MULTICENTER VALIDATION OF FULLY AUTOMATED CAPILLARY ELECTROPHORESIS METHOD FOR DIAGNOSIS OF THALASSEMIAS AND HEMOGLOBINOPATHIES IN THAILAND

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Abstract. Thalassemias and hemoglobinopathies are highly prevalent in Thailand and other Southeast Asian countries. Accurate and precise separation of hemoglobin types, together with reliable quantitation, are essential for differential diagnosis of these diseases. Presented in this study is a multicenter validation of a fully automated capillary electrophoresis (CE) method for hemoglobin separation and quantitation involving four reference laboratories in Thailand. Analytical performance characteristics, including precision and accuracy were compared with existing validated HPLC and LPLC methods using 412 blood samples from unrelated subjects. Coefficient of variance of Hb A_2 quantitation was 1.80-2.86, 1.26-5.13 and 1.08-6.66% for within run, between run and inter-laboratory comparison, respectively. Results of Hb A_2 and Hb F quantitated by the CE method correlates well with those of the two comparative methods (r = 0.98-0.99). The CE method correctly determined the genotypes (thalassemias and hemoglobin variants) of all blood samples tested. The major advantage of the CE system is its ability to separate and quantitate Hb A_2, Hb E, Hb F, Hb H and Hb Bart's, which are important parameters required for diagnosis of thalassemias and hemoglobinopathies.

Keywords: capillary electrophoresis, thalassemia, hemoglobinopathy, validation

INTRODUCTION

Capillary electrophoresis (CE) is one of the analytical separation techniques, which has found extensive use in clinical laboratories (Petersen *et al*, 2003; Magaña *et al*, 2009). Numerous methods for detecting clinically relevant analytes have been developed using CE to detect thalassemia and hemoglobinopathy, including Hb separation and quantitation of the fraction of Hb F in peripheral blood (Clarke and Higgins, 2000; Hartwell *et al*, 2005) as the increase in Hb F level is an important

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feature associated with β-thalassemia disease, δβ-thalassemia, and hereditary persistence of Hb F (HPFH), although a number of drug treatments can result in elevation of Hb F levels (Mosca *et al*, 2009). The presence of Hb Bart's or Hb H is the hallmark of α-thalassemia.

In 2007, the CE system was approved by USA Food and Drug Administration for the evaluation of hemoglobinopathies (Keren *et al*, 2008). The system uses CE to separate and quantitate Hb A_2, Hb F and other abnormal Hbs. This completely automated system with multiple capillaries provides several potential advantages for clinical laboratories, including improvement in turnaround time, high throughput, minimal sample manipulation, requirement of small sample volume, and reasonable cost. Studies have shown that CE is a suitable method for the diagnosis of thalassemias and hemoglobinopathies (Cotton *et al*, 1999; Shihabi *et al*, 2000; Jenkins and Ratnaike, 2003; Louahabi *et al*, 2006; Boonkant *et al*, 2008; Winichagoon *et al*, 2008; Delft *et al*, 2009; Higgins *et al*, 2009; Yang *et al*, 2009; Srivorakun *et al*, 2011).

In order to comply with national and international regulations in clinical laboratory diagnosis, all laboratory tests must be validated before being introduced for testing in patients so as to ensure that the results reported meet the clinical expectations with a desired degree of reliability (Westgard, 1999; Thompson *et al*, 2002; ICH Expert Working Group, 2005). This study reports the results of a multicenter validation of the Capillarys 2 System (Sebia, France) for diagnosis of thalassemias and hemoglobinopathies in Thailand. The analytical performance characteristics, including accuracy and precision were evaluated. Criteria for making a decision for acceptance of the method were considered based on both performance and application characteristics.

MATERIALS AND METHODS

Blood samples and study sites

The study was conducted at four reference laboratories of the Department of Medical Sciences (DMSc), Ministry of Public Health, Thailand, namely, Clinical Research Center (Lab #1) and Regional Medical Sciences Center (RMSc) at Chiang Rai Province (Lab #2), Udon Thani Province (Lab #3) and Surat Thani Province (Lab #4) during November 2009 to January 2010. All laboratories were required to perform an internal quality control and to participate in a proficiency testing program, in order to monitor their analytical performances along with the study.

A total of 412 routine EDTA blood samples sent for diagnosis of thalassemias and hemoglobinopathies were used in this study. These samples were examined using LPLC at Lab#1 and HPLC at Lab # 2, 3 and 4. Overall, 116 samples were obtained from normal individuals, 66 samples from β-thalassemia carriers, 57 samples from thalassemia patients with different genotypes and 173 samples from subjects with Hb variants. The presence of β-thalassemia and Hb variants were confirmed by direct DNA sequencing (Sangkitporn *et al*, 2009).

