Detection of Hemoglobin Constant Spring by Capillary Electrophoresis and High-Performance Liquid Chromatography: A Study in Kelantan, Malay

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Abstract

***Objectives*:** Haemoglobin Constant Spring (Hb CS) is one of the most common non-deletion types of alpha (α) thalassemia in Southeast Asia. This also includes our place of interest, Kelantan, Malaysia. The nature of this abnormal globin gene is that it is unstable, labile, and present in a minute amount in the peripheral blood, thus leading to underdiagnoses of the disease. This study was conducted to (1) determine the prevalence of Hb CS among the Kelantan population, (2) compare the different levels of Hb CS detected by capillary electrophoresis (CE) for three different groups of Hb CS (heterozygous, homozygous, and compound heterozygous) and (3) determine the efficacy of CE and HPLC for detection of Hb CS.

***Methods*:** A cross-sectional study involving secondary data collection taken from Form 4 students in schools all over Kelantan who were involved in a thalassemia screening program conducted by the Ministry of Health (MOH) Malaysia. The haemoglobin (Hb) analysis was performed using an automated CE system (CAPILLARYS2 Flex-Piercing System Sebia) and high-performance liquid chromatography (HPLC) (VARIANT II, Bio-rad Laboratories). DNA analysis used multiplex polymerase chain reaction (PCR) and multiplex amplification refractory mutation system (ARMS) to detect both deletion and non-deletion α-thalassemia.

***Results*:** 376 samples (99.5%) with presence of peak value on Zone 2 of CE were confirmed to have termination codon CS mutation. Heterozygous Hb CS is the most common type detected in 344 samples (91.5%), followed by compound heterozygous Hb CS which was 31 samples (8.2%) and only 1 sample (0.3%) of homozygous Hb CS.

***Conclusion*:** The diagnosis of Hb CS will be accurately diagnosed by combining CE and HPLC methods before confirmation by DNA molecular study, which is far more expensive.

Keywords: Alpha thalassemia, Hb constant spring, capillary electrophoresis, molecular study

Introduction

Thalassemia is a public health problem in Malaysia. Current estimation shows that 6.8% of Malaysians are thalassemia carriers who might be affected by various degrees of anaemia.[1] Alpha thalassemia is caused by

deletions or non-deletions / mutations within the α-globin gene complex, leading to a decrease or absence of α-globin chain production.[2]

Haemoglobin Constant Spring (Hb CS) is the most prevalent non-deletion α-thalassemia among the Southeast Asian population.[3,4] In Malaysia, the frequency is higher in Malays (2.24%) compared to Chinese and Indian (0.66% and 0.16% respectively).[5,6] Hb CS involves a (TAA>CAA) base pair substitution in the terminal codon of the α2 globin gene resulting in the elongation of the α chain up to another 31 amino acid residues.[7] The natural features of Hb CS lead to the decrease in the rate of normal α-globin synthesis due to its mRNA instability.

Generally, heterozygote Hb CS has normal clinical and haematological features. On the other hand, homozygotes Hb CS may present as thalassemia intermediate with mild anaemia, jaundice, and hepatosplenomegaly.[8] However, the interaction of Hb CS gene with deletion type of α-thalassemia is the leading cause of non-deletion Hb H (β_4) (--/$\alpha^{cs}\alpha$). This non-deletion α-thalassemia is more severe than deletion α-thalassemia, where some patients became transfusion-dependent.[7]

Hb CS is frequently missed by routine laboratory testing usually employed in Malaysian laboratories, especially in the heterozygote state, because Hb CS is unstable and presents at a low level in peripheral blood.[9] In this study, the diagnosis of Hb CS was determined by automated capillary electrophoresis (CE). CE was chosen as the method of choice for Hb analysis as it is believed to be superior to high-performance liquid chromatography (HPLC) in detecting of Hb CS trait. In CE, this haemoglobin will give a peak at the Zone 2 (Hb C/Hb CS zone). Another common variant that also shares the same peak is Hb Pakse. Whereby in HPLC, Hb CS gives a very small peak at the C window with a retention time of 4.90 - 5.30 minutes.[10] However, in our previous experience using HPLC, sometimes there was no peak seen in this region for heterozygotes, which may lead to misdiagnosis. The gold standard for diagnosis is still based on molecular analysis, which is costly and tedious.[3] In Malaysia, only several centers offer these molecular tests.

