The accurate prediction of rare hemoglobin variants using a combination of high performance liquid chromatography, retention time and isoelectric focusing electrophoresis position

Mohamed S. Khalil, MSc, MD, Adele T. Molyneux, BSc, Samy Marouf, MSc, MD, Ghazy A. Eldamanhory, FRCPath, Anna H. Schuh, PhD, FRCPath, Shirley J. Henderson, BSc, PhD, John M. Old, PhD, FRCPath.

ABSTRACT

الاهداف: تقييم دقة تشخيص الانواع النادرة من الهيموجلوبين وذلك باستخدام نتيجة كل من وقت الاحتجاز بواسطة التحليل الكروماتوجرافى عالى الجودة (HPLC) للسوائل وموضع بؤرة (IEF) تساوى الجهد الكهربائى بواسطة فصل الهيموجلوبين الكهربائى.

الطريقة: تم تشخيص 40 مريض مصاب بانواع مختلفة من الهيموجلوبين باستخدام كل من التحليل الكرماتوجرافي عالى الاداء HPLC وفصل الهيموجلبين IEF باستخدام نقطة التعادل الكهربائية مع مقارنة النتائج بالتحليل الجينى. تم إجراء الدراسة مختبر أمراض الهيموجلوبين – أكسفورد خلال الفترة أغسطس 2008 إلى اكتوبر 2008.

النتائج: تم تطابق نتائج التحليل ل 13 من 14 متغير في 39 حالة (97.5%) مقارنة بحالة واحدة ل HPLC و IEF عندما يستخدم بمفرده. تم تطوير اختبارARMS–PCR الفريد للهيموجلوبين وتم استخدامه بنجاح وذلك لإعطاء تشخيص بسيط و سريع و رخيص.

خاتمة: يمكن اعتبار استخدام كل من التحليل الكرماتوجرافي عالى الاداء HPLC مع فصل الهيموجلبين (IEF) استخدام نقطة التعادل الكهربائية معا ذو كفائة عالية فى تشخيص الانواع النادرة من الهيموجلبين. يعطي اختبارARMS–PCR الفريد للهيموجلوبين طريقة تشخيص بسيطه و سريعه و رخيصه للأنواع المختلفة للهيموجلوبين.

Objectives: To investigate the predictive accuracy of using a combination of the high pressure liquid chromatography (HPLC) retention time and the relative isoelectric focusing (IEF) position to diagnose rare hemoglobin variants.

Methods: A selected group of 40 patients with a rare beta-chain variant were assigned a presumed diagnosis following HPLC and IEF screening and then the variant identified in each case by DNA analysis. The study was conducted at the National Hemoglobinopathy Reference Laboratory, Oxford, United Kingdom, from August 2008 to October 2008.

Results: Thirteen out of 14 different variants were predicted accurately in 39 (97.5%) cases, compared to only one each for HPLC and IEF when used individually. A novel amplification refractory mutation system-polymerase chain reaction test was developed for Hb J-Baltimore and used successfully, to provide a simple, rapid, and inexpensive diagnosis.

Conclusion: The use of both HPLC retention time and isoelectric focusing position provides an accurate presumed diagnosis of a rare hemoglobin variant in the majority of cases. Amplification refractory mutation system-polymerase chain reaction test can provide a simple, rapid and inexpensive molecular diagnostic method for rare beta-chain variants.

Saudi Med J 2009; Vol. 30 (9): 1158-1164

From the National Hemoglobinopathy Laboratory (Khalil, Molyneux, Henderson, Old, Marouf, Schuh) Molecular Hematology, Churchill Hospital, Oxford, United Kingdom, Department of Clinical Pathology (Khalil), Assiut University, Egypt, and the Department of Hematology (Marouf, Eldamanhory), King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia.

Received 21st February 2009. Accepted 13th May 2009.