Hb separation and quantitation by automated CE

Hb separation and quantitation of Hb types were performed using Capillarys 2 System (Sebia, France) equipped with 8 capillaries according to the manufacturer's instructions. In brief, blood sample was added to lysis solution and an aliquot was introduced by aspiration at the anodic end into the capillary tube before applying a voltage of 9,800 V. Detection

of Hbs was conducted by measuring absorbance at 415 nm at the cathodic end of the capillary tube. At the end of the analysis, Hb A fraction was adjusted to be in zone 9 at the middle of the window. Relative quantity (percent of total Hb) and presumptive identification of the Hb types (located in various zones from zone 1 to 15) were recorded from the resulting electrophoregram. Positions of different Hbs are identified in the pertinent zones.

Hb separation and quantitation by other chromatography methods

The automated cation-exchange HPLC (Beta-Thalassemia Short Program, Variant™ Hb Testing System; Bio-Rad Laboratories, Hercules, CA) and the automated cation-exchange LPLC (Hb Gold analyzer, Drew Scientific, Cumbria, UK) were used as comparative methods for Hb typing and Hb A_2/Hb F quantitation following the manufacturers' instructions.

Validation study

Precision. Precision of Hb A_2, Hb F and Hb E quantitation was subdivided into within run precision, between run precision and inter-laboratory precision. Within run precision was evaluated under constant conditions as much as possible by the same scientists using the same instruments in each laboratory. Each sample was analyzed in 8 consecutive replicates for each single run. Between run precision was determined by comparing the run results within the same laboratory over 3 weeks. Inter-laboratory comparison was performed using 6 different blood samples, which were delivered to all reference laboratories within 3 days after blood collection. Laboratory results were subsequently sent to CRC within 1 week for statistical analysis.

Accuracy. Due to a lack of certified reference materials, comparisons of the methods were conducted to estimate the accuracy of Capillarys 2 System. LPLC and HPLC were used as the comparative methods at CRC laboratory and 3 reference RMSc laboratories. EDTA blood of normal subjects, Hb H disease patients and β-thalassemia carriers were selected as normal Hb A_2, low Hb A_2 and high Hb A_2 samples, respectively. These samples were selected so as to cover the entire working range of the three methods.

Statistical analysis

Mean, standard deviation (SD) and coefficient of variation (%CV) were used to estimate precision. Correlation of percent Hb A_2 and percent Hb F quantitated by Capillarys 2 System and those of the two comparative methods were determined by linear regression analysis. The strength of association between the two variables was based on the value of correlation coefficient (r).

RESULTS

Performance characteristics of Capillarys 2 System

Results of inter-laboratory studies demonstrated that all blood samples were clearly differentiated by all laboratories (Table 1). Overall %CV of Hb A_2 quantitation was 1.80-2.86%, 1.26-5.13% and 1.08-6.66% for within run, between run and inter-laboratory comparison, respectively. Poorer precision was observed in samples with normal Hb F level (< 1%).

As regards Hb separation results, all samples had consistent results at all 3 levels of precision: Hb A, Hb A_2 and Hb F was always identified in zone 9, zone 3 and zone 7, respectively. Comparisons of the 3 methods performed to estimate accuracy of percent Hb A_2 and percent Hb F showed good linear correlation (Fig 1).

Table 1
Precision of Hb A_2, Hb F and Hb E quantitation using Capillarys 2 System.

Precision level	Hb A_2		Hb F		Hb E	
	Percent Hb A_2	Percent CV	Percent Hb F	Percent CV	Percent Hb E	Percent CV
Within run precision						
Quality control material						
Normal Hb A_2 control	2.24-2.56	1.85-2.86	-	-	-	-
AFSC control	2.44-2.56	1.80-2.24	21.7-22.35	0.71-1.86	-	-
Between run precision						
Quality control material						
Normal Hb A_2 control	2.45-2.53	2.21-4.11	-	-	-	-
AFSC control	2.44-2.56	2.13-3.11	21.44-21.74	3.59-4.86	-	-
EDTA blood sample						
Normal subject	2.81	2.28	0.32	9.97	-	-
β-thalassemia carrier	5.60	1.26	-	-	-	-
Hb E carrier	3.50	2.02	-	-	29	3.02
β-thalassemia/ Hb E	5.25	1.90	20.38	2.02	38.86	3.03
β-thalassemia homozygote	2.92	5.13	46.05	5.35	-	-
Inter-laboratory precision						
EDTA blood sample						
Normal subject	2.7	2.18	-	-	-	-
β-thalassemia carrier	5.34	1.08	-	-	-	-
Hb E carrier	3.47	6.66	1.5	6.42	22.4	4.46
Hb E carrier	3.60	2.78	-	-	22.25	2.45