Thus, this study aims to determine the prevalence of Hb CS among the Kelantan population, compare the different levels of Hb CS detected by CE for three groups of Hb CS (heterozygous, homozygous, and compound heterozygous) and determine the efficacy of CE and HPLC method for detection of Hb CS. In order to develop effective prevention and control programs and treatment plans, it is crucial to understand the prevalence of Hb CS and how it interacts with other hemoglobinopathies.

Methods

This study collected secondary data from thalassemia registry/database haematology laboratory, Hospital Raja Perempuan Zainab II (HRPZII) from 2017 to 2018. Study subjects were Form 4 students all over Kelantan involved in the thalassemia screening program conducted by the Ministry of Health (MOH) Malaysia. Their blood samples were taken in EDTA containers and sent for haemoglobin analysis in HRPZ II. This study was approved by Medical Research and Ethics Committee of the MOH Malaysia (approval number NMRR-18-3787-44516 (IIR)) and Universiti Sains Malaysia Research Committee (USM/JEPeM/18120785). The sample size was 386, calculated based on One-way ANOVA using G-Power software. A total of 13 895 samples were sent for haemoglobin analysis during this study period, and 835 samples turned out to have a peak in Zone 2 CE. However, due to budget constraints, only 378 samples were randomly chosen for DNA analysis.

Quantifying and identifying hemoglobinopathy was performed using an automated CE system (CAPILLARYS2 Flex-Piercing System Sebia) and HPLC (VARIANT II, Bio-Rad Laboratories, Hercules, CA, USA). The samples were analyzed within 24 hours, first with HPLC, followed by CE, and performed according to the manufacturer's instructions. The principle HPLC uses is the separation of molecules with net positive charges being separated into different fractions through adsorption onto a negatively charged static phase in a chromatography column. The haemoglobin molecules can be identified optically in the eluate, provisionally distinguished by their retention time, and measured by area under the peak after separation. While the Sebia Capillarys 2 system, software version 6.2, uses the principle of CE in which charged molecules are separated at alkaline pH by their electrophoretic mobility, electrolyte pH, and electroosmotic flow. Runs were done within 24 hours after collection, and quality control was monitored using the Hb A2 commercial control materials (Sebia).

We have outsourced these samples to a reference molecular laboratory for DNA analysis and molecular study. In Malaysia, the molecular laboratory in Hospital Kuala Lumpur (HKL) is the central laboratory that offers DNA analysis for both deletion and non-deletion α-thalassemia. For the common deletion α-thalassemia, multiplex Gap

PCR is being used as the method of choice and can identify these α-gene deletions, i.e., single-gene deletion: α3.7, -α 4.2 and two gene deletions: -- SEA, -- FIL, -- MED, -- (α)20.5, -- THAI. As for the non-deletion α-thalassaemia, a multiplex ARMS PCR-based method is employed to identify point mutation at the initiation codon, Codon 30 and Codon 35 for Hb Evora, Codon 59 for Hb Adana, Codon 125 for Hb Quang Sze and point mutation at termination codon for Hb CS (TAA→ CAA).

Statistical analysis used Statistical Package for the Social Science (SPSS) version 26.0. The peak value of Zone 2 on CE was analyzed using an independent t-test that compares heterozygous Hb CS and compound heterozygous Hb CS. The data were presented in mean (SD) with a p-value of <0.05, considered statistical significance. The correlation between CE and HPLC was determined using the Pearson correlation coefficient test. The strength of the association between the two variables is based on the correlation coefficient (r) value. The study design, from data collection to data analysis, is depicted in the flowchart (Figure 1).

