An assessment of techniques suitable for the diagnosis of sickle-cell disease and haemoglobin C disease in cord blood samples

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SYNOPSIS Agar gel, cellulose acetate, and starch gel electrophoresis are all capable of diagnosing sickle-cell anaemia, sickle-cell haemoglobin C disease, and haemoglobin C disease in cord blood samples. Of these three electrophoretic techniques, agar gel is the easiest to interpret.

Paper electrophoresis can reliably and rapidly detect sickle haemoglobin and haemoglobin C in cord blood samples. Being incapable of differentiating foetal and normal adult haemoglobin, the value of paper electrophoresis is limited to an initial screening procedure.

Hospitals with patients of West Indian or African origin will find cases of sickle-cell anaemia, sickle-cell trait, sickle-cell thalassaemia, and sickle-cell haemoglobin C disease. These conditions are collectively known as sickle-cell disease. In addition cases of haemoglobin C trait and haemoglobin C disease may be found. Of these disorders, sickle-cell anaemia, the homozygous sickling state (SS), is clinically the most severe.

The early diagnosis of sickle-cell anaemia is advantageous to the patient, the child being more likely to survive with adequate nutrition and prompt treatment of infection (Trowell, Raper, and Welbourn, 1957). Also, as many of these patients subsequently go into hospital as surgical emergencies, early diagnosis may prevent unnecessary operations.

Because mothers may not attend postnatal clinics, the early diagnosis of sickle-cell anaemia should be achieved before the mothers' discharge after delivery. The cord blood is a convenient source of material but diagnosis at birth presents technical difficulties. In the newborn, the diagnosis of sickle-cell disease and haemoglobin C disease is dependent upon the recognition and differentiation of small quantities of normal adult

haemoglobin (HbA), sickle haemoglobin (HbS) and haemoglobin C (HbC) in the presence of large quantities of foetal haemoglobin (HbF).

The purpose of the investigation was the assessment of techniques suitable for the screening of large numbers of cord blood samples for sickle-cell disease, in particular sickle-cell anaemia and also haemoglobin C disease.

Materials

A total of 50 selected cord bloods have been examined; 38 were obtained from deliveries in the St Thomas' Hospital Group, London, and were collected from the offspring of Negro mothers known to have sickle-cell or HbC trait. The remaining 12 were also received from mothers known to have sickle-cell trait attending the Korle-Bu Hospital, Accra, Ghana.

Methods

SICKLING TEST

The sickling test using freshly made 2% aqueous sodium metabisulphite was undertaken in all

samples (Daland and Castle, 1948). The unsealed slides were incubated for 30 minutes at 37°C.

ELECTROPHORETIC TECHNIQUES

Each sample was converted into haemolysate following the method described in the ACP Broadsheet no. 33 (Lehmann and Ager, 1965). The following electrophoretic techniques were employed.

Paper electrophoresis

A vertical tank and Tris buffer pH 8.9 (Cradock-Watson, Fenton, and Lehmann, 1959) were used.

Cellulose acetate electrophoresis

The method of Kohn (1969) was used with a discontinuous barbitone-Tris buffer (Graham and Grunbaum, 1963). Electrophoresis was conducted for one hour at 350 V and subsequently the cellulose acetate was fixed in a solution of 5% sulphosalicylic acid in 3% trichloroacetic acid for about one hour before staining with benzidine (Smith, 1968).

Vertical starch gel electrophoresis

The gel was made from Electrostarch (Electro-starch Co., Madison, Wisconsin, USA) and poured into a Boyer tray (Buchler Instruments, New Jersey, USA). The haemolysate was converted to 2 g% cyanmethaemoglobin solution before electrophoresis by dilution of the haemolysate in a potassium cyanide-potassium ferricyanide solution. The EDTA, boric acid, NaOH buffer used for the tray and the tanks were as described by Jonxis and Huismann (1968) and the postelectrophoretic orthotolidine staining was as described by Huehns (1968).

Agar gel electrophoresis

The buffer solutions described by Robinson, Robson, Harrison, and Zuelzer (1957) and the method described by Marder and Conley (1959), both modified, were employed.