Application of Capillarys 2 System for diagnosis of thalassemias and hemoglobinopathies

Capillarys 2 System was employed in the analysis of 412 unrelated Thai subjects. In homozygous β-thalassemia, samples, electrophoregrams revealed the prominence of Hb F levels and decreased percent Hb A. The electrophoregrams also demonstrated the presence of Hb A_2, Hb E and Hb F in blood samples from β-thalassemia/Hb E individuals (Fig 2B). In Hb H disease, Hb Bart's and Hb H were clearly separated from each other and could be readily quantitated (Fig 2C). The genotypes of all thalassemia subjects (n = 123) in this study were correctly identified by Capillarys 2 System in comparison with the two other chromatographic methods (Table 2).

Presumptive identifications of the common Hb variants present in the Thai population, namely, Hb E and Hb Constant Spring (CS), were clearly possible due to their different positions from those of Hb A, Hb A_2 and Hb F in the electrophoregrams of Capillarys 2 System (Fig 2B and C). Three rare Hb variants (Hb J-Bangkok, Hb G-Makassar and Hb C) were also detected (Fig 2D, E and F).

Table 2
Capillarys 2 System results obtained from blood samples of thalassemias and hemoglobinopathies frequently observed in Thailand.

Group	*N*	Hb pattern	Percent Hb A_2	Percent Hb F	Other Hbs		Interpretation	Degree of agreement
					Type	%		
1	116	A_2A	2.8 ± 0.3	0.3 ± 0.5	-	-	Normal Hb typing	100%
2	66	A_2A	5.8 ± 0.7	1.2 ± 1.0	-	-	β-thalassemia carrier	100%
3	2	A_2FA	3.1 ± 0.2	57 ± 15	-	-	β-thalassemia homozygote	
4	120	EA	4.0 ± 0.4	0.8 ± 1.1	E	24 ± 3	Hb E carrier	100%
5	30	EF	5.5 ± 1.1	44 ± 18	E	49 ± 16	β-thalassemia/ Hb E	100%
6	25	A_2ABart'sH	1.2 ± 0.3	0.7 ± 0.3	H	3 ± 2	Hb H disease	100%
					Bart's	1.0 ± 1.1		
7	50	EE	7 ± 9	6 ± 6	E	87 ± 9	Hb E homozygote	100%
8	1	Abnormal Hb	3.7	2.2	Hb J Bangkok	47.4	Hb J Bangkok carrier	100%
9	1	Abnormal Hb	2.8	5.7	Hb G Makassar	38.0	Hb G Makassar carrier	100%
10	1	Abnormal Hb	3.3	0.2	Hb C	36.0	Hb C carrier	100%

Degree of agreement represents percent agreement between final interpretation of Capillarys 2 System and those of HPLC and LPLC techniques used as comparative methods.

