Screening for Intermediate and Severe Forms of Thalassaemia in Discarded Red Blood Cells: Optimization and Feasibility

Elizabeth George, FRCPA*, Mei I Lai, Phd*, Lai Kuan Teh, MSc*, Rajesh Ramasamy, PhD*, Ern Huei Goh, BSc*, Kamalan Asokan, BSc***, J A M A Tan, PhD**, Maithili Vasudevan, BSc***, Sharon Low, BSc*****

*Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, **Faculty of Medicine, University of Malaya, ***StemLife Berhad, Kuala Lumpur, Malaysia

SUMMARY

Detection and quantification of Hb subtypes of human blood is integral to presumptive identification of thalassaemias. It has been used in neonatal screening of thalassaemia and Hb variants. The use of discarded red blood cells following processing of the cord blood for stem cells provides readily available diagnostic material for thalassaemia screening. In this study, we determined the range of Hb subtypes in 195 consecutive cord blood samples collected for cord blood banking. The `cord blood samples' analysed were those of the remaining red blood cells after the cord blood was processed for stem cell storage. Quantification of Hb subtypes by high performance liquid chromatography (HPLC) was done on BioRad Variant II Hb testing system. Only 73 (36.5%) of the samples could be analyzed neat without dilution. With a 1:300 dilution with wash solution the acceptable area as recommended by the manufacturer for reading of a C-gram within the 1 to 3 million ranges were achieved in all. Eighteen (9%) 12 showed classical Hb Barts (γ4) prerun peaks were confirmed by Sebia Hydrasys automated Hb gel electrophoresis and quantified by Sebia Capillarys 2 capillary electrophoresis. Only 1 (0.5%) was presumptively identified with HbH disease. Due to the limited number of samples no beta-thalassaemia major, Hb E beta-thalassaemia and Hb Barts hydrops fetalis were found. The HPLC assay was possible at a cost US$ 5 per sample and a turnover time of 10 samples per hour without technical difficulties. This study reports an effective and valuable protocol for thalassaemia screening in red blood cells which would otherwise be discarded during cord blood processing. Cord blood with severe and intermediate forms of thalassaemia can be preselected and not stored.

KEY WORDS:
Thalassaemia screening, Discarded red blood cells, Cord blood banking

INTRODUCTION

The thalassaemias represent the most common autosomal recessive disorders in the world. Thalassaemias are classified into 3 main clinical phenotypes: trait, intermedia and major. Beta-thalassaemia major results from severe transfusion dependent anaemia and alpha thalassaemia major or Hb Barts hydrops foetalis is incompatible with life[1]. In Malaysia, 3.5-4.5% of population are carriers of thalassaemia[2]. The thalassaemia trait is usually asymptomatic with many carriers unaware of their status in the absence of a blood test for screening.

After a baby is born, the umbilical cord is cut and most often thrown away. However, it is a recognized fact that the remaining blood in the cord is rich in stem cells which can be collected. Cord blood is a source of `haemopoietic' cells that can be used in stem cell transplants to treat a range of diseases and disorders. In cord blood banking, cord blood is processed to separate the blood cells and collect the stem cells. These stem cells are frozen and stored at minus 196 degrees Celsius. Red blood cells separated out in the process are discarded.

Red cell indices

Neonatal blood cell indices show that the red blood cells are larger than that of adults and at birth the mean corpuscular volume (MCV) is 105-125 fl[3]. In neonates, thalassaemia trait is manifested by a decrease MCV (usually less than 95fl) compared to normal infants[4]. In Hb Barts hydrops foetalis, numerous nucleated red blood cells are seen in the peripheral blood smear.

Hb subtypes at birth

The haemoglobin composition in normal cord blood shows the predominant Hb subtype is HbF ($\alpha_2\gamma_2$) (60-85%) with some HbA ($\alpha_2\beta_2$)(15-40%) and no measurable HbA2 ($\alpha_2\delta_2$). Hb Barts (γ4) is less than 0.5%.

