

Haemoglobinopathies are now more frequently encountered in the routine laboratory as a result of increased ethnic diversity. Here, Dr Adrian Stephens follows up a previous article on the spectrum of disease and its effects with a comprehensive look at laboratory screening and diagnosis.

# Haemoglobinopathies

## Screening and diagnosis

Over the past 25 years there has been a huge increase in haemoglobinopathy investigations undertaken in many of the laboratories in the UK. This increase is partly due to immigration into the UK of people at risk of having sickle cell and/or one of the thalassaemia syndromes, and partly due to our better understanding of these conditions and the clinical advantages to be gained from both antenatal and newborn screening and diagnosis. Amalgamation of work from different laboratories has resulted in some laboratories doing 25,000 antenatal samples per year and some doing 25,000 – 50,000 newborn samples/year. For these reasons many laboratories now make use of automated high-performance liquid chromatography (HPLC) for antenatal samples and automated HPLC or automated isoelectric focusing (IEF) for newborn screening.

Prenatal diagnosis has been introduced to investigate the genotype of a fetus when both parents have been found to carry one of the clinically important haemoglobinopathies

such as sickle cell or thalassaemia. Several national and international bodies have published guidelines in recent years<sup>1-6</sup> and protocols are being developed for England that are being published on the web.<sup>7</sup>

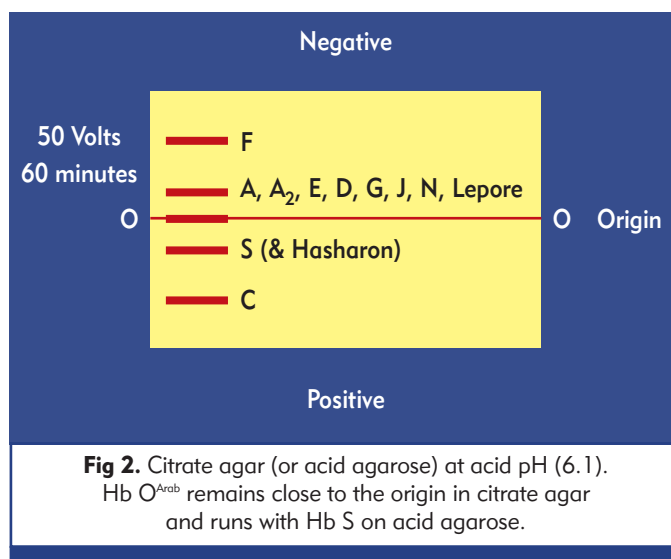
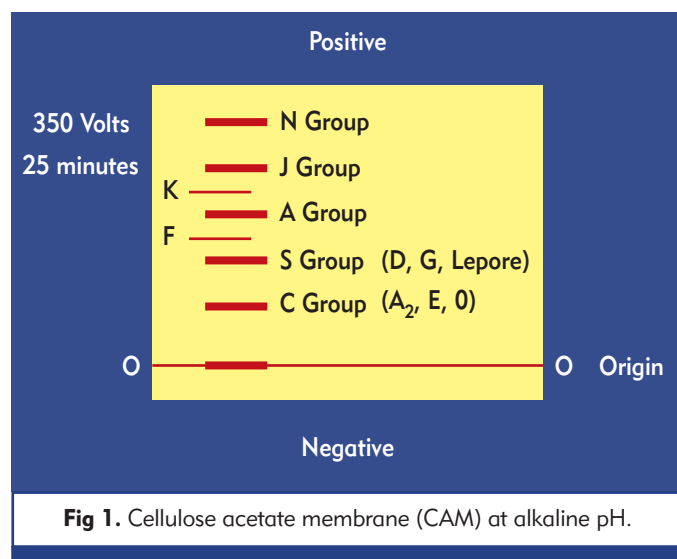
It is important to remember the difference between screening (which is usually testing fit people by a simple test) and definitive diagnosis (which usually follows the investigation of a patient with a medical problem and involves several, often detailed tests, and sometimes more than one blood or tissue sample). Most analytical work in the haemoglobinopathy laboratory relates to screening (prior to general anaesthesia, antenatal screening or newborn screening) and the follow up of people known, or thought to have, sickle cell disease or thalassaemia. Occasionally, it will be necessary to analyse samples for the presence of one of the unstable haemoglobins, high-affinity haemoglobins or haemoglobin Ms.