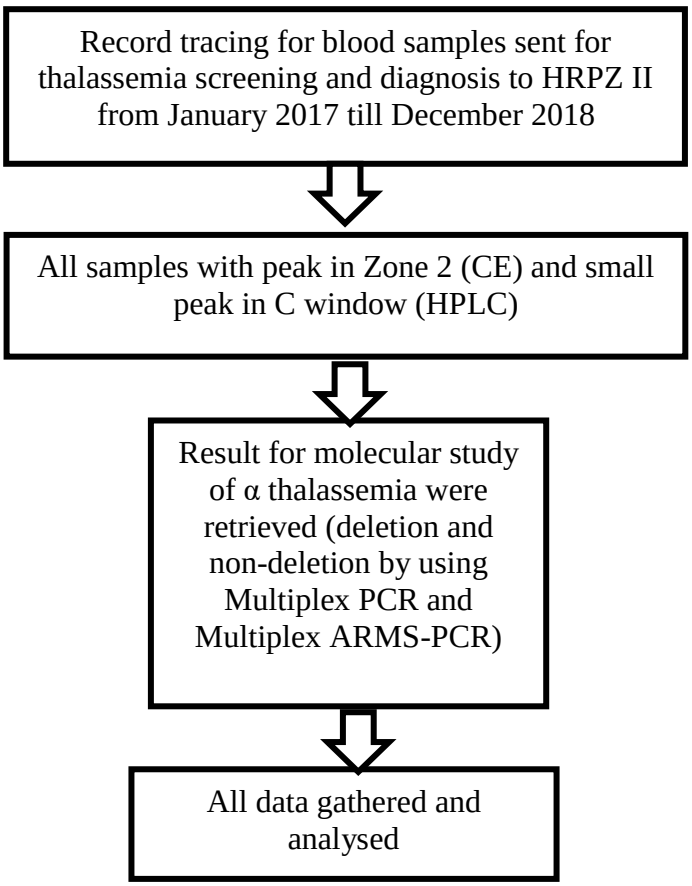

Figure 1: The flow-chart showing study design on detection of Hb CS by CE and HPLC.

Results

A convenient sampling of 835 samples that showed peaks at Zone 2 of CE were recruited in this study. The target group of participants were teenagers. The major age group found in this study were 16 years old, 442 (52.9%), followed by 15 years old, 391 (46.8%) and 17 years old, 2 (0.3%). There were 457 (55%) female participants, whereas the remainder of 378 (45%) were male participants. Since the Malay ethnic population predominantly occupies Kelantan, the vast majority of the ethnic included were 829 (99.3%) Malays, and the balance was from other ethnic groups such as 3 (0.35%) Chinese and 3 (0.35%) Siamese (Table 1).

Table 1: Demographic data (N = 835).

Demographic data	**n (%)**
Age group (years)	
15	391 (46.8%)
16	442 (52.9%)

17	2 (0.3%)
Gender	
Male	457 (55.0)
Female	378 (45.0)
Ethnic group	
Malay	829 (99.3)
Chinese	3 (0.35)
Siamese	3 (0.35)

From 835 that have a peak in Zone 2 CE, only 378 samples were randomly chosen to proceed with DNA analysis. Hb CS was confirmed in 376 samples, and 2 samples were normal. Among the Hb CS cases, 344 samples were heterozygous Hb CS (91.5%), followed by 31 samples of compound heterozygous Hb CS (8.2%), and only 1 sample was homozygous Hb CS (0.3%) (Table 2). The level of Hb CS in heterozygotes ranged from 0.3% to 1.1%, whereas in the compound heterozygous was 0.2% to 1.6%, and a level of 4.9% was seen in the homozygous Hb CS (Table 3).