Beta-thalasssaemia

Adults who are carriers of classical beta-thalassaemia have HbA2 levels that are elevated but this parameter is not useful in the newborn for identification of carriers as no measurable HbA2 is present. Lack of expression of one or both beta alleles will result in reduced formation of HbA. HbA of <15% has been used to identify beta-thalassaemia in newborn screening[5]. In beta-thalassaemia intermedia, the predominant Hb is Hb F and HbA is <15%. In beta-thalassaemia major transfusion dependent with severe mutations (β^0/β^0) the cord blood will show no HbA.

Alpha-thalassaemia

A relationship between Hb Barts levels in cord blood and number of α-globin genes has been established,[6,7,8,9,10]. In Hb Barts hydrops foetalis, the predominant Hb will be Hb Barts and there will be no HbA present. The peripheral blood film in Hb Barts hydrops foetalis, will show numerous nucleated red blood cells.

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Corresponding Author: Elizabeth George, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Dept of Pathology-hematology, Serdang, Selangor 43400, Malaysia Email: elzageorge@hotmail.com

International Standards of Cord Banking requires cord blood units used for transplantation to exclude the presence of hemoglobinopathy. The hemoglobinopathy of importance in these countries being HbS[11]. In regions where thalassaemia is a public health problem, there is a need to screen cord blood that is to be stored for intermediate and severe forms of thalassaemia.

In this feasibility study, Hb subtypes were identified and quantified in the discarded red blood cells in cord blood banking for the presumptive screening of thalassaemia.

MATERIALS AND METHODS

Sample

One hundred ninety five consecutive cord blood units collected in citrate-phosphate-dextrose (CPD) blood bags for stem cell storage formed the study group. Each bag had 35ml of CPD and a capacity to hold 250 ml of blood. Data of volume of cord blood collected and complete blood counts (CBC) were provided. The CBC was determined by using an automated blood count analyzer (CellDyn 3200, Abbott Laboratories, USA) prior to cord blood processing. An aliquot of 3.5ml of red blood cells separated out in the process of stem cell collection from each of these cord blood units were screened for thalassaemia following institutional clearance; the study protocol was in agreement with local ethics standards and the Helsinki Declaration of 1975, as revised in 1989. A schematic diagram for thalassemia screening is shown in Figure 1.

High Performance Liquid Chromatography (HPLC)

Quantification of Hb subtypes was performed using the BioRad cation-exchange HPLC Hb analyser using the β-thalassaemia short program and adhering to the manufacturer's instructions (BioRad Laboratories, Hercules, USA). The instrument accurately measures HbA, HbF, HbA_2 and Hb variants. Hb Barts and HbH appear as pre-run peaks in the chromatogram (C-gram) with approximate retention time (RT) values of 0.2 and 0.5 respectively.

Capillary electrophoresis

Hb Barts was quantified by high voltage and resolution Hb separation technique using the capillary electrophoresis system (Sebia Capillarys 2, Sebia Inc, Georgia, USA). HbA, HbF and Hb Barts fractions were identified in zones 9, 7 and 12 respectively[10].

Automated Gel electrophoresis

Hb electrophoresis (Sebia Hydrasys, Sebia Inc., Georgia, USA) at alkaline pH8.5 was done to confirm the presence of HbA, HbF and Hb Barts. Hb Barts runs faster than HbA (5mm anodal to HbA[11].

RESULTS

Quantification of Hb subtypes on the BioRad Variant II Hb testing system was only possible directly with 73 (36.5%) samples. Dilution with wash solution in 1:300 (5µl blood / 1.5ml wash solution) showed that the area acceptable for chromatography fell in the recommended 1 to 3 million ranges in all samples.