More than 1000 mutations have been described which cause the haemoglobinopathies (>800 variants, >100  $\alpha$ -thalassaemias and >200  $\beta$ -thalassaemias).<sup>8</sup>

The clinical effects have been discussed previously.<sup>9</sup> However, it is fortunate that all the common clinically important variants can be detected (but not identified) by cellulose acetate electrophoresis at alkaline pH, or by HPLC or IEF. The presence of sickle haemoglobin can be shown by a positive sickle solubility test. Hence, if these tests are negative, no further investigations will normally need to be undertaken. An anticoagulated liquid sample will last several weeks at 4°C and in exceptional circumstances, if it is not possible to obtain another sample, enough haemoglobin can usually be retrieved from a blood clot for haemoglobin analysis.

Although most cases of  $\beta$ -thalassaemia trait in people over one year of age can be detected by the presence of a raised Hb A<sub>2</sub> level, there is only a small numerical difference in the Hb A<sub>2</sub> level between those with and without  $\beta$ -thalassaemia trait and thus it is extremely important that the Hb A<sub>2</sub> is measured precisely (duplicates should be  $\pm$  0.2% of each other, equivalent to an SD of 0.05%).

Haemoglobin F can be quantitated to a



similar degree of accuracy, although, at levels of 10% or greater, an SD of 0.5% is good enough for clinical purposes. Similarly, for clinical purposes, quantitation of Hb S and other variants to this degree of accuracy and precision is satisfactory (duplicates within 2% of each other). It is important that the clinicians know (and inform their patients) that although the techniques available for screening will detect most of the significant genotypes they will not detect all cases (Hb S Providence is not detected by electrophoresis but is detected by the sickle test).

As more than 800 variants have already been described it is necessary to use second-, third- and sometimes fourth-line tests to elucidate the nature of any haemoglobin variant detected with sufficient accuracy for clinical purposes, and these different techniques should rely on different analytical principles.

For instance, if electrophoresis (or HPLC) shows two bands (or peaks) with Hb A and Hb S (with that for Hb A being greater than that for Hb S), the next test should be the sickle test and if this is negative then sickle trait can be excluded. If the sickle test is positive and the red cells on the blood film show normal morphology, then it is reasonable to conclude that the person has sickle cell trait (Hb A + Hb S). However, if the red cells are abnormal, and in particular if sickle cells or target cells are seen, it is likely that the blood sample came from a person with sickle cell anaemia or sickle  $\beta$ -thalassaemia who has been transfused. If electrophoresis (or HPLC) shows two bands with Hb S and Hb C, a sickle test is positive and the blood film shows red cell changes typical of Hb SC disease, then it is reasonable to conclude that the person has Hb SC disease, but if there is any doubt electrophoresis on commercial acid agarose (or in agar) at acid pH should be undertaken to clarify the situation.

Further examples of the genotypes commonly encountered in the UK and their laboratory results can be found on the internet,<sup>7</sup> and if these recommendations are followed it will allow standardised reporting of all normal results and 99% of abnormal results in the UK. With good laboratory practices the error rates for detection should be less than 1% and are often less than 0.1%, with the most common errors being due to mislabelling and other causes of specimen mix up.

The unstable haemoglobins, the haemoglobins with altered affinity for oxygen, and the haemoglobin Ms all require a different approach because many are not detected by the above techniques.

### Investigation prior to general anaesthesia

Prior to general anaesthesia, patients whose ancestors originated from those parts of the world where sickle haemoglobin is common (Table 1) should be screened for haemoglobin S and if present it is important to determine

whether the patient is a carrier (Hb A + Hb S) or has one of the genotypes associated with sickle cell disease such as homozygous sickle cell anaemia (Hb SS) or Hb SC disease (Hb S + Hb C) (Table 2). The reason for this is that if a patient has one of the genotypes of sickle cell disease it is important that the anaesthetist knows this because it may affect the peri-operative or operative management of that patient. If a patient has sickle cell trait (Hb A + Hb S) it will rarely affect the peri-operative or operative clinical management of the patient but it is medico-legally prudent for the anaesthetist to know their phenotype. There is no need to test for any of the thalassaemia syndromes prior to general anaesthesia.

