

Cord Blood Screening for Hemoglobin Abnormalities by Thin Layer Isoelectric Focusing

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Hemoglobin variants can be successfully identified in cord blood samples. The methods most commonly used include cellulose acetate (CAC) and citrate agar (CAG) electrophoresis. Recently thin layer isoelectric focusing (TLIF) has been shown to be an excellent method for identifying hemoglobin variants. To determine the applicability of TLIF for cord blood screening, we compared the results of 835 samples obtained by TLIF with that obtained by CAC, CAG, and the combination of both CAC and CAG. In 100 of these samples we detected an abnormal hemoglobin pattern using TLIF. In contrast, we detected only 80 abnormal samples by CAC, 70 by CAG, and 80 by using the combination of CAC and CAG. Due to the increased resolution provided by TLIF, we correctly diagnosed two sickle cell trait samples by TLIF that were incorrectly suspected to be homozygous for sickle cell disease by CAC and CAG. We identified 41 samples containing Bart's hemoglobin by TLIF in contrast to only 21 using CAC and 14 using CAG. The time and cost of TLIF was comparable to that using the combination of both methods. We, therefore, conclude that TLIF is the method of choice for cord blood screening.

SCREENING cord blood samples for hemoglobin variants is the ideal approach to early detection of sickle cell disease and alpha thalassemia trait.¹ Although Hb F is the major hemoglobin in cord blood, small quantities of other hemoglobins such as A, S, C, and Bart's can be detected. In the absence of Hb A, the detection of Hb S, C, and SC is consistent with the diagnosis of a clinically significant sickle cell disorder. Due to the transient appearance of Bart's hemoglobin in the newborn period, cord blood screening is also an effective method for detection of alpha thalassemia syndromes.^{2,3}

Techniques used to identify abnormal hemoglobins in cord blood have included electrophoresis on cellulose acetate at pH 8.4,⁴ citrate agar at pH 6.2,⁵ the combination of both of these methods and microcolumn chromatography.⁶ Electrophoresis on cellulose acetate (CAC) is the most frequently used method. Although this technique readily identifies Bart's hemoglobin, resolution and separation of Hb F from Hb S may be poor and small amounts of Hb S may go undetected. Electrophoresis on citrate agar (CAG) gives better separation of Hb F from Hb S than CAC, but the citrate agar method is not ideal for detection of Bart's hemoglobin. Microcolumn chromatography, used in a few cord blood screening programs, is an effective method for sickle cell screening, but is not

useful for detection of Bart's hemoglobin or hemoglobin variants other than Hb S and Hb C.

Thin layer isoelectric focusing (TLIF) has recently been shown to be an extremely accurate method for identification of abnormal hemoglobins.^{7,8} Because of the potential this method offers for cord blood screening, we compared the results of TLIF with that of CAC and/or CAG electrophoresis. Our findings indicate several advantages of TLIF over the other techniques.

MATERIALS AND METHODS

Informed consent was obtained prior to cord blood testing. Blood samples were collected from the placenta in EDTA and stored at 4°C. Eight hundred and thirty-five specimens were randomly selected from a mixed racial population containing 567 blacks, 209 whites, and 59 others. Specimens were separated into two parts. One part was tested at Children's Hospital Medical Center, Oakland, California by CAC and CAG electrophoresis.^{4,9} The other part was mailed on ice to Créteil, France, where TLIF was performed according to methods described by Bassett et al.⁷ In brief, 5 μ l of whole blood was placed in the wells of a microtitration plate containing 0.1 ml of 0.05% KCN. The samples were transferred with a multiple syringe (Desaga) to a line of 48 squares of Whatman filter paper placed on a strip of cellophane tape. The tape bearing the samples was placed on the cathodal side of the gel. The gel (250 \times 115 \times 0.5 mm) was prepared as described with several modifications: KCN (12 mg) was added before polymerization of the gel to avoid the formation of methemoglobin during the migration; the glass plate was replaced by a plastic sheet (Gelbond film 53 700, Marine Colloids, Rockland, Maine) to allow the densitometric measurement of the Hb fractions after the migration, and the prerun of 10 min before the addition of the samples was omitted. The duration of the migration at constant power (18 W) was 75 min. When focusing had reached completion the plate was placed in 20% trichloroacetic acid for 5 min and rinsed with water. The densitometric measurement of abnormal samples was performed at 415 nm (Supercellomatic, Issy les Moulineaux, France). The gel was transferred to a sheet of filter paper and dried for storage.