Thirty-nine (20%) of the 195 cord blood red cell samples had MCV less than 105 fl. The mean MCV was 95.9fl. Eighteen (9.2%) of those with a MCV less than 105 fl had prerun peak of Hb Barts (Table II) (Figure 2) (Figure 3). These were confirmed by capillary electrophoresis and automated gel electrophoresis. There was significant difference ($p<0.05$) in the MCV and MCH of those with Hb Barts peaks and those without. Twenty-one (10.7%) with MCV less than 105 fl and no Hb Barts peak. This group was presumptively identified to have other forms of thalassaemia.

There was a positive correlation of cord blood volume and Hb levels ($p<0.01$, $r=0.765$) (Figure 4). There was no significant difference in the haemoglobin levels in those with normal MCV and those with thalassaemia (Table I).

DISCUSSION

Fetal blood (umbilical vein) is only 50-60% saturated with oxygen, and this relative hypoxia accounts for the magnitude of active erythropoiesis observed *in utero* [3]. At birth this is manifested by a cord blood haemoglobin concentration of 14-22 g/dl, presence of nucleated red blood cells and reticulocytosis (3-7%) and elevated erythropoietin levels. In this study, the mean Hb seen was 10.2 gm/dl and there was a positive correlation between the Hb levels and cord blood volume indicating that haemodilution occurred from excess CPD to blood ratio.

In the adult, screening for thalassaemia involves a step by step process utilising the BHES protocol. B refers to a scrutiny of the complete blood counts and an examination of the blood film; H refers to HPLC to quantify Hb subtypes; E refers to Hb electrophoresis and S refers to stability tests with the most important of ones being the H-inclusion and Sickle cell tests. Cutoff values to screen for thalassaemia being MCV less than 80fl and MCH less than 27pg[12,13,14]. These values cannot be used in cord blood screening for thalassaemia as the normal neonate has a MCV of 105-125fl[3].

Newborn screening methods for haemoglobinopathies include alkaline electrophoresis, isoelectric focusing (IEF), automated high performance liquid chromatography (HPLC) and capillary electrophoresis (CE)[15,16,17]. The samples studied were cord blood. In this study, discarded red cells derived from cord blood processing for stem cells were screened for severe and intermediate forms of thalassaemia. The use of discarded red cells for screening of thalassaemia has not been reported before.

In this study, thirty-nine (20%) have MCV less than 105fl and were presumptively identified with thalassaemia. A relationship between Hb Barts levels in cord blood and number of α-globin genes has been established[5,6,7,9,10]. Hb Barts was noted in eighteen (9%) and possible presence of alpha-thalassaemia in 12 with levels >0.5 %. Studies suggest Hb Barts less than 0.5% may be seen in normal neonates. One sample showed Hb Barts level of 19.8% in keeping with the possible presence of HbH disease. In mild alpha –thalassemia mutations presenting as alpha thalassemia trait, Hb Barts levels may not be detectable[18]. Accurate identification of

Table I: Mean of Hb,HbF, HbA, MCV and MCH in 195 Cord blood samples

		MCV > 105fl (n=156)			MCV <105 fl (n=39)			P value	Significance
		Minimum	Maximum	Mean ± 2SD	Minimum	Maximum	Mean ± 2 SD		
FBC	Hb	5.51	15.20	10.24 ± 3.483	6.28	12.20	9.99 ±3.132	.404	n.s.
	MCV	105.00	128.00	112.85 ± 10.609	84.80	104.00	97.36 ±12.532	.000*	sig.
	MCH	31.70	46.30	35.53 ± 3.799	24.50	38.50	30.87 ±6.999	.000*	sig.
HPLC	HbF	55.60	98.70	84.82 ± 13.984	69.30	95.20	82.85 ± 11.909	.108	n.s.
	HbA	3.80	36.30	15.53 ± 10.396	0.30	31.10	17.27 ± 12.274	.073	n.s.