The simplest tests for sickle haemoglobin are the sickle solubility test and the sickling test,<sup>10,11</sup> both of which only require a very small amount of blood and simple reagents, and the result can be available in a few minutes. False-negative results may occur if whole blood is used for the sickle solubility test in a patient who is very anaemic, and false positives may occur if the patient has opaque plasma as occurs in parenteral feeding and some cases of hyperlipidaemia. For these reasons it is much better to use packed red cells instead of whole blood and to achieve this it is usual to use 10  $\mu$ L of packed red cells to 2 mL of reducing buffer (or half the amount recommended for whole blood). As oxidation can inactivate the buffer it is important that the buffer storage conditions are carefully controlled and that both positive and negative controls are included with each batch of tests. If these precautions are undertaken it will avoid virtually all false negatives and false positives apart from those related to specimen mix up.

The sickle solubility test should be positive if at least 15% of the haemoglobin is sickle haemoglobin and for this reason it may be negative after transfusion, even if the patient has homozygous sickle cell anaemia (Hb SS). The sickling test is an alternative to the sickle solubility test and has the advantage that it can detect very small amounts of Hb SS or Hb SC red cells in a transfused patient because detection is by microscopic examination of a wet film looking for sickled red cells. However, it is more time consuming than the sickle solubility test, especially if large numbers of samples need to be tested.

Both the sickle solubility test and the sickling test will usually be negative in newborns with sickle cell trait or even with sickle cell disease, partly due to the small amount of haemoglobin S that is present at birth and partly due to the presence of large amounts of fetal haemoglobin which inhibits sickling. These tests only show the presence or absence of sickle haemoglobin and, if positive, further tests must be undertaken to differentiate between sickle cell trait and sickle cell disease. Haemoglobin electrophoresis at alkaline pH or HPLC will usually be sufficient for this but electrophoresis on commercial acid agarose

(or in agar) at acid pH will sometimes be needed.

Examination of the blood film should always be undertaken if the sickle test is positive, as this may be the only means to differentiate a sample from someone with sickle cell trait or from someone with sickle cell disease who has been transfused. In some laboratories where large numbers of samples have to be tested, it may save time if electrophoresis at alkaline pH or automated HPLC is used as an initial screen and then the sickle tests need only be carried out on those samples in which a haemoglobin band (or peak) runs with haemoglobin S.

### Antenatal and preconceptional screening

In antenatal and preconceptional screening, the goal is to detect the presence of haemoglobin variants or thalassaemia states that can result in a baby with one of the sickling syndromes or a clinically significant form of  $\alpha$  or  $\beta$ -thalassaemia. This is because some parents will want to consider prenatal diagnosis if a pregnancy is at risk of resulting in a baby with serious disease. It is also important for the obstetrician to know if the mother has sickle cell disease, or that she may be carrying a fetus with Hb Barts Hydrops, as both these situations will affect the clinical management of the pregnancy.

The mutations that can result in sickle cell disease and should be screened for are Hb S, Hb C, Hb D<sup>Punjab</sup>, Hb O<sup>Arab</sup>, Hb Lepore and  $\beta$ -thalassaemia. Those that are important for  $\beta$ -thalassaemia are  $\beta$ -thalassaemia, Hb E, Hb Lepore and Hb O<sup>Arab</sup>. The  $\alpha$ -thalassaemia mutation is the only common genotype that can result in the severe form of  $\alpha$ -thalassaemia known as Hb Barts Hydrops and the only way to confirm (or exclude) its presence is by DNA analysis. Non-deletional  $\alpha$ -thalassaemia can also result in Hb Barts Hydrops but it is thought to be much rarer than deletional  $\alpha$ -thalassaemia and is also much more difficult to detect and diagnose.