RESULTS

A typical hemoglobin electrophoretic pattern of TLIF is shown in Fig. 1. A mixture of known hemoglo-

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Supported in part by Grant No. HL20985 from the National Heart, Lung and Blood Institute, National Institutes of Health, DHEW.

Submitted July 9, 1980; accepted August 7, 1980.

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0006-4971/80/5606-0019\$01.00/0

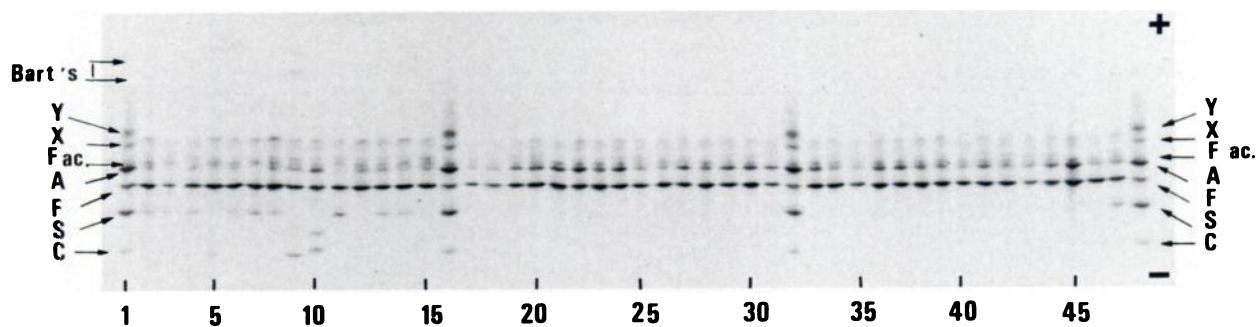


Fig. 1. Isoelectric focusing pattern of cord blood samples. X - Hb J Calabria, Y - Hb J Broussais. Control track: 1, 16, 32, 48.

bins has been placed at intervals (Tracks 1, 16, 32, and 48). Several abnormal samples have been gathered on the left. Unselected samples are on the right. Acetylated Hb F, which represent 10% of Hb F is seen on the anodic side of Hb A. Hemoglobin bands seen between acetylated Hb F and Bart's hemoglobin are called aging bands because they increase upon blood storage. These bands are not methemoglobin and their chemical nature has not been determined. They are not present in fresh samples. The distinction between FS (Samples 7 and 11) and FAS hemoglobin patterns (Samples 2, 3, 4, 6, 13, 14, and 47) are clearly shown. In sample 10, two abnormal fractions are observed. The distance between them and their relative proportions are similar to that of Hb F and Hb A. This pattern suggests an α chain mutation. This result was confirmed by globin chain electrophoresis (not shown). For final diagnosis, peptide analysis was required. Hemoglobin Bart's split into several bands, proportions of which change upon storage.

A comparison between cord blood electrophoresis on CAC and CAG and TLIF is shown in Fig. 2. In contrast to TLIF (A), resolution of individual hemoglobin bands, especially Hb F and Hb A, by CAC and CAG (B) is poor. On CAC the presence of Hb S is questionable in the first sample. We were unable to identify Hb S in this sample by CAC even with applications of higher concentration. In the third sample, it is impossible to exclude Hb A. In general, hemoglobin bands showed up more distinctly on CAG than on CAC. However, Hb S occasionally migrated on or close to the application point. This caused a problem in interpretation, since membrane-bound hemoglobin, not removed during hemolysate preparation, also appeared to remain at the origin. In sample 8, a clear distinction between Hb D and Hb S is seen on TLIF. The migration distance between Hb A and D is different from Hb A and S.

The results we obtained on 835 cord blood samples are shown in Table 1. The final diagnosis, correct diagnosis, and incorrect diagnosis are indicated for