Address correspondence and reprint request to: Dr. John Old, Consultant Clinical Scientist, National Haemoglobinopathy Reference Laboratory Molecular Haematology, Oxford Haemophilia Centre, Churchill Hospital, Oxford, OX3 7LJ, United Kingdom. Tel. +44 (1865) 225329. Fax. +44 (1865) 857095. E-mail: john.old@orh.nhs.uk

The majority of hemoglobin (Hb) variant results from amino acid substitution due to a single nucleotide change. More than 700 variants have been described to date of which the most clinically important ones are Hb S, Hb C, Hb E, Hb D-Punjab, and Hb O-Arab.[1] The mutations for these 5 abnormal Hbs can be diagnosed by simple DNA analysis techniques.[2] However, the majority of the other abnormal variants are not diagnosed routinely by simple DNA analysis methods and either

high performance liquid chromatography (HPLC) or gel electrophoresis is used to obtain a presumed diagnosis.[3] This is not very accurate as several variants may have the same HPLC retention time (rt) or run in the same position on gel electrophoresis or isoelectric focusing electrophoresis (IEF) (personal observation). However, we have observed that variants running with similar retention times have different IEF positions and vice versa. Thus, the combination of both HPLC rt and IEF position may provide a more accurate procedure to obtain a presumed diagnosis without recourse to identification by DNA sequencing. A precise identification of a Hb variant can be achieved only by DNA analysis, mass spectrometry or amino acid sequencing.[4] However, this is not carried out in most hematology laboratories and the identity of a Hb variant is generally inferred from either the electrophoretic mobility, HPLC rt, its quantity, IEF position and patient's ethnic origin.[5] This data leads to a presumed diagnosis of variable accuracy.

Alkaline and acid Hb electrophoresis are the most widely used methods for investigating Hb variant and hemoglobinopathy and making a presumed diagnosis. Alkaline electrophoresis are capable of separating common Hb variant as Hb A, Hb F, Hb S, Hb C, but Hb D, Hb G, Hb Lepore are unresolved from each other as Hb A_2, Hb C, Hb O-Arab. In addition, there are other variants with identical electrophoretic mobilities similar to Hb C or S.[6] Isoelectric focusing of Hb variants gives much better resolution than alkaline and acid Hb electrophoresis, and it was first used for detecting abnormal Hbs in cord blood samples.[7] This method is now used in the majority of large-scale screening programmes, as the resolution is much better than acid/alkaline electrophoresis and only a small quantity of blood is needed. However, a number of variants have the same iso-electric points as each and co-migrate to the same positions. Thus, as observed with other forms of gel electrophoresis, the method does not separate all variant Hbs from each other, and it is recommended by screening programmes that further tests are always carried out to confirm the presumed identity of an abnormal variant.[8] For example, although there is some variation in the separation of Hbs by different IEF systems,[9] Hb E and Hb O-Arab, Hb G-Philadelphia and Hb Lepore are commonly found variants that co-migrate.[10] Hemoglobin fraction analysis by cation exchange HPLC has the advantages of quantifying of Hb A_2 and F along with Hb variant screening in a single highly reproducible step.[11] However, many variants run in the same position causing diagnostic confusion, such as Hb A_2, Hb E, and Hb Lepore.[3] Thus, the rt cannot be used alone for an accurate presumptive diagnosis, and a diagnosis needs to be made on a combination of electrophoresis and HPLC data. Much of published literature on the use of HPLC or IEF for investigating hemoglobinopathies and thalassemia syndromes has evaluated their effectiveness in newborn screening program,[12,13] their performance in comparison with other technologies,[14,15] the analysis of complicated α- and ß-thalassemia syndromes in Southeast Asia[16] and in analysis of small patients population and a reference collection of rare Hb variants.[17] However, there are few reports on the sensitivity, specificity and predictive value of using a combination of HPLC rt and IEF position for a presumed diagnosis.

To evaluate the predictive value of using HPLC rt and relative IEF position for the accurate diagnosis of rare Hb variants, we report here the results of predictions using both HPLC and IEF screening data in small selected cohort of referred samples from patients identified as carrying a rare β-chain variant. Predictions were based on data from variants previously identified by DNA sequencing and then a definitive identification made for each patient by DNA analysis techniques. In addition, we report here the development of a new ARMS-PCR method[18] for the molecular diagnosis of Hb J- Baltimore, the most common rare variant in our cohort.

Disclosure. The Oxford Partnership Comprehensive Biomedical Research Centre supported this work with funding from the Department of Health's NIHR Biomedical Research Centres funding scheme. The investigations were also supported by the European Commission grant for the project "Infrastructure for Thalassaemia Research Network", Co-ordination Action, ITHANET, RI-2004-026539.