The stock buffer is made up as follows:

Trisodium citrate147 g
Citric acid4.3 g
Dissolve and make up to 1 litre with distilled water.
20% (w/v) citric acid in distilled water.

Both solutions tend to grow moulds and should be refrigerated.

One hundred ml of stock buffer was diluted to 1 litre with distilled water. One hundred ml of this diluted buffer (pH 6.6) was used in making 1% agar (Difco Bactoagar) solution, which was then allowed to cool to about 60°C and poured into a glass plate, bordered to give a gel area of 15 × 22 cm. The remaining 900 ml was adjusted to pH 6.2 by the addition of 2.2 ml of 21% citric acid and used in the buffer compartments of the tank.

Approximately 1 cm-long slits were cut at

intervals across the gel about 5 cm from the anodic end. (A second row of slits could be made 15 cm from the anodic end.) Pieces of 10 mm × 3 mm Whatman no. 42 paper were soaked in 2 g% haemoglobin solutions. The excess haemoglobin was removed by gently blotting, and the paper introduced into the agar slits: 50 m amps (approximately 100 v) was applied for half an hour at 4°C and the paper strips were gently removed. The slits were 'closed up' by readjusting the gel and electrophoresis was continued for a total of six hours. The gel was fixed and stained with benzidine (Smith, 1968).

Results

SICKLING TEST

Providing the samples were received within 24 hours, it was found possible to predict correctly the presence or absence of sickle haemoglobin in all the cord blood samples. Two several-day-old samples of cord blood from babies carrying

Fig. 1 Paper electrophoresis of cord bloods.

From left to right: 1 C disease; 2 C trait; 3 SC disease; 4 sickle-cell anaemia; 5 S trait; 6 normal cord blood control.

Note that haemoglobins S and C do not clearly separate, but HbC tends to be more cathodal in position.

Fig. 2 Cellulose acetate electrophoresis of cord bloods with one adult sickle-cell trait control.

From left to right: 1 normal cord blood; 2 S trait; 3 S-thalassaemia; 4 sickle-cell anaemia; 5 SC disease; 6 adult S trait control.

The apparent presence of HbA in the SC sample (no. 5) is an artefact resulting from the age of this specimen (see text).

Fig. 3 Vertical starch gel electrophoresis of cord bloods with one adult sickle-cell trait control.

From left to right: 1 normal cord blood; 2 S trait; 3 sickle-cell anaemia; 4 adult S trait control.

The trace of haemoglobin anodal to HbA in the S trait sample (no. 2) represents γ_4 (Bart's).

Fig. 4 Agar gel electrophoresis of cord blood samples.

From left to right: 1 SC disease; 2 C disease; 3 C trait; 4 sickle-cell anaemia; 5 S trait; 6 normal cord blood control.

Fig. 5 Agar gel electrophoresis of cord blood samples.

From left to right: 1 sickle-cell anaemia; 2 S-thalassaemia; 3 S trait; 4 normal cord blood control.

The trace of haemoglobin anodal to HbF in the S trait and normal cord blood control samples (nos. 3 and 4) represents γ_4 (Bart's).

the sickle trait were falsely reported as negative. The samples from Ghana were, because of age, found unsatisfactory for the sickling test.

ELECTROPHORESIS

Paper

Figure 1 demonstrates the electrophoretic pattern from cord blood of the newborn children with varying forms of sickle-cell disease and also HbC trait and HbC disease.

Cellulose acetate

Figure 2 shows the electrophoretic pattern of cord blood from a normal baby and babies with sickle-cell trait, a sickle-cell thalassaemia, sickle-cell anaemia, and sickle-cell haemoglobin C disease with an adult sickle-cell trait control.

Vertical starch gel

Figure 3 shows the electrophoretic pattern from cord blood of a normal baby and babies with sickle-cell trait and sickle-cell anaemia and an adult sickle-cell trait control.

Agar gel

Figures 4 and 5 show the electrophoretic pattern from cord blood of newborn children with varying forms of sickle-cell disease and also HbC trait and HbC disease.