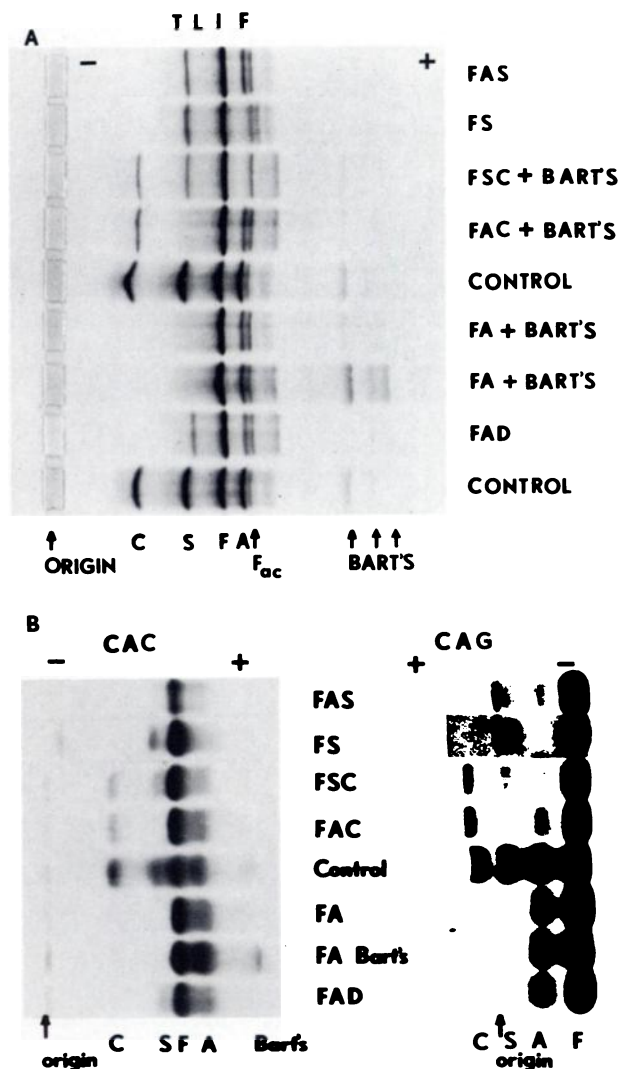


Fig. 2. Hemoglobin pattern obtained with TLIF (A), CAC and CAG (B) of selected cord blood samples. Control consists of the mixture of known A, F, S and C hemoglobins. The anodal bands seen in lanes 3, 4, control and 7 represent Bart's hemoglobin.

Table 1. Results of Cord Blood Screening With CAC, CAG, and TLIF

Diagnosis	Phenotype	N	CAC		CAG		Combined CAC and CAG		TLIF	
			Interpretation		Interpretation		Interpretation		Interpretation	
			Correct	Incorrect	Correct	Incorrect	Correct	Incorrect	Correct	Incorrect
Sickle cell anemia	FS	2	2	0	2	0	2	0	2	0
Sickle cell trait	FAS	35	33	2 (FS)	33	2 (FS)	33	2 (FS)	35	0
Hb SC disease	FSC	1	1	0	1	0	1	0	1	0
Hb C trait	FAC	15	15	0	15	0	15	0	15	0
Hb D trait	FAD	1	0	1 (FAS)	0	1 (FA)	1 (D/G)	0	1	0
Hb E trait	FAE/O	1	0	1 (FAC)	0	1 (FA)	1	0	1	0
Possible alpha-thal syndrome	FA + Barts	41	21	20 (FA)	14	27 (FA)	21	20 (FA)	41	0
Unusual variants		4	3	1 (FAS)	2	1 (FA) 1 (FAC)	4	0	4	0
Normal	FA	735	735	0	735	0	735	0	735	0
Total		835	810	25	802	33	813	22	835	0

The number of correct and incorrect interpretations for each method is indicated. The hemoglobin patterns in parentheses represent the interpretation that was subsequently shown to be incorrect. None of the methods gave an abnormal result which was shown to be normal.

each method. For this study, the final diagnosis was established in each case by repeat testing at age 6 mo, using all three electrophoretic methods, as well as a solubility test on samples containing Hb S. All samples were correctly diagnosed by TLIF. In contrast, 25 samples were misdiagnosed with CAC alone and 33 were misdiagnosed with CAG alone.

The incorrect results on CAC were due to fetal hemoglobin obscuring Hb A resulting in two false positive FS patterns. Comigration of Hb S with Hb D, and comigration of Hb C with Hb E also gave false FAS and FAC results. Inability to detect small quantities of Bart's hemoglobin accounted for the major discrepancy between CAC and TLIF results. One unusual variant was not detected by CAC.

Incorrect diagnosis on CAG was also due to poor resolution of Hb F and Hb A accounting for the two false positive FS results. Electrophoresis on CAG did not detect the Hb D and Hb E variant. An alpha chain variant gave an incorrect FAC interpretation on CAG. There were 27 samples containing Bart's hemoglobin not detected by CAG. Finally two variant hemoglobins were not correctly recognized by CAG (alpha and gamma chain variants).