The variant haemoglobins listed above can all be detected by cellulose acetate electrophoresis at alkaline pH<sup>2</sup> or by automated HPLC,<sup>12</sup> although if a variant is detected then further tests will have to be undertaken to elucidate whether the variant is one of the clinically important variants or one of the variants that have no clinical implications. Typical separations for some of the common Hb variants have been published for electrophoresis,<sup>8,13-17</sup> IEF<sup>18</sup> and HPLC.<sup>19</sup> However, it must be realised that a different buffer gradient, temperature or a different column can result in different retention times with HPLC. Thus, errors are likely to occur if retention times from one piece of equipment are used with different manufacturers' equipment. If a band (or peak) runs with haemoglobin S it is important to undertake a sickle solubility test (or sickling test) to see whether or not sickle haemoglobin is present. If HPLC is used as the first-line test and a variant is detected but

**Table 1.** Occurrence of sickle cell disease.

People whose ancestors came from one of these regions
West, Central and East Africa
Southern Italy and Sicily
Greece, Turkey and Cyprus
Middle East
India

the pattern does not indicate sickle cell trait (Hb A + Hb S) then both cellulose acetate electrophoresis at alkaline pH and on commercial acid agarose (or in agar) at acid pH should be undertaken.

Cellulose acetate electrophoresis will indicate to which broad group the variant belongs (such as the S, C, J, N groups; Fig 1) and in the case of possible sickle cell disease will confirm whether or not any Hb A is present. This is important because some of the post-translational adjuncts of Hb S (Hb S<sub>i</sub>) elutes with, or close to, Hb A on HPLC so that Hb A may appear to be present when it is absent (so that a person with homozygous sickle cell anaemia may appear to have Hb S β<sup>+</sup>-thalassaemia). Another Hb S adjunct (an 'ageing' band, sometimes called S<sub>id</sub>) elutes with Hb A<sub>2</sub> on HPLC and this leads to an erroneously 'raised' Hb A<sub>2</sub>. Neither of these post-translational adjuncts separate from Hb A on electrophoresis at alkaline pH and so interpretation is less likely to be erroneous with electrophoresis.

Electrophoresis on commercial acid agarose (or in agar) at acid pH will allow differentiation of Hb S from Hb D, G, and Lepore; and Hb C from Hb O (Fig 2). If any variant is present, it is important to inspect a stained blood film as this will usually indicate if the patient has been recently transfused (the red cells will usually be dimorphic). If a person is found to have haemoglobin D or G it is important to elucidate whether or not the variant is haemoglobin D<sup>Punjab</sup> or one of the other 'Ds' or 'Gs', as there are many other haemoglobin variants that run in similar positions on electrophoresis or HPLC, but it is only Hb D<sup>Punjab</sup> that is clinically significant. Similarly, if a person is found to have Hb O it is important to elucidate whether or not it is Hb O<sup>Arab</sup>, as it is only the Hb O<sup>Arab</sup> that is clinically significant.

In thalassaemia trait, mean cell volume (MCV) and mean cell haemoglobin (MCH) will normally be reduced and the red cell count will be higher than expected for the haemoglobin concentration (unless the patient also has some other condition that increases the MCV such as vitamin B<sub>12</sub> or folate deficiency or liver disease). Various formulae have been suggested that make use of these properties, but it should be remembered that they can never be diagnostic. However, they can be a useful guide as to when more diagnostic testing is indicated, especially in situations where

**Table 2.** Sickle cell disease genotypes.

Homozygous sickle cell anaemia (Hb SS)
Hb SC Disease (Hb S + Hb C)
Hb SD <sup>Punjab</sup> Disease (Hb S + Hb D <sup>Punjab</sup> )
Hb SO <sup>Arab</sup> Disease (Hb S + Hb O <sup>Arab</sup> )
Hb Sβ-thalassaemia Disease (Hb S + β-thal)
Hb S Lepore disease (Hb S + Hb Lepore)
Hb S + Hb 'X' where 'X' is an unusual sickling haemoglobin such as: Hb C <sup>Harlem</sup> , Hb C <sup>Ziguinchor</sup> , Hb S <sup>Travis</sup> , Hb S <sup>Antilles</sup> , Hb S <sup>Oman</sup> , Hb S <sup>Providence</sup> , Hb Jamaica Plain All these have two mutations, one being the sickle mutation
<b>NOTE</b> Individuals with any of the above may also have α-thalassaemia and some will also have G6PD deficiency.

resources are limited, as can the one-tube 0.36% buffered saline osmotic fragility test for thalassaemia,<sup>20</sup> which is sometimes called the NESTROF (Naked Eye Single Tube Red Cell Osmotic Fragility) test.