The results of 50 samples examined showed 17 normal cases, 24 cases of sickle-cell traits, three of sickle-cell anaemias, one of sickle-cell thalassaemia, and one of sickle-cell HbC disease. In addition there were three samples with HbC trait and one with HbC disease.

It was possible to confirm the diagnosis in 28 of the 38 London cord blood samples by a later haematological examination. Two of the three cases of sickle-cell anaemia, the case of sickle-cell thalassaemia, and that of HbC disease were all confirmed. In 22 cases it was possible also to examine the fathers, and in these cases the diagnosis of the baby was compatible with the parental haemoglobin pattern.

No follow-up or family study was possible on the 12 samples received from Ghana, which included the case of sickle-cell HbC disease and one case of sickle-cell anaemia.

Discussion

About 70% of the total cord blood haemoglobin is HbF. The differentiation between a normal baby and a baby with the haemoglobin disorders investigated in this paper depends on the recognition of the constituent haemoglobins making up the residual small adult fraction. These constituents are shown in the Table.

A recommended technique for the diagnosis of

Sample	Haemoglobin
Normal	A
Sickle-cell trait	A & S
Sickle-cell anaemia	S
Sickle-cell thalassaemia	S ($\pm$ A)
Haemoglobin C trait	A & C
Haemoglobin C disease	C
Sickle-cell haemoglobin C disease	S & C

Table Haemoglobins found in adult fraction of cord blood, sickle-cell disease, and haemoglobin C trait and haemoglobin C disease¹

¹Note that the adult fraction constitutes only a minority (about 30%) of the cord blood haemoglobin. The majority of haemoglobins found in the cord blood of all these conditions is foetal haemoglobin.

sickle-cell disease in cord bloods must be reliable, easy to perform and interpret, and capable of coping with large numbers of specimens.

With the exception of the sickling test and paper electrophoresis, which merely demonstrate the presence of HbS, the techniques reviewed were all found potentially capable of diagnosing sickle-cell anaemia in cord blood samples. Although the sickling test and paper electrophoresis cannot distinguish sickle-cell trait from anaemia, the ease with which they are performed made them worthy of consideration as screening procedures capable of eliminating normal samples.

It is known that the sickle test even in adults may be unreliable (Schneider, Alperin, and Lehmann, 1967). Previous workers (Diggs, Ahmann, and Bibb, 1933; Watson, 1948) found the sickle test unreliable in cord blood, but these authors did not use a reducing agent. However, Schneider and Haggard (1955) using metabisulphite as a reducing agent, found that sickling was detectable in 8.3% of American Negro cord bloods. This figure is similar to the level of sickling found in American Negro adults (Diggs *et al*, 1933) and suggests that in their hands the sickling test was probably reliable in cord blood samples.

In our hands, and provided the sample was less than 24 hours old, it was possible after careful scrutiny to predict correctly if cord blood samples contained any HbS. It was not possible to differentiate sickle-cell anaemia from the sickle-cell trait and it was common for many fields to be searched before a single sickle cell was found. It is therefore not surprising that whilst the sickling test may be useful to confirm the presence of HbS, the small numbers of sickle cells present make it unwise to rely upon sickling as a screening procedure, when, in any case, HbC disease would not be detected.

Paper electrophoresis using a Tris buffer system was successful in reliably demonstrating sickle haemoglobin in all cases in this survey. Our findings are at variance with those of Schneider and Haggard (1955) who found paper electrophoresis unreliable when used to detect

HbS in cord bloods. However, this was probably due to the use of a different buffer system, the Tris buffer system used in this survey being particularly capable of detecting minor haemoglobin fractions.

Figure 1 shows that a paper electrophoretic system is incapable of differentiating the sickle-cell trait from sickle-cell anaemia and also HbC trait from HbC disease.

Figure 2 shows that cellulose acetate electrophoresis is capable of differentiating normal cord samples from cord samples with sickle-cell trait and sickle-cell anaemia. The presence of HbS and HbC was always obvious but in our hands the separation between HbF and HbA is inadequate and this makes it difficult to recognize the absence of HbA. Older specimens (eg, the sickle-cell haemoglobin C sample) tend, by blurring, to make the distinction even harder. The differentiation between sickle-cell anaemia and sickle-cell trait, which depends upon the absence or presence of HbA, may therefore be difficult with cellulose acetate.