Methods. Forty ethylenediaminetetraacetic acid blood samples referred from different laboratories in UK for diagnosis of an unusual or rare variant were selected for this study. A full blood count was performed on all samples and each was screened by HPLC and IEF. Informed consent was obtained from each subject for the investigation of an abnormal haemoglobin variant. The observations reported here from part of the patient's consented hemoglobinopathy diagnostic investigations and thus no ethical approval was required.

All specimens were analyzed on the Bio-Rad Variant II HPLC system with use of the Variant II Thalassemia Short Program (Bio-Rad Laboratories Hercules, California, USA), and the rt for each variant Hb was noted.

All specimens were subjected to IEF electrophoresis, in which Hbs migrate to characteristic fixed position in the gel according to their isoelectric point at which the

net charge on the molecule is zero (isoelelectric point). Hemoglobin bands were visualized on the gel using a protein stain and their positions were measured from the Hb A band. Variants focusing below Hb A (towards the anode) were given positive values and variants focusing above Hb A (towards cathode) were given negative values. All variant band distances were adjusted for assay variation using a correction factor calculated from the position of observed Hb S control band relative to its standard position of <8.5 mm Hb A.[19]

The DNA was extracted from the whole blood sample using the standard phenol/chloroform method.[20] For DNA sequencing, amplification of β–globin gene was performed by polymerase chain reaction (PCR) using primers designed to amplify the entire gene. The PCR amplification was performed in 25 mM magnesium chloride in a standard ammonium sulphate buffer on a Biometra Uno 11 machine. Cycle sequencing of the product was then performed using Beckman CEQ TM DTCS -Quick Start kit (Beckman High Wycombe, UK) and the sequence analyzed on a Beckman Coulter CE8000 Genetic Analysis System.

For identification of Hb J-Baltimore by the amplification refractory mutation system (ARMS), a mutant ARMS primer was designed to detect the mutation for Hb J-Baltimore at codon 16 (GGC>GAC) as previously described.[21] The mutant primer sequence was CACCACCAACTTCATCCACGTTCAC, together with the previously published common primer (ACCTCACCCTGTGGAGCCAC, HUMHBB: 62028-62047), amplified the mutant DNA sequence to produce a product of 240bp. The assay also contains 2 control primers, (GAGTCAAGGCTGAGAGATGCAGGA, HUMHBB: 64084-64061; CAATGTATCATGCCTCTTTGCACC, HUMHBB: 63124-63247), which produce a control band of 861bp in all samples. Standard ARMS-PCR conditions were used for the amplification: 25 cycles with an annealing temperature of 65°C.

Results. *Phenotypic analysis.* A full blood count revealed that 30 patients had a normal blood count and 10 patients were anemic, 9 of which had a normocytic normochromic anemia and one patient had a microcytic hypochromic anemia (Hb 9.7 gm/dl, MCV 69 fl, MCH 21.8 gm), most probably due to co-inherited ß-thalassaemia. Two of the patients were compound heterozygotes for a common ß-globin chain variant (Hb C and Hb E) plus a rare variant. Excluding Hb S and Hb C, HPLC analysis of each patient revealed 14 abnormal peaks with different retention times, and IEF electrophoresis revealed 10 different abnormal bands in 39 patients. One patient carried a variant which separated from Hb A by HPLC, but co-migrated with Hb A on IEF. A presumed diagnosis could be made in each case for the rare variant based on the observed HPLC retention time and IEF relative position, using data obtained in our laboratory for previous cases in which variants with the observed times and positions had been identified by DNA sequencing. The presumed diagnosis and HPLC rt plus IEF relative position data for each of the 40 cases are listed in **Table 1**. Four variants were presumed to be Hb Osu Christiansborg, 4 as Hb Hope, 2 as Hb N-Baltimore, 20 as Hb J-Baltimore, 2 as Hb Richmond, and one case each of Hb Rothschild, Hb J-Iran, Hb K-Woolwich, Hb Tyne, Hb E-Saskatoon, Hb G-Makassar, Hb K-Ibadan, Hb J-Bangkok and Hb D-Punjab.