Combination of the CAC and CAG methods resulted in 22 misdiagnosed samples. This combination failed to detect 20 samples containing Bart's hemoglobin, and gave incorrect results on 2 FAS samples.

The four unusual variants were clearly identified on TLIF, but none were clearly identified on either CAC or CAG, alone. The combination of both CAE and CAG gives a presumptive diagnosis in all four cases. Peptide mapping was required for final identification of these samples.

DISCUSSION

Cord blood screening is a useful tool for identification of sickle cell disease and alpha-thalassemia syndromes. Our results indicate the technical superiority of TLIF over the more conventionally used CAC and CAG methods. TLIF separates proteins on the basis of their isoelectric points. This permits clear resolution and identification of individual hemoglobin variants. Comparison of the migration pattern of the sample being tested to that of the control provides a ready diagnosis in most cases. When an unusual sample is identified by TLIF, it is possible, when one has a pool of characterized abnormal hemoglobins, to establish a definite diagnosis by comigration and mixing studies.⁷ In contrast, neither CAC nor CAG methods have similar sensitivity for detection of small quantities of Bart's hemoglobin, for clear resolution of Hb F and Hb A bands, and for simple identification of variants which comigrate with Hb S and Hb C. Due to the technical superiority of TLIF compared to CAC or CAG, TLIF reduces the overall frequency of retesting on the original cord blood samples.

A time and material cost analysis for processing 48 samples on TLIF compared with CAC and CAG electrophoresis indicates that the preparation time required for CAC and CAG is 20–25 min, whereas the preparation time required to prepare the gel for TLIF is 45 min. Electrophoretic time is 25 min for CAC, 1 h for CAG, and 75 min. for TLIF. Staining takes 25 min for CAC and 10 min for CAG. Fixing takes 5 min for TLIF. Therefore, the total time for CAC is 70 min, CAG 90 min, and TLIF 125 min. The material costs for this analysis is \$5.76 for CAC plates, \$18 for citrate agar plates, and \$10 for TLIF gels. However,

since cord blood samples containing either Hb S or Hb C required both CAC and CAG electrophoresis for correct identification, the total time and cost required to perform TLIF is less than that for the combination of the other 2 methods. In addition the gel can be scanned, dried, and stored without staining.

Our experience with these methods for cord blood screening indicates the following limitations. The technical skills required to perform TLIF are greater than those required for CAC or CAG. CAC frequently gives poor resolution of Hb F and Hb A thereby obscuring minor Hb A components; thus, repeat CAC is frequently required to improve resolution. Electrophoresis on CAG is complicated by variation in individual batches of CAG gel. This variation effects the migration of hemoglobins and causes Hb S to remain at the origin. Since membrane-bound hemoglobin also appears to remain at the origin, small quantities of Hb S can go undetected. CAG is also an inadequate method for detection of Bart's hemoglobin. Bart's hemoglobin is detected more often by TLIF than by CAC. TLIF may be more sensitive for the detection of some thalassemia-2 than CAC, although the clinical significance of this finding in the black population is questionable.

An effective cord blood screening program for hemoglobinopathies depends not only on accurate laboratory methods, but also on comprehensive education and counseling for identified families.¹⁰ This is important for the newborn with sickle cell disease, as

well as the parents of a child with sickle cell trait. In the latter case, identification of a newborn with sickle cell trait may provide the opportunity to identify that family that is at risk of having a child with sickle cell disease.

Regardless of the method used, the following laboratory precautions must be considered for cord blood screening: (1) All samples diagnosed as sickle cell disease should be retested as soon as possible to confirm the diagnosis. Family studies should be performed. In order to avoid paternity issues, parents should fully understand the implication of such testing. (2) It should be remembered that the differential diagnosis of an FS pattern includes sickle cell-hereditary persistence of fetal hemoglobin and sickle cell Beta⁰-thalassemia trait.¹¹ (3) All samples having an FAS or FAC pattern must be repeated by 1 yr of age, since the diagnosis of Sickle Beta⁺-thalassemia trait or C Beta⁺-thalassemia trait cannot be excluded on the basis of the cord blood results even by TLIF. (4) Red cell indices should be determined on samples where Hb Bart's has been identified. Mean corpuscular volume (MCV) values below 94 fl and mean corpuscular hemoglobin (MCH) values below 29.5 μg are consistent with alpha thalassemia trait.¹²

ACKNOWLEDGMENT

The authors wish to thank Maggie Garfield for typing the manuscript and Phillip Lomas for technical assistance.

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