If the Hb A<sub>2</sub> is raised (3.5–7.0%) in a person with thalassaemic red cell indices then it is reasonable to conclude that the patient has β-thalassaemia trait. If an Hb A<sub>2</sub> variant is present it is the total Hb A<sub>2</sub> that must be assessed. If the person has thalassaemic red cell indices but the Hb A<sub>2</sub> is in the normal range then it is likely that the person has α-thalassaemia trait, although δβ-thalassaemia is an uncommon alternative explanation. It is clinically important to know whether an individual has α<sup>0</sup>- or α<sup>+</sup>-thalassaemia trait because, as stated above, it is only α<sup>0</sup>-thalassaemia trait that can result in Hb Barts Hydrops.

When screening in this situation, the first step is to know the person's ethnic origin, as α<sup>0</sup>-thalassaemia is only common in people whose ancestors came from SE Asia (east of Bangladesh) or from the Eastern Mediterranean (Cyprus, Greece and Turkey) (Table 3).<sup>21</sup> If the person falls into one of these groups it is important to assume that the individual has α<sup>0</sup>-thalassaemia until proved otherwise. α<sup>+</sup>-thalassaemia occurs widely in populations that originate from those parts of the world in which malaria is or was prevalent (Table 4). As there is a high incidence of both α-thalassaemia and β-thalassaemia in SE Asia, a person from this region with β-thalassaemia may also have α-thalassaemia, and this possibility should always be considered. If it is considered that both members of an expectant couple may have α<sup>0</sup>-thalassaemia they should be referred to a centre that can undertake the appropriate DNA investigations because this is the only way that the diagnosis can be elucidated accurately.

**Table 3.** Occurrence of α<sup>0</sup>-thalassaemia.

People whose ancestors came from one of these regions
Thailand, Burma Laos, Cambodia, Vietnam, Malaysia
South China (including Hong Kong)
Philippines
Cyprus, Greece, Turkey
Sporadic anywhere

**Table 4.** Prevalence of α<sup>+</sup>-thalassaemia.

People whose ancestors came from one of these regions	
Mediterranean	5 – 15 %
Saudi	60 %
South Asia (India, Pakistan, Bangladesh, Nepal, Sri Lanka)	15 – 50 %
South East Asia (as in Table 2, but also including Indonesia)	15 – 80 %
West Africa	5 – 40%
Jamaican Blacks	25 %
Sporadic anywhere	

If a pregnant woman is shown to have one of the clinically important haemoglobin variants, β-thalassaemia trait or α-thalassaemia trait then it is important to test the partner as soon as possible so that if he also carries one of these genes the couple can be offered prenatal diagnosis. If a pregnant woman has a variant of unknown significance but her partner can be shown not to have any evidence of a haemoglobinopathy there is no clinical urgency to determine the precise nature of the variant. If the partner does have, or carries, a clinically significant haemoglobinopathy then it is important to decide if the woman's haemoglobin variant is clinically significant as soon as possible.

The Hb A<sub>2</sub> and Hb F levels can be measured by traditional manual methods<sup>22,23</sup> or by automated HPLC.<sup>12</sup> Automated HPLC will normally only give reliable results for both Hb A<sub>2</sub> and Hb F as long as no variant haemoglobin is present because if a variant is present then it or one of its adjuncts may elute in the same place as Hb A<sub>2</sub> or Hb F. Hb S is a common example of this interference with Hb A<sub>2</sub> quantitation.

It is not usually necessary to quantitate Hb A<sub>2</sub> in the presence of sickle haemoglobin but if an accurate level of Hb A<sub>2</sub> is needed in this situation then either cellulose acetate electrophoresis with elution, or microcolumn chromatography with columns and buffers specially designed for this situation, should be used.

Both IEF and HPLC are able to separate not

only the glycosylated haemoglobins (known as Hb A<sub>1</sub>, S<sub>1</sub>, C<sub>1</sub> etc) from the non-glycosylated 'parental' haemoglobins (known as A<sub>0</sub>, S<sub>0</sub>, C<sub>0</sub> etc), but also acetylated Hb F (known as F<sub>1</sub>) from the main Hb F band or peak (known as Hb F<sub>0</sub> or Hb F<sub>II</sub>). Depending on the buffer gradient used, the Hb A<sub>1</sub> may be subdivided into four smaller fractions (A<sub>1</sub> a, b, c, d)<sup>24</sup> and there are similar 'fast eluting' fractions for most, if not all, variant haemoglobins (eg S<sub>1</sub> a, b, c, d), some of which may elute at the same time as other normal or variant haemoglobins.