Although the HPLC retention times were different for each variant, if used on their own, they could only predict one of the 14 variants-Hb Tyne. The other 13 variants had retention times, which were the same range as that observed by a number of other variants. The rt ranges and number of candidate variants for each range were as follows: Hb J-Iran, rt: 1.1-1.2 min, (5 candidate variants); Hb K-Woolwich rt: 1.2-1.3;[3] Hb Hope rt: 1.4-1.5;[4] Hb N-Baltimore, rt: 1.5-1.6,[5] Hb J-Baltimore, rt: 1.7-1.8,[10] Hb K-Ibadan, rt: 2.0-2.1;[4] Hb J-Bangkok, rt: 2.4-2.5,[3] Hb Tyne, rt 2.6-2.7,[1] Hb Osu Christiansborg, rt: 3.7-3.8,[5] Hb D-Punjab, rt: 4.0-4.1,[5] Hb E-Saskatoon, rt: 4.3-4.4,[5] Hb Richmond, rt: 4.4-4.5,[9] Hb G-Makassar, rt: 4.5-4.6,[6] and Hb Rothschild, rt: 5.2-5.3.[2]

The IEF data on its own could also only give a presumed diagnosis for one variant band-Hb Rothschild. The other band positions had more than one candidate variant that focused at the same position according to our previous results. The data was as follows: Hb N-Baltimore and Hb J-Iran focused at >9 mm Hb A (4 candidate variants); Hb J-Baltimore, Hb K-Ibadan, and Hb J-Bangkok all focused at >6 mm Hb A;[9] Hb K-Woolwich at >3 mm A,[3] Hb Tyne co-migrated with Hb A, Hb Richmond at <3.6 mm Hb A,[4] Hb D-Punjab at <7.0 mm Hb A,[19] Hb G-Makassar at <8.5 mm Hb A,[15] Hb Osu Christiansborg at <9.0 mm Hb A,[3] Hb E-Saskatoon at <15.0 mm Hb A,[2] Hb Rothschild at <26.0 mm Hb A.[1]

However, the data in **Table 1** shows that candidate variants with the same HPLC rt range usually have different IEF positions, and candidate variants that focus at the same IEF position usually have different HPLC retention times. This allowed the combination of the 2 parameters to give a single presumed candidate for all 14 variants.

The results of the DNA investigations for each variant are also described in **Table 1**. Forty patients were studied, 38 heterozygotes and 2 cases which were

Table 1 - Comparison between results of Hb variants identified by HPLC and IEF (presumed diagnosis) with the diagnosis by ARMS PCR or DNA sequencing analysis