*Sig. = P value < 0.01 (Analysis was done using One way Anova , SPSS 17)
n.s. = no significance

Table II: Cord blood samples with Hb Barts

	No.	race	Hb	MCV	MCH	High performance liquid chromatography (HPLC)			Capillary electrophoresis genotype			Sebia hydrasys	Assumed
						Hb F	Hb A	Hb Barts	Hb F	Hb A	Hb Bart (Z 12)		
1.	25371	C	9.23	87.0	24.5	73.5	28.0	+	52.2	25.3	19.8	+	- - / - α
2.	25458	C	7.86	96.0	28.0	71.4	25.3	+	69.2	25.8	4.3	+	- - / αα
3.	25381	M	7.79	93.1	26.9	86.1	15.6	+	82.4	15.7	1.7	+	- α / αα
3.	25385	C	6.28	93.2	27.1	75.3	25.5	+	73.0	25.4	1.3	+	- α / αα
4.	25397	C	10.1	87.7	26.9	85.9	16.0	+	84.0	15.1	0.7	+	- α / αα
5.	25422	C	7.58	95.0	26.5	76.9	23.6	+	74.6	22.5	2.7	+	- α / αα
7.	25460	C	8.21	91	26.6	79.7	16.7	+	81.8	16.3	1.9	+	- α / αα
8.	25461	C	11.2	104	30.0	90.6	6.9	+	90.9	5.7	3.4	+	- α / αα
9.	25569	C	9.70	87.9	26.1	73.2	26.3	+	71.4	26.9	1.5	+	- α / αα
10.	25601	C	12.1	85.3	27.4	78.8	21.4	+	76.8	19.4	3.4	+	- α / αα
11.	25616	C	10.7	88.5	27.4	77.9	22.1	+	77.5	19.0	3.3	+	- α / αα
12.	25627	C	8.89	84.8	26.8	79.9	19.6	+	79.4	17.3	3.1	+	- α / αα
13.	25653	C	7.79	89.6	27.1	78.4	22.4	+	76.5	21.1	2.3	+	- α / αα
14.	25365	C	10.5	85.8	27.0	82.1	18.5	+	80.1	19.5	0.4	+	αα / αα
15.	25435	C	9.44	96.1	29.2	82.2	16.8	+	85.3	14.3	0.3	+	αα / αα
16.	25565	C	10.7	99	33.2	87.8	12.3	+	88.3	11.7	0.3	+	αα / αα
17.	25621	C	10.8	101	32.1	84.3	15.4	+	84.5	15.2	0.3	+	αα / αα
18.	25659	C	8.78	104	32.9	82.8	16.3	+	82.9	16.1	0.3	+	αα / αα
	Mean			92.72	28.09	80.38	19.37		78.38	18.46	2.83		

+positive [HPLC= prerun peak; Sebia hydrasys= fast run band] Sebia Capillarys Z-12=zone 12; C=Chinese; M=Malay

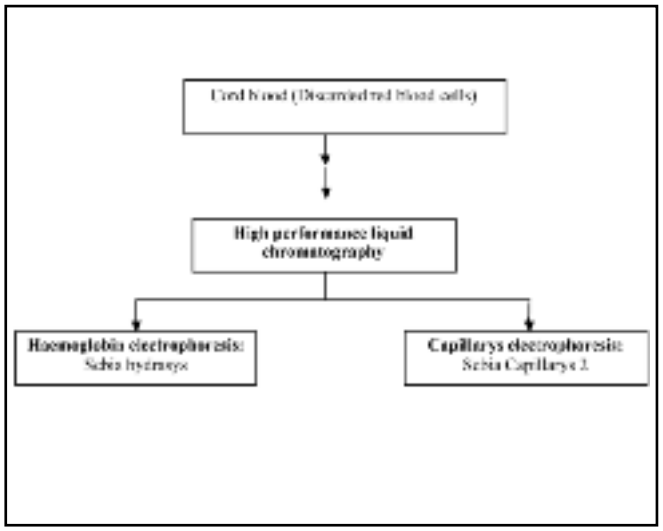

Fig. 1: Schematic diagram for thalassaemia screening in cord blood.