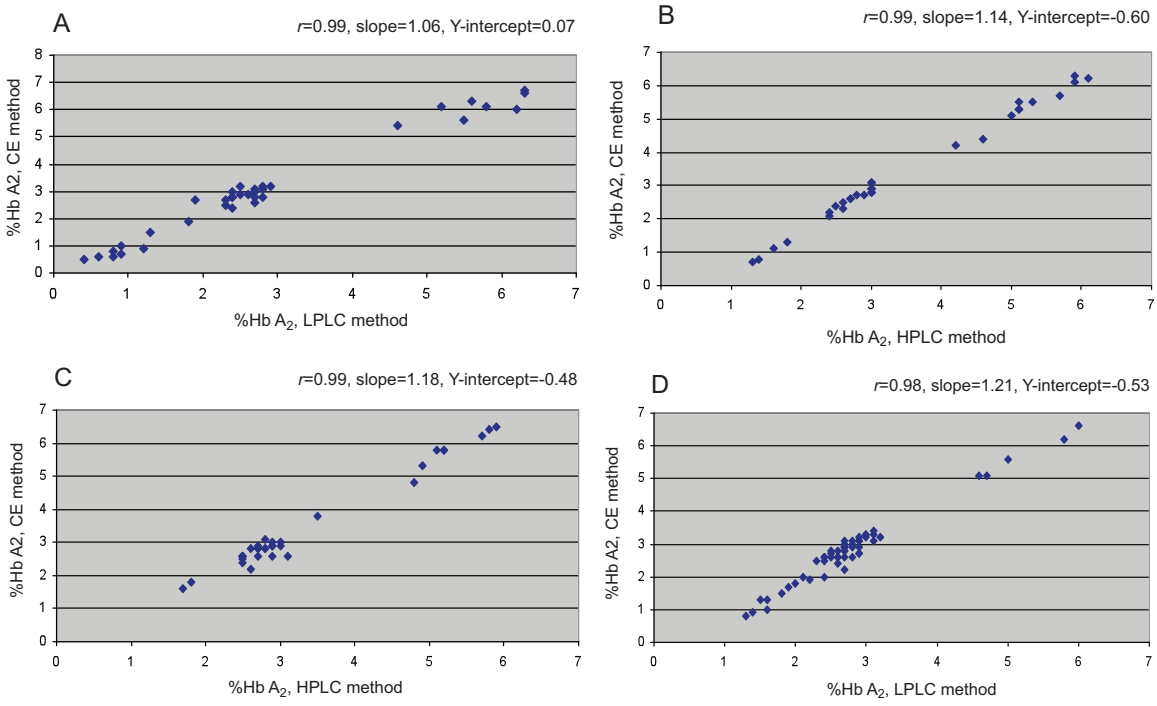

Fig 1–Comparison of Hb A_2 levels between CE and HPLC or LPLC methods at four reference laboratories in Thailand. Hb A_2 levels in EDTA blood samples were determined as described in Materials and Methods. A, Lab #1; B, Lab #2; C, Lab #3; D, Lab #4.

DISCUSSION

In Thailand and other Southeast Asian countries, α- and β-thalassemias are highly prevalent, including Hb variants such as Hb E and Hb CS, the co-inheritance of which can have a profound impact on the severity of the thalassemia syndromes (Fucharoen and Winichagoon, 2001; Fucharoen *et al*, 2004; Nuntakarn *et al*, 2008; Colah *et al*, 2010). The Capillarys 2 System (Boonkant *et al*, 2008; Keren *et al*, 2008) has been developed for diagnosis of these genetic diseases to provide a rapid and fully automated system, with a throughput of 34 samples per hour. Other advantages of the system include the ability to separate and quantitate Hb A_2, Hb H and Hb Bart's, competitive cost and minimal sample manipulation.

In order to determine that the Capillarys 2 System is suitable and capable of providing useful analytical data for diagnosis of thalassemias and hemoglobinopathies in the setting in a developing country, validation experiments were carried out as a multicenter study at 4 reference laboratories on 412 blood samples from unrelated subjects. Our studies showed that within run and between run precisions of Hb A_2, Hb F and Hb E quantitations were comparable with previous reports (Fucharoen *et al*, 1998; Sangkitporn *et al*, 2002; Paleari *et al*, 2007), whereas inter-laboratory precision was quite low. The narrow scatter of the results confirmed the reproducibility of the assay.

In agreement with the previous studies (Winichagoon *et al*, 2008; Higgins *et al*, 2009), a higher variation was found for