Case	Hb %	HPLC rt (min)	IEF (mm)	Presumed diagnosis	DNA diagnosis	Mutation	DNA analysis method
1	43.4	3.72	9.5	Hb Osu Christiansborg	Hb Osu Christiansborg	b52 GAT→AAT	Sequenced
2	46.0	3.76	9.2	Hb Osu Christiansborg	Hb Osu Christiansborg	b52 GAT→AAT	Sequenced
3	43.1	3.82	9.0	Hb Osu Christiansborg	Hb Osu Christiansborg	b52 GAT→AAT	Sequenced
4	35.2	3.83	9.0	Hb S + Hb Osu Christinansborg	Hb S + Hb Osu Christiansborg	b52 GAT→AAT	Sequenced
5	31	5.23	26.0	Hb C + Hb Rothschild	Hb C + Hb Rothschild	b37 TGG→CGG	Sequenced
6	41.2	1.4	-1.0	Hb Hope	Hb Hope	b136 GGT→GAT	Sequenced
7	40.9	1.4	-1.0	Hb Hope	Hb Hope	b136 GGT→GAT	Sequenced
8	48.5	1.4	-1.3	Hb Hope	Hb Hope	b136 GGT→GAT	Sequenced
9	38.0	1.41	-1.1	Hb Hope	Hb Hope	b136 GGT→GAT	Sequenced
10	47.2	1.58	-9.2	Hb N-Baltimore	Hb N-Baltimore	b95 AAG→GAG	Sequenced
11	46.6	1.62	-9.2	Hb N-Baltimore	Hb N-Baltimore	b95 AAG→GAG	Sequenced
12	44.6	1.71	-6.1	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	ARMS
13	44.8	1.74	-6.4	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	ARMS
14	46.0	1.74	-6.5	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	ARMS
15	43.8	1.78	-6.7	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	ARMS
16	42.2	1.79	-6.4	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	ARMS
17	42.3	1.80	-6.4	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	ARMS
18	43.1	1.80	-5.7	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	ARMS
19	45.8	1.82	-6.1	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	Sequenced
20	45.5	1.83	-7.1	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	Sequenced
21	46.1	1.84	-5.9	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	Sequenced
22	46.5	1.84	5.4	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	Sequenced
23	43.1	1.86	-6.5	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	Sequenced
24	44.8	1.86	-7.4	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	Sequenced
25	36.1	1.86	-6.5	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	Sequenced
26	44.4	1.87	-6.7	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	Sequenced
27	23.2	1.87	-5.6	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	Sequenced
28	46.1	1.89	6.2	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	Sequenced
29	46.0	1.78	-5.6	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	Sequenced
30	45.0	1.7	-7.2	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	Sequenced
31	45.6	1.7	-6.5	Hb J-Baltimore	Hb J-Baltimore	b16 GGC→GAC	Sequenced
32	36.4	2.07	-6.4	Hb K-Ibadan	Hb Ulm	b66 AAA→AAT	Sequenced
33	48.9	1.21	-9.1	Hb J-Iran	Hb J-Iran	b77 CAC→GAG	Sequenced
34	37.1	1.3	-3.0	Hb K-Woolwich	Hb K-Woolwich	b132 AAA→CAA	Sequenced
35	37.8	2.7	0.0	Hb Tyne	Hb Tyne	b5 CCT→TCT	Sequenced
36	43.5	4.3	15.5	Hb E-Saskatoon	Hb E-Saskatoon	b22 GAA→AAA	Sequenced
37	38.8	4.55	8.5	Hb G-Makassar	Hb G-Makassar	b6 GAG→GCG	Sequenced
38	42.3	2.49	-5.5	Hb J-Bangkok	Hb J-Bangkok	b56 GGC→GAC	Sequenced
39	43.0	4.05	7.1	Hb D-Punjab	Hb D-Punjab	b121 GAA→CAA	ARMS
40	41.5	4.47	3.6	Hb Richmond	Hb Richmond	b102 AAC→AAA	Sequenced

Hb - hemoglobin, HPLC - high pressure liquid chromatography, IEF - isoelectric focusing, ARMS - amplification refractory mutation system, PCR - polymerase chain reaction.

Figure 1 - Results of the ARMS-PCR assay developed for the detection of the mutation for Hb J-Baltimore. Tracks 1 and 2, 6 and 7, contain the amplified products of normal DNA (negative result), tracks 2 and 3 show Hb J-Baltimore control DNA (positive result), and tracks 4 and 5 show DNA from case 12 with a presumed diagnosis of Hb J-Baltimore.

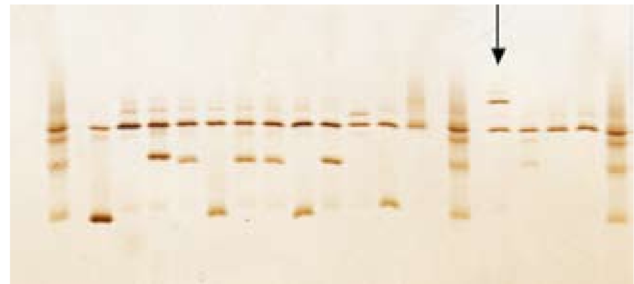

Figure 2 - Isoelectric focusing electrophoresis gel results showing the position of Hb Ulm. The variant is in track 15, labeled by an arrow, and focuses at >6.4 mm Hb A.