Haemoglobins A<sub>1</sub> a, b, and c are all glycosylated and increase in poorly controlled diabetes mellitus. Hb A<sub>1d</sub> is not glycosylated but is a combination of glutathione with the cysteine residue at β<sup>93</sup> and increases with storage of the sample. This separation can be useful when quantitating the glycosylated haemoglobins while monitoring someone with diabetes mellitus, but there is a disadvantage in haemoglobinopathy analysis because it makes the patterns much more complicated and hence more difficult to interpret than electrophoresis on cellulose acetate or commercial acid agarose (or citrate agar).

Haemoglobin H bodies (supravital stained with brilliant cresyl blue) are usually present in the two- or three-gene deletional types of α-thalassaemia. In the two-gene deletional type they may only be seen in 1 in 1000–10,000 red cells and so may only be seen after prolonged examination (eg 20 minutes) of the preparation under a x40 objective, and not always then. In Hb H disease they are usually seen in 20–60% of the red cells and so can be detected very easily. Commercial alkaline agarose can be used instead of cellulose acetate, and commercial acid agarose can be used instead of citrate agar, but, as may be expected, these materials are usually more expensive and the separations obtained may be slightly different, especially from published data that refers to citrate agar.

### Problems with HPLC and IEF

Apart from newborn screening and the quantitation of glycosylated Hb A<sub>1</sub>, HPLC and IEF have little clinical or scientific advantage over traditional manual techniques such as cellulose acetate electrophoresis for the initial screen, cellulose acetate electrophoresis and elution for the quantitation of Hb A<sub>2</sub> and variants, or the two-minute alkali denaturation test as modified by Pembrey *et al.*<sup>22</sup> for the quantitation of Hb F. However, managerially, automated HPLC has a huge advantage when screening large numbers of samples and usually at least 100 samples can be run overnight and further samples run during the day. The same equipment can often be used to measure Hb A<sub>1c</sub> but usually different reagents will be needed.

When selecting HPLC equipment in this situation it is essential to choose equipment that can clearly separate Hb A<sub>2</sub> from Hb A<sub>0</sub>, as it is clinically important to measure the Hb A<sub>2</sub> to a much higher degree of accuracy and precision than is required for the Hb A<sub>1c</sub>.

Isoelectric focusing has recently been automated but this equipment is very expensive and is probably only appropriate for laboratories undertaking a very large workload such as some regional newborn screening laboratories.

### Prenatal diagnosis

Prenatal diagnosis (PND) should be considered whenever both members of a couple have been shown to carry the gene for one of the clinically important haemoglobinopathies such as β-thalassaemia major, sickle cell disease or α<sup>0</sup>-thalassaemia. Initially, the couple must have a discussion with an experienced counsellor (usually with medical or nursing qualifications) to discuss the condition at risk (its natural history and genetics), the tests available, known error rates, the possibility of non-informative results, and any complications of the PND procedure that might affect the mother or the fetus. The couple should normally also be given written information to take away with them.

Before undertaking the test, it is important to know precisely what condition needs to be tested for and therefore blood samples must be taken to confirm the phenotype and genotype of both parents. PND analysis is usually undertaken by DNA techniques on a tissue sample (chorionic villus sampling [CVS]) at 11–12 weeks or from the placenta (placentesis) at any time later in pregnancy. Hence, the analysis of samples from both parents should be completed as soon as possible so that the PND can be undertaken during the first trimester. Occasionally, amniotic fluid is used but in this situation it is often necessary to culture the sample prior to analysis and there will then be some delay in obtaining the result. If the pregnancy has reached 19–20 weeks, globin biosynthesis may be undertaken on a sample of fetal blood obtained from the umbilical cord by fetoscopy.