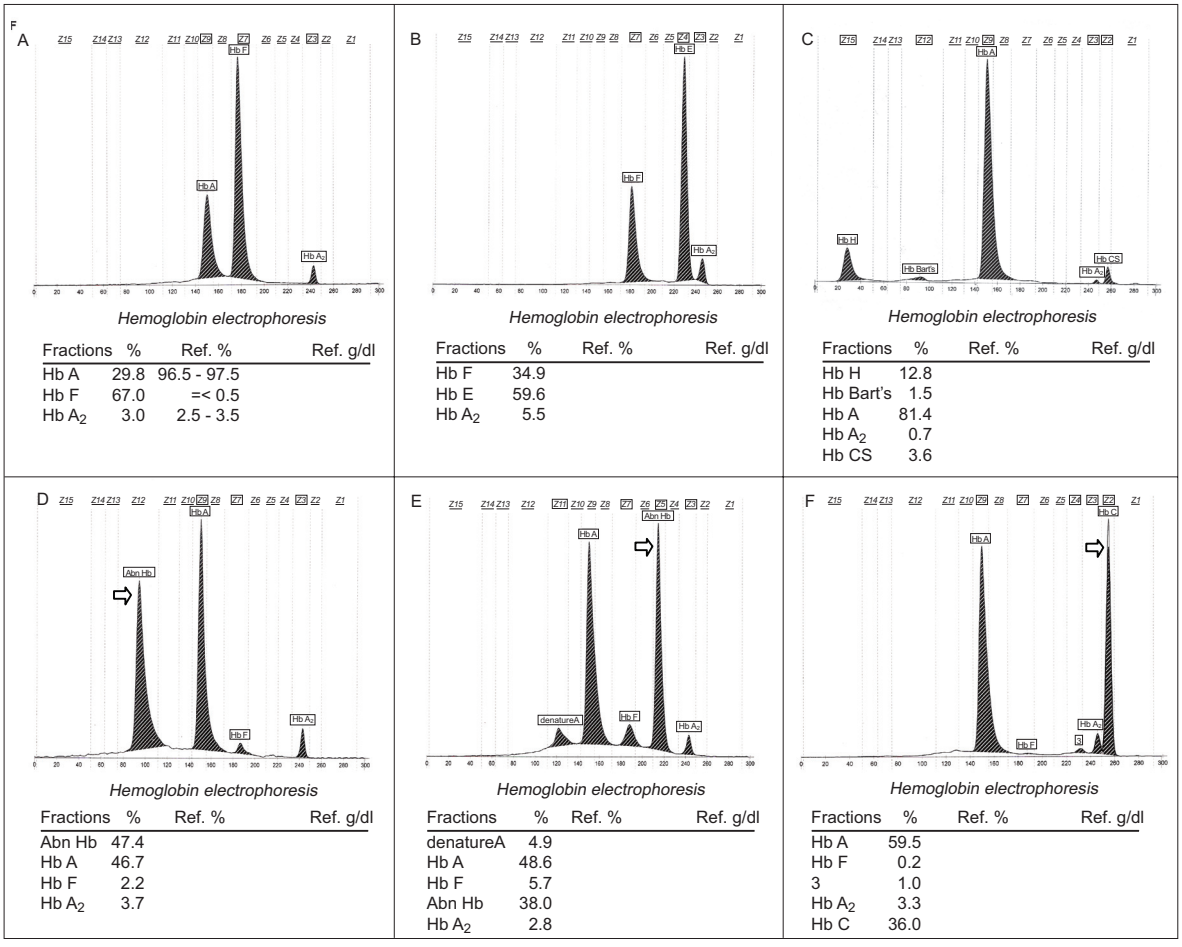

Fig 2–Typical Capillarys 2 System electrophoregram of homozygous β-thalassemia (A), β-thalassemia/ Hb E (B), Hb H/CS (C), Hb J Bangkok (D), Hb G Makassar (E) and Hb C (F). Blood sample was introduced by aspiration at anodic end into the capillary tube before applying a voltage of 9,800 V. Detection of Hb was by measuring absorbance at 415 nm at cathodic end of the capillary tube. At the end of the analysis, Hb A fraction was adjusted to be at the middle of the window.

Hb F quantitation, especially at low levels when present in normal subjects. This variation decreased to an acceptable level when Hb F levels were increased in pathological conditions, such as β-thalassemia/ Hb E and homozygous β-thalassemia. The higher variations in cases of low Hb F (≤ 1%) might not be of importance as low Hb F level has no or is of minimal clinical significance.

Two other chromatographic techniques, HPLC and LPLC, were employed as comparative procedures as these methods have previously been validated (Fucharoen *et al*, 1998; Sangkitporn *et al*, 2002). Our results indicated that percent Hb A_2 and percent Hb F determined by Capillarys 2 System correlated well with both comparative methods (r = 0.98-0.99).

The Capillarys 2 System has the ability to separate Hb A_2 from Hb E and also to quantify Hb Bart's and Hb H in Hb H subjects. In both HPLC and LPLC methods, Hb E co-elutes with Hb A_2, making it impossible to separate and quantitate Hb A_2 in such samples. In addition, in the latter two techniques Hb H and Hb Bart's are both eluted early from the columns and in many cases they are not readily resolved from one another. It is worth noting that the Capillarys 2 System allows facile detection of Hb CS, as well as other rarer Hb variants, making it suitable for diagnosis of thalassemias and hemoglobinopathies in Thailand and other regions where α-thalassemia, β-thalassemia, Hb E and Hb CS are prevalent.

In summary, this study showed that Capillarys 2 System provides a more advantageous alternative for diagnosis of thalassemias and hemoglobinopathies than other existing HPLC and LPLC chromatographic techniques. Its characteristics regarding precision, accuracy and cost are comparable to these latter methods. The main advantage of the Capillarys 2 System is the ability to separate and quantitate Hb A_2, Hb E, Hb F, Hb H and Hb Bart's, important parameters for the accurate diagnosis of thalassemias and hemoglobinopathies.

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