Table 2: Types of Hb CS based on molecular analysis (N = 376).

Types of Hb CS	**n (%)**
Heterozygous	344 (91.5%)
Homozygous	1 (0.3%)
Compound heterozygous	31 (8.2%)

Table 3: Mean and range of Zone 2 peak on CE for different types of Hb CS (N = 376).

Group	N	Mean± SD	Range of Zone 2 peak on CE (%)	*p* value[a]
Heterozygous Hb CS	344	0.61 ± 0.13	0.3-1.1	<0.001
Homozygous Hb CS	1	4.90*	4.9*	
Compound heterozygous Hb CS	31	0.77 ± 0.34	0.2-1.6	

**no standard deviation (SD) because constant value, only one participant in this group.*
[a] p value significant at p*<0.001.*
*Note: Independent t-test CE [a]*p *< 0.001*

From those 31 samples of compound Hb CS, it was further divided into Hb CS with 3.7 deletion, Hb CS with 4.2 deletion, Hb CS with concurrent Hb E, Hb CS with concurrent Hb E and 3.7 deletion and Hb CS with concurrent Hb E and 4.2 deletion (Table 4).

Table 4: Distribution of compound heterozygous Hb CS according to genotypes of Hb CS and level in CE (N = 31).

Types and co-inheritance	**n (%)**	**Hb CS level in CE (mean ± SD)**
Hb CS with α-$^{3.7}$	16 (51.6%)	0.94(0.26)
Hb CS with α-$^{4.2}$	3 (9.7%)	1.07(0.50)
Hb CS with Hb E trait	7 (22.6%)	0.41(0.18)
Hb CS with α-$^{3.7}$ and Hb E	4 (12.9%)	0.58(0.05)
Hb CS with α-$^{4.2}$ and Hb E	1 (3.2%)	0.50*

**no SD because constant, only one sample involved.*

Of the 344 cases of heterozygous Hb CS, all (100%) were detected by CE, whereas only 290 (84.3%) samples were detected by HPLC. As for compound heterozygous Hb CS, 28 (90.3%) samples were detected by HPLC compared to 31 (100%) that CE can detect. Only 1 sample of homozygous Hb CS in this study can be detected by both methods (Table 5). However, using HPLC, only a small hump was observed at the C window without quantification, especially in the case of heterozygous Hb CS.

Table 5: Detection of Hb CS by CE and HPLC in study samples (N = 376).

Types of Hb CS	**CE**	**HPLC**	
	n	**No peak n (%)**	**Peak n (%)**
Heterozygous Hb CS	344	54 (15.7)	290 (84.3)
Homozygous Hb CS	1	0 (0.0)	1 (100.0)

Compound heterozygous Hb CS	31	3 (9.7)	28 (90.3)

Pearson correlation coefficient test was used to examine the relationship between HPLC and CE findings of Hb CS by comparing the peak value in Zone 2 of CE with the small peak detected on HPLC at C-window (n= 376). Table 6 shows a significant positive direct correlation between HPLC and CE value ($p<0.001$). The correlation strength was good, with a positive linear relationship (Figure 2).

Table 6: Correlation HPLC and CE value.