### Newborn screening

Newborn screening is undertaken to detect newborns with sickle cell disease or β-thalassaemia major. However, it must be realised that the present techniques are likely also to detect many carrier states such as sickle cell trait and Hb C trait, but none of the present non-DNA techniques for newborn samples will detect β-thalassaemia trait in newborns.

Historically, newborn haemoglobinopathy screening has often been undertaken on liquid blood samples obtained from the umbilical cord at birth; however, following recent government decisions, newborn screening in England (and already in the USA) will be undertaken on the same dried blood spot (the Guthrie spot) that is already used to screen for phenylketonuria (PKU) and congenital hypothyroidism. It has been decided<sup>7</sup> that from April all newborns in England will be screened in this way for sickle cell disease and β-thalassaemia major unless

the mother declines for her baby to have the test. Decisions have not yet been made for Scotland, Wales or Northern Ireland.

As the results of this test may take a few weeks to reach the mother, hospital laboratories may still be asked to test blood samples from newborn babies of couples at risk of having a child with one of these conditions because the parents may want to know whether or not their child is affected as soon as possible. Newborn screening laboratories will initially use HPLC or IEF and confirm abnormal results with the alternative technique, but it is likely that other techniques (eg automated mass spectroscopy) will be introduced in the future.<sup>25</sup> If liquid blood samples are used, cellulose acetate or citrate agar electrophoresis<sup>2</sup> are quite satisfactory alternatives to HPLC or IEF and these liquid samples will be stable for several weeks. However, satisfactory results cannot be obtained with these techniques on dried blood spots more than 36–48 hours old.

When interpreting the results it is important to remember that at birth approximately 80–90% of the haemoglobin will be fetal haemoglobin and so the technique chosen must be able to clearly separate small amounts (approximately 2–20%) of Hb A and Hb S (or Hb C or D<sup>Punjab</sup> or O<sup>Arab</sup> or E) from the Hb F. As stated above, neither the sickle solubility test nor the sickling test can be relied upon to give satisfactory results in newborns, and neither will it be possible to get satisfactory results if a baby has been transfused before the sample has been obtained. It is also not possible to differentiate Hb SS from Hb S β<sup>0</sup>-thalassaemia or from Hb S/hereditary persistence of fetal haemoglobin (HPFH) in neonates because in all these conditions the only haemoglobins present will be Hb F and Hb S. Retesting at a later time or family studies will elucidate the diagnosis, as will DNA analysis.

### Unstable haemoglobins

If a person has a haemolytic anaemia and it is considered that an unstable haemoglobin<sup>26</sup> may be the cause, a stability test such as the heat stability or isopropanol stability test should be undertaken. If it is positive then the degree of stability can be assessed by thermal precipitation of methaemoglobin.

### High-affinity haemoglobins

If a person has a high level of haemoglobin of unknown cause then it is important that the presence of a high-affinity haemoglobin is sought before the person is labelled as having polycythaemia rubra vera (PRV) and treated with radioactive phosphorus (<sup>32</sup>P). To detect this it is necessary to measure the oxygen affinity because many of these variants do not separate from Hb A on electrophoresis, HPLC or IEF. This can be achieved by dedicated equipment or by manual techniques.<sup>27</sup>

### Haemoglobin M

If a person is clinically cyanotic but has normal blood gases and there is no evidence

of oxidative poisoning, the presence of methaemoglobin due to a Hb M should be considered.<sup>28</sup> As these are due to a mutation affecting histidine they are not usually detectable by electrophoresis at alkaline or acid pH, and electrophoresis must be undertaken at pH 7 in order to detect them.<sup>10</sup>

### Definitive diagnosis

The precise nature of a haemoglobin variant can only be determined by detailed protein analysis. In the past, this was done by first purifying the haemoglobin by column chromatography, then separating the globin chains by further column chromatography followed by enzymatic digestion of the protein (usually by trypsin) to produce peptides (14 in the  $\alpha$ -chain and 15 in the  $\beta$ -chain) before separating the peptides by two-dimensional high-voltage electrophoresis and chromatography, or sometimes by HPLC (known as fingerprinting). It was often also necessary to undertake amino acid analysis of the separated peptides.