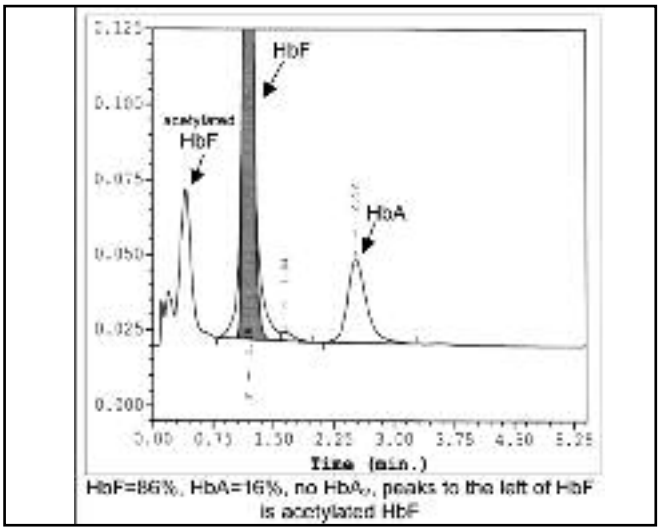

Fig. 2: C-gram of normal cord blood sample.

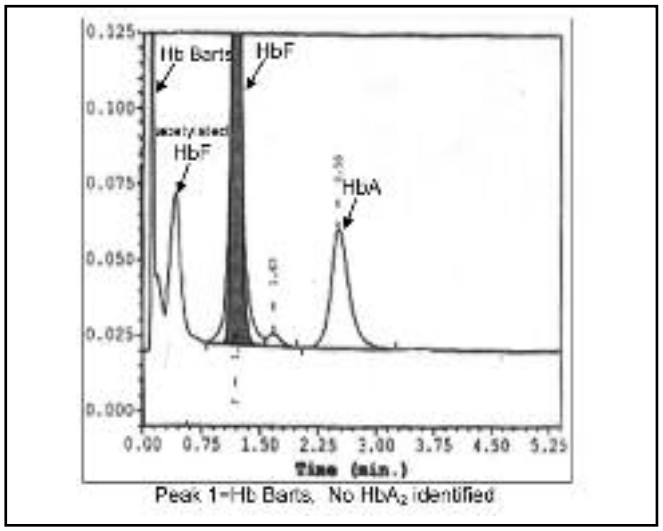

Fig. 3: C-gram of HbH disease with Hb Barts peak.

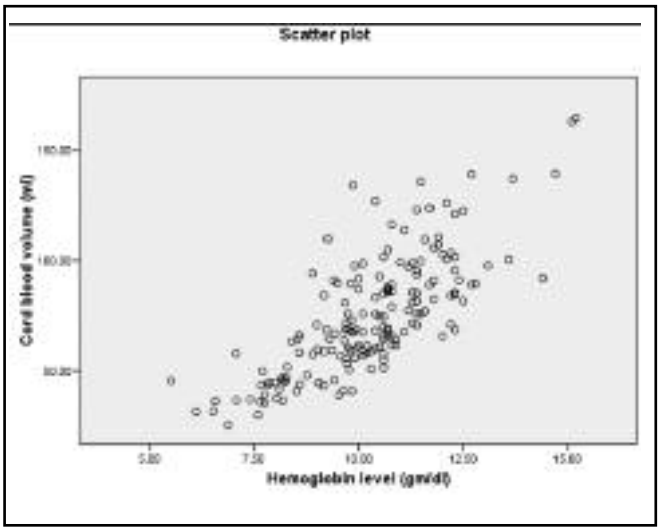

Fig. 4: Scatter plot of cord blood volume and Hb level.