Figure 3 shows that the starch gel system employed is capable of distinguishing HbF, HbA, and HbS. Whereas HbC separates clearly, HbS separates relatively poorly from HbF. In addition, the absence of HbA may be difficult to recognize if the pattern is blurred as in old samples. Experience in interpretation appears to be necessary.

Figures 4 and 5 show that agar gel electrophoresis is capable of differentiating all the sickling diseases encountered in this survey as well as HbC trait and HbC disease. Figure 5 shows that it is possible to differentiate some cases of sickle-cell thalassaemia from sickle-cell anaemia, by the detection of HbA in the former condition. However, as HbA is not detectable in all cases of sickle-cell thalassaemia (Weatherall, Clegg, Nakorn, and Wasi, 1969) a diagnosis in some cases cannot be made without a family study.

Occasionally, particularly in overloaded gels, benzidine-staining material may be found at the point of application after the electrophoretic run. So that this does not cause confusion, it is essential in a satisfactory separation that the HbA should be on the cathodal side and HbS on the anodal side of the point of application. Marder and Conley (1959) investigated the influence of haemoglobin concentration and pH on the mobility of haemoglobin fractions after agar gel electrophoresis. In our experience, with a haemoglobin concentration of 2 g% and with a gel buffer of pH 6.6, neither HbA nor HbS were at the origin after electrophoresis. The results obtained on agar gel electrophoresis are clear and easy to interpret. Care, however, should be taken that a standard amount of haemolysate is introduced to ensure reproducible results.

Cellulose acetate, agar gel, and vertical starch gel electrophoresis are all widely used techniques, each capable of diagnosing sickle-cell anaemia and

cord samples. If sickle-cell anaemia is to be recognized in cord blood samples, the most suitable of these three electrophoretic techniques is likely to be the one with which the laboratory has had previous experience.

In many cases a laboratory will not routinely be using any of these three electrophoretic techniques. The starch gel technique will be found to be adequate but it is tedious to perform if large numbers of specimens are involved and interpretation is sometimes difficult. Cellulose acetate, with its advantage of rapidity, would also be an acceptable technique, and would be capable of coping with large numbers of specimens, but again interpretation may not always be easy.

Agar gel electrophoresis was suggested for the diagnosis of sickle-cell anaemia by Fessas (1965) and has been used by Centa and Sciaratta (1967) to follow up a sickle-cell trait baby from birth to 6 months old. On a larger scale Van Baelen, Vandepitte, and Eeckels (1968) used agar gel for the diagnosis of sickle-cell anaemia in Congolese neonates. In our hands, the ease of interpretation of the agar gel makes this the most suitable electrophoretic technique for the diagnosis of sickle-cell anaemia in cord blood. Because agar gel electrophoresis is a reasonably time-consuming procedure, the possible use of a previous screening technique to eliminate normal samples was investigated.

If one only examines the cord bloods of babies with sickle-positive mothers, one would expect, in a community with a 20% sickling rate, that over 50% of the babies would have the sickle-cell trait and approximately 5% sickle-cell anaemia. If cord bloods of all babies are examined, about 20% of babies would have the sickle-cell trait and approximately 1% sickle-cell anaemia. Particularly in the latter case, in order to eliminate the high percentage of normal cord bloods, it appears desirable to adopt an initial rapid screening procedure to detect the presence of HbS.

Two of the techniques considered, paper and cellulose acetate electrophoresis, are potential screening procedures because they are capable of rapidly and reliably detecting HbS in cord blood samples. Of these two techniques, paper electrophoresis is the cheaper and requires less operational skill.

If large numbers of cord blood samples are to be examined for sickle-cell and HbC diseases, the most suitable technique appears to be an initial

screening with paper electrophoresis. All samples where HbS and HbC are detected are subsequently submitted to agar gel electrophoresis.

We are grateful to Dr F. S. Boi-Doku of The Health Laboratory Services, Ghana, who kindly sent us the samples from the Korle-Bu Hospital, Ghana.

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