Development of mass spectroscopy has simplified this procedure and also means that only a very small specimen is needed.<sup>25,29</sup> The haemoglobin and globin chains no longer have to be isolated prior to analysis and any tryptic digestion has been reduced to approximately 30 minutes. This means that results can be available in a few hours instead of several weeks, provided the equipment is available. Analysis of DNA can be used to calculate which protein has been synthesised by determining what mutation has occurred. This is a good approach if it is only necessary to determine whether or not an Hb D is Hb D<sup>Punjab</sup>, or an Hb O is Hb O<sup>Arab</sup>. However, it is much more time consuming if all that is known is that a variant is present and not even whether it is the  $\alpha$ - or  $\beta$ -globin chain that has been affected, and it may be necessary to sequence both  $\alpha$  genes and the  $\beta$  gene to elucidate the mutation. As stated above, DNA analysis is essential for the diagnosis of  $\alpha^0$ -thalassaemia.

### Quality control and assessment

As with all analytical procedures it is very important to run controls with each batch of tests and to participate in external quality assessment (EQA) programmes. Control samples may be obtained from volunteers or commercial sources, and the accuracy of Hb A<sub>2</sub> and Hb F quantitation can be checked against international standards held and supplied by the National Institute of Biological Standards and Controls (NIBSC).

### Reporting

Appropriate conclusions on laboratory reports are an integral part of haemoglobinopathy analysis. It is important to separate analytical data from the conclusions and from any recommendations. Consideration should be given to including a 'footer' on all reports, indicating that the analytical results may be misleading if the person has had a blood transfusion in the

previous four months. In the past, confusion has often resulted from different ways of reporting the same analytical data and the national screening programme has produced detailed guidelines with a view to minimise such confusion.<sup>7</sup>

The common, clinically important variants Hb S, C, D<sup>Punjab</sup>, E, O<sup>Arab</sup> and Lepore are  $\beta$ -chain variants and all migrate more slowly than Hb A on electrophoresis at alkaline pH (they are cathodal to Hb A), and elute later than Hb A on HPLC. Hence, a variant that migrates faster than Hb A on electrophoresis (or elutes earlier than Hb A on HPLC) is unlikely to be clinically significant when considering sickle cell and thalassaemia, but it may interfere with the quantitation of Hb F on HPLC.  $\alpha$ -chain variants usually comprise 20–25% of the total haemoglobin, whereas  $\beta$ -chain variants usually comprise 45–50% of the total haemoglobin. However, three common variants do not follow this generalisation: sickle haemoglobin a  $\beta$ -chain variant, comprises 25–40%; Hb E, another  $\beta$ -chain variant, 25–30%; and Hb G<sup>Philadelphia</sup>, an  $\alpha$ -chain variant, 30–40% of the total haemoglobin. The  $\beta$ -chain variants are fewer in the presence of  $\alpha$ -thalassaemia and the  $\alpha$ -chain variants are more in the presence of  $\alpha$ -thalassaemia.  $\alpha$ -chain variants that migrate more slowly than Hb A on electrophoresis are usually associated with a visible Hb A<sub>2</sub> variant, but the Hb A<sub>2</sub> variant is less likely to be noticeable with variants that migrate anodally (or faster than Hb A) as it may then co-migrate with Hb A.

When examining HPLC chromatograms it is important to inspect the shape of the peaks, which should normally be symmetrical. If a peak is not symmetrical, or if it is 'split', or if there is a shoulder to the peak, it often means that two haemoglobins are present but that their physicochemical properties are not sufficiently different to produce two separate peaks. If a different buffer gradient is available it may be possible to achieve complete separation but it is likely that a completely different technique (eg electrophoresis, IEF or mass spectrometry) will be needed to clarify the situation.

### In summary

Most analytical work in the haemoglobinopathy field can be undertaken by fairly simple manual or automated techniques. However, it is essential to take great care over the detailed methodology (such as temperature of buffers and other reagents), the precise time that reactions are allowed to continue (heat stability and alkali denaturation) and that controls are always included with each batch of analyses. If automated chromatographic analysers are used, the chromatograms must be inspected very carefully and any peaks that have been labelled automatically should be checked carefully against the chromatogram because small changes in the ambient temperature or buffer may lead to misleading peak identification. For this reason, the

chromatograms need to be authorised by an experienced analyst before allowing transfer by interface to pathology computers. If this is not carried out then significant errors in reporting may occur. ■

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