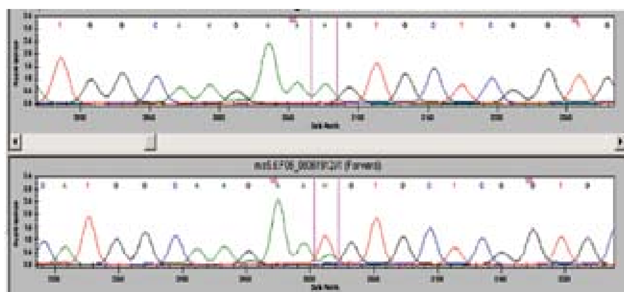

Figure 3 - DNA sequencing chromatogram showing the mutation A→T at codon 66 of the β-globin gene for case 32, identified as Hb Ulm (codon 66, AAA→AAT). The upper chromatogram shows normal DNA sequence and the lower chromatogram shows DNA heterozygous for Hb Ulm.

compound heterozygotes of a variant with Hb S in one case and Hb C in the other. The DNA sequencing analysis revealed 5 patients carried the mutation for the variant Hb Christiansborg, 4 patients carried the mutation for Hb Hope, 2 patients carried the mutation for Hb N-Baltimore, 13 carried the mutation for Hb J-Baltimore, and one patient each carried the mutations for Hb Ulm, Hb J-Iran, Hb J-Bangkok, Hb Richmond, Hb D-Punjab, Hb G-Makassar, Hb Tyne, Hb E-Saskatoon, and Hb K-Woolwich.

Thirteen patients with a presumed diagnosis of Hb J-Baltimore showed a G>A mutation at codon β16 (GGC→GAG) by DNA sequence analysis. To screen the remaining cases assigned a presumed diagnosis of Hb J-Baltimore with a quicker and cheaper method than DNA sequencing, it was decided to develop an ARMS PCR assay, similar to the ones developed previously for the common β-chain variants Hb D-Punjab, Hb S, Hb C, and Hb E.[8] A mutant ARMS primer was designed to detect specifically the mutation for Hb J-Baltimore and tested successfully using DNA from a patient previously identified to carry Hb J-Baltimore by DNA sequencing. The DNA samples from the known case of Hb J-Baltimore yielded the expected specific amplification product of 240 bp, while normal DNA control samples did not amplify with the mutant ARMS primer (**Figure 1**). The ARMS-PCR assay was then used to screen the remaining 7 presumed cases, all of which were found to give a positive result for the Hb J-Baltimore mutation.

Discussion. The screening techniques of HPLC and IEF are very reproducible and provide reliable, accurate data of retention times and focusing positions of variants. The HPLC retention times were very reproducible, varying by only approximately 0.1 minutes for the same variant. For IEF, the same variants always focused to the same position with only approximately 0.5mm variation. However, the retention times and IEF positions have a limited usefulness on their own as a diagnostic tool, as many variants have the same times or focusing positions. The results of our study reported here show that HPLC identified 14 abnormal Hb peaks with significantly different retention times compared to IEF electrophoresis, which revealed only 10 different abnormal Hbs. Thus, HPLC was slightly more sensitive than IEF, in that it detected the larger number of different variants. In terms of specificity, both techniques were approximately the same. Used individually, HPLC, and IEF data could only give a presumed diagnosis for Ione variant for each technique. For the 14 different variants diagnosed by HPLC, there were a total of 64 candidate variants observed previously by us to have same retention times. Thirteen of the different retention times had more than one candidate variant; only Hb Tyne had a unique retention time. For the 10 abnormal band positions revealed by IEF, there were a total of 61 different variants confirmed by our previous studies that could focus to these positions. Nine of these positions had more than one candidate variant; only Hb Rothschild had a unique band position. However, the combination of HPLC rt and IEF position was able to give a presumed diagnosis for each of 14 different

variants, which was then tested by mutation analysis. Of the 40 patients that were given a presumed diagnosis for rare Hb variant by the use of the combination of the HPLC rt and IEF position, 39 were confirmed to carry the predicted variant by using ARMS-PCR or DNA sequencing analysis. Only one case was predicted incorrectly by HPLC and IEF, giving an inaccuracy of 2.5% in our cohort of samples. Thus, 13 different variants in 39 cases were predicted correctly by using a combination of HPLC rt and IEF position. A wrong prediction was made for Case 32. The HPLC showed an abnormal Hb of 36.4% with a rt of 2.07 minutes, and IEF showed an abnormal band at 6.4 mm above the Hb A band (**Figure 2**). Previous diagnostic studies showed this data was consistent with Hb K-Ibadan. However, DNA sequencing identified the point mutation A→T at codon 66 [AAA→AAT] (**Figure 3**), which is the mutation for the rare β-chain variant Hb Ulm.[22] Thus, the HPLC rt and IEF position for Hb Ulm of 2.07 minutes and -6.4 mm, are indistinguishable from Hb K-Ibadan (2.06 minutes and -6.7mm). However, these 2 variants have been reported in different racial groups (Hb Ulm in a German patient, Hb K-Ibadan in an African patient), and so ethnic origin may be used as a final check to differentiate between the 2 variants.