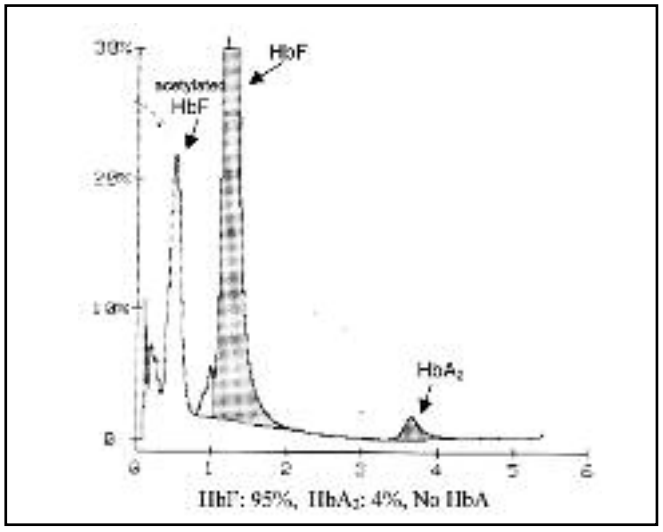

Fig. 5: C-gram of β-thalassaemia major.

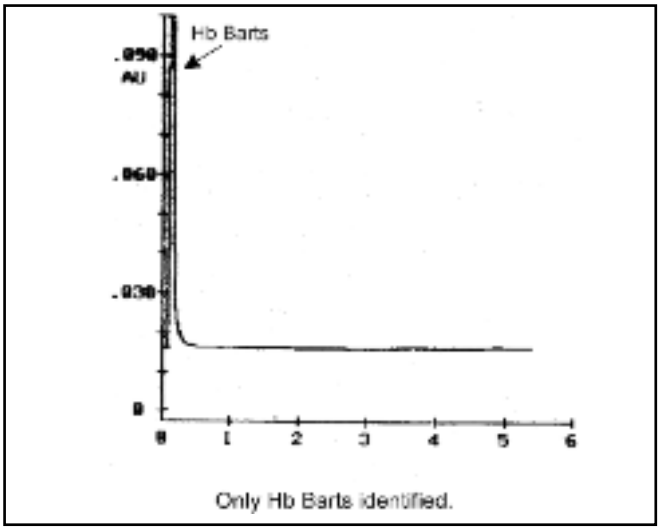

Fig. 6: C-gram of Hb Barts hydrops fetalis (newborn).

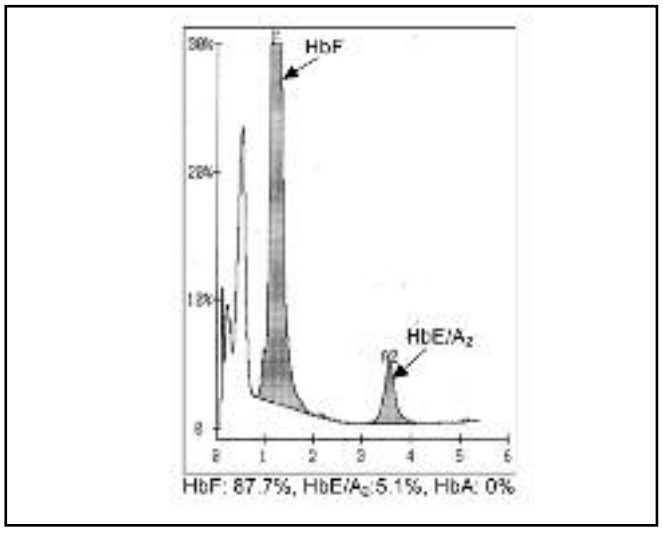

Fig. 7a: Cord Blood newborn.

