold@hammer.imm.ox.ac.uk.

Further testing is also available from the following: Dr Mary Petrou, Perinatal Centre, University College London Medical School, Department of Obstetrics and Gynaecology, 86–96 Chenies Mews, London WC1E 6HX. Telephone 0171 388 9246; fax 0171 380 9864. Dr D. M. Layton, South Thames Regional Centre for Prenatal Diagnosis of Blood Disorders, Department of Haematological Medicine, King's College Hospital, Denmark Hill, London SE5 9RS. Telephone 0171 346 3242; fax 0171 346 3514.

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