Our results showed that HPLC and IEF demonstrate excellent sensitivity and specificity in predicting 13 different β-chain variant genotypes: Hb J-Baltimore, Hb N-Baltimore, Hb Osu Christiansborg, Hb Hope, Hb J-Iran, Hb Osu Christiansborg, Hb D-Punjab, Hb G-Makassar, Hb Tyne, Hb E-Saskatoon, Hb K-Woolwich, Hb Rothschild, and Hb Richmond. However, the data failed to identify one variant, Hb Ulm, which had not been observed by us before in other patients. The Hb Ulm has the same HPLC rt and IEF position as Hb K-Ibadan, and illustrates that this method still only gives a presumed diagnosis, as a novel variant is always waiting to be discovered with the same possible diagnostic parameters as a rare known variant.

Our results show that once a variant has been defined by molecular analysis, the HPLC rt and IEF position are reliable and reproducible parameters for the identification of rare variants found in a local population. Used individually, there are too many other variants with either a similar rt range, or the same IEF position, to make an accurate presumed diagnosis. The results presented here demonstrate that when used in combination, they can give a presumed identification in new carriers with acceptable accuracy. The limitations of the study, are that there will always be just a few variants, known or novel, that exhibit both the same HPLC retention times and the same IEF positions, as this study showed in the case of Hb K-Ibadan and Hb Ulm. Thus, this approach will always be limited by the fact that it can only give a presumed diagnosis. However, the use of a third defining factor, the ethnic origin of the patient may help to raise the accuracy of the prediction even further without recourse to DNA sequencing in most cases where 2 variants are found to have the same HPLC and IEF parameters. If DNA analysis is required for confirmation of important cases, for example in antenatal screening, the simple, cheap and quick method of ARMS-PCR can be developed for confirmation of the variant mutation. This was carried out successfully for the most common variant in our cohort of patients, Hb J-Baltimore, and thus in principle the technique can be developed for all other β-chain variants, using the same assay conditions as previously developed for diagnosis of the common β-chain variants Hb's S, C, D-Punjab, and E.

Acknowledgment. *The views expressed in this publication are those of the authors and not necessarily those of the Department of Health.*

References

1. Baysal E. The β- and δ-thalassemia repository. ***Hemoglobin*** 1995; 19: 213-236.
2. Old JM. Hemoglobinopathies. In: Rodeck CH, Whittle MJ, editors. Fetal Medicine: Basic Science and Clinical Practice. 2nd ed. London (UK): Churchill Livingstone; 2009. p. 331-343.
3. Old J, Traeger-Synodios J, Galanello R, Petrou M, Angastiniotis A, editors. In: Prevention of thalassaemias and other haemoglobin disorders; Volume 2. Thalassaemia International Federation (TIF), Nicosia, Cyprus, 2005
4. Clarke G, Higgins TN. Laboratory investigation of hemoglobinopathies and thalassemia: review and update. ***Clin Chem*** 2000; 46: 1284-1290.
5. Worthington D. NHS Sickle Cell and Thalassaemia Screening Programme: Handbook for laboratories, 1st ed. London: NSC; 2006.
6. Bain BJ. Hemoglobinopathy diagnosis. Oxford (UK): Blackwell Science Ltd; 2001. p. 260.
7. Galacteros F, Kleman K, Caburi-Martin J, Rosa J, Lubin B. Cord blood screening for haemoglobin abnormalities by thin layer isoelectric focussing. ***Blood*** 1980; 56: 1068-1078.
8. Old JM. Screening and genetic diagnosis of hemoglobinopathies. ***Scand J Clin Lab Invest*** 2007; 67: 71-86
9. Mohammed AA, Okorodudu AO, Bissell MG, Dow P, Reger G, Meier A, et al. Clinical application of capillary isoelectric focusing on fused silica capillary for determination of Hb variants. ***Clin Chem*** 1997; 43: 1798-1799.
10. Working Party of the General Haematology Task Force of the British Committee for Standards in Haemotology. Guideline: the laboratory diagnosis of haemoglobinopathies. ***Br J Haematol*** 1998; 101: 783-792.
11. Ou CN, Rognerud CL. Diagnosis of hemoglobinopathies: electrophoresis vs. HPLC. ***Clin Chem Acta*** 2001; 313: 187-194.
12. Eastman JW, Lorey F, Arnopp J, Currier RJ, Sherwin J, Cunningham G. Distribution of hemoglobin F, A, S, C, E and D quantities in 4 million newborn screening specimens. ***Clin Chem*** 1999; 45: 683-685.