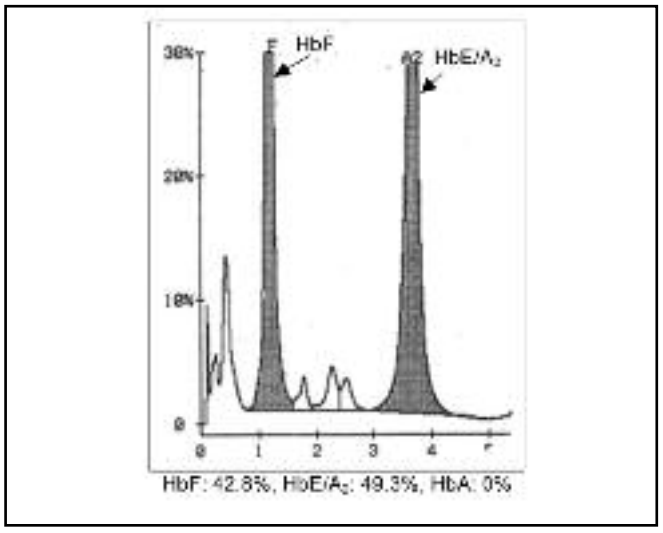

Fig. 7b: At age 8 months (HbE-β^{o} thalassaemia).

alpha thalassemia carriers in newborns requires molecular studies. In cord blood banking it is the identification of HbH disease that is of importance rather than carriers.

The BioRad Variant II Hb analyzer demonstrates Hb Barts as a prerun peak with a retention time of 0.2 in the chromatogram. The Sebia Capillarys 2 system identifies Hb Barts in zone 12 and quantifies it as a percentage. The limitations of quantifying Hb Barts levels are due to the lack of reference materials, controls or specific calibrators in the current techniques. However the pattern of the chromatogram generated by the BioRad Variant II Hb analyzer (prerun peaks) and the high levels as seen in HbH disease and Hb Barts hydrops fetalis are able to presumptively identify both these conditions. Current data on HbH disease indicates this condition can result in morbidity and should be identified[17,18,19]. Hb Barts hydrops fetalis is a fatal condition and results in the death *In-utero* or within 6 hours of birth. DNA studies in those infants whose cord blood showed Hb Barts are necessary to confirm alpha-thalassaemia and identify the number of α-globin genes affected in HbH disease.

There were no cord blood samples in this study with beta-thalassaemia major or Hb Barts hydrops foetalis. However we were able in an earlier study to identify these severe thalassaemia conditions easily by HPLC as they would show total absence of HbA in both; in beta-thalassaemia major, the predominant Hb is HbF with no HbA; in Hb Barts hydrops foetalis Hb Barts is the predominant Hb and no HbA is seen[14,20,21] (Figure 5, Figure 6). In Hb E beta-thalassemia, there will be measurable `HbA2'. Normal neonates do not have measurable HbA2 [4,22,23,24]. Any `HbA2' seen in the chromatogram is Hb E on the BioRad Variant II Hb analyzer in the newborn (Figure 7).

In this study, to quantify and identify Hb subtypes 3 instruments were used: BioRad Variant II Hb analyzer (HPLC), Sebia Hydrasys (Hb electrophoresis) and Sebia Capillarys 2 (Hb capillary electrophoresis). The presumptive identification of beta-thalassemia major, Hb E beta-thalassemia and Hb Barts hydrops fetalis are easily done by HPLC. A prerun peak of Hb Barts together with the presence of HbF and HbA in the chromatogram warrants molecular analysis for HbH disease. In cord blood banking, the identification of alpha or beta-thalassemia carriers is not crucial.

CONCLUSION

A cutoff value of MCV less than 105fl presumptively identifies thalassaemia in cord blood samples. This novel approach using discarded red blood cells from cord blood has the potential to identify Hb Barts hydrops fetalis, beta-thalassemia major, Hb E beta-thalassemia and HbH disease. Screening for thalassemia in discarded red cells available following cord blood processing for stem cell storage is an effective method to identify severe forms of thalassemia. In countries where there is a high risk of thalassemia, it is important to screen for severe and intermediate forms of thalassemia prior to cord blood stem cells being stored.

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