13. Rugless MJ, Fisher CA, Stephens AD, Amos RJ, Mohammed T, Old JM. Hb Bart's in cord blood: an accurate indicator of α-thalassaemia. ***Hemoglobin*** 2006; 30: 57-62.
14. Mario N, Baudin B, Aussel C, Giboudeau J. Capillary isoelectric focusing and high-performance cation-exchange chromatography compared for qualitative and quantitative analysis of hemoglobin variants. ***Clin Chem*** 1997; 43: 2137-2142.
15. Cotton F, Lin C, Fontaine B, Gulbis B, Janssens J, Vertongen F. Evaluation of a capillary electrophoresis method for routine determination of hemoglobins A2 and F. ***Clin Chem*** 1999; 45: 237-243.
16. Fucharoen S, Winichagoon P, Wisedpanichkij R, Sae-Ngow B, Sriphanich R, Oncoung W, et al. Prenatal and postnatal diagnoses of thalassemias and hemoglobinopathies by HPLC. ***Clin Chem*** 1998; 44: 740-748.
17. Riou J, Godart C, Didier H, Mathis M, Bimet C, Bardakdjian-Michau J, et al. Cation-exchange HPLC evaluated for presumptive identification of hemoglobin variants. ***Clin Chem*** 1997; 43: 34-39.
18. Abraham R, Thomas M, Britt R, Fisher C, Old J. Hb Q-India: an uncommon variant diagnosed in three Punjabi patients with diabetes is identified by a novel DNA analysis test. ***J Clin Path*** 2003; 56: 296-299.
19. Basset P, Beuzard Y, Garel MC, Rosa J. Isolectric focusing of human hemoglobin: Its application to screening, to the characterization of 70 variants, and to the study of modified fractions of normal hemoglobins. ***Blood*** 1978; 51: 971-982.
20. Old JM, Higgs DR. The thalassemias In: Weatherall DJ, editor. Methods in Haematology. Edinburgh: Churchill Livingstone; 1983. p. 74-102.
21. Old J, Khan S, Verma I, Fucharoen S, Kleanthous M, Ioannou P, et al. A multi-centre study in order to define further the molecular basis of β-thalassemia in Thailand, Pakistan, Sri Lanka, Mauritius, Syria, and India, and to development a simple molecular diagnostic strategy by ARMS-PCR. ***Hemoglobin*** 2001; 25: 397-407.
22. Friess U, Beck A, Kohne E, Lehmann R, Koch S, Haring HU, et al. Novel haemoglobin variant (B66 (E10) Lys-Asn)) with decreased oxygen affinity, causes falsely low haemoglobin A1c values by HPLC. ***Clin Chem*** 2003; 49: 1412-1415.

References

* References should be primary source and numbered in the order in which they appear in the text. At the end of the article the full list of references should follow the Vancouver style.

* Unpublished data and personal communications should be cited only in the text, not as a formal reference.

* The author is responsible for the accuracy and completeness of references and for their correct textual citation.

* When a citation is referred to in the text by name, the accompanying reference must be from the original source.

* Upon acceptance of a paper all authors must be able to provide the full paper for each reference cited upon request at any time up to publication.

* Only 1-2 up to date references should be used for each particular point in the text.

Sample references are available from:
http://www.nlm.nih.gov/bsd/uniform_requirements.html