Variables	Pearson correlation, r	P value
CE vs HPLC	0.73	<0.001

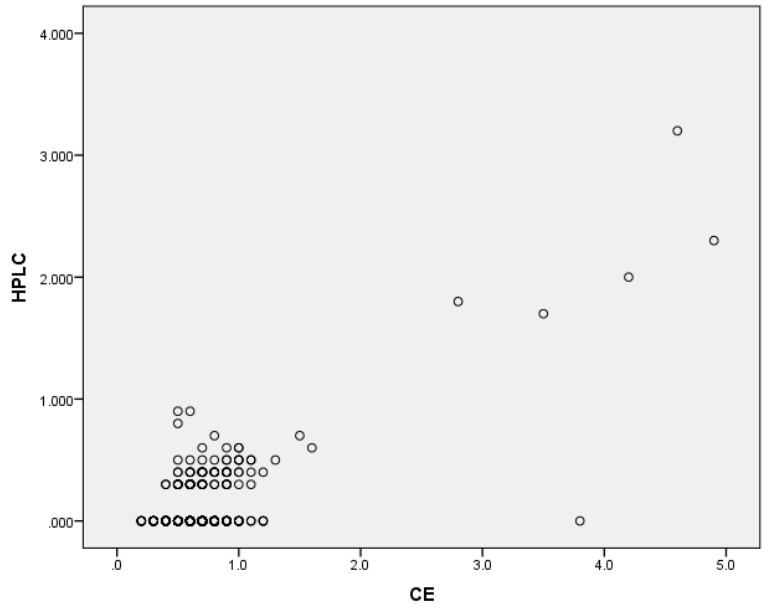

Figure 2: Scattered plot showed the correlation between HPLC and CE

Discussion

The study was performed to determine the prevalence of Hb CS responsible for non-deletion α-thalassemia in our region. Kelantan is in the northeast Peninsular of Malaysia, and the majority of the population is Malay.[11] HRPZ II is the tertiary hospital in Kelantan that caters all the samples from Form 4 Thalassemia screening program in Kelantan. Thus, the samples included in this study are a good representation of the population in Kelantan. Through the report, comprehensive data on age groups, gender, and ethnicity can be obtained. Based on 835 samples that showed peak at Zone 2 CE, which can be only presumptive for diagnosing Hb CS, Malay (99.3%) is the major population affected by thalassemia, followed by female gender (55%).

This study observed that heterozygous Hb CS was the most common and occurred in high frequency in Kelantan (91.5%). A similar finding was reported by Liao C *et al*., where this type of Hb is majority in southern China.[12] An amount of 0.1 to 1.0% of total Hb, with an average of 0.6 ± 0.1%, is usually observed in a heterozygote, and this finding was quite similar to our study.[7]

Homozygote Hb CS shows a clinical picture of thalassemia intermedia phenotype associated with overt haemolytic anemia.[13,14] In a study conducted in 2012 by Pornpraset *et al.*, the findings showed that the quantification of mean± standard deviation (SD) Hb CS level was significantly higher in homozygous group than that of heterozygous group (1.9 ± 1.8 vs 0.4 ± 0.2, $p = 0.007$).[9] This finding was similar to other studies, in which the homozygous group had much higher level in CE than heterozygous group. The limitation in our study was that only 1 sample was confirmed to be homozygous Hb CS and showed level of 4.9% in CE. However, this method might facilitate laboratory diagnosis of heterozygous and homozygous Hb CS.

Compound heterozygous Hb CS showed a lower level of Zone 2, ranging from 0.2 to 1.6%, and similar finding was also reported by Ramli M et.al[15]. Then they were further analysed and classified based on the genotypes; Hb

CS with α-$^{3.7}$, Hb CS with α-$^{4.2}$, Hb CS with heterozygous Hb E, Hb CS/α-$^{3.7}$ with heterozygous Hb E and Hb CS/α-$^{4.2}$ with heterozygous Hb E.

The value of Hb CS varies depending on whether it was compounded with beta-thalassemia or deletion α-thalassemia. This study showed that the Hb CS value in CE is lower if compounded with beta variant (Hb E) than in compounded with deletion α-thalassemia. The results were similar to a study by Sanchaisuriya *et al*. that detected low level of Hb CS (0.2 ± 0.1) for compound heterozygous Hb CS with heterozygous Hb E group ($\alpha^{CS}\alpha/\alpha\alpha$; β/β^{E}) than the group of compound heterozygous Hb CS with heterozygous α^{+} thalassaemia [($\alpha^{CS}\alpha/-\alpha^{3.7}$; β/β^{E}) and ($\alpha^{CS}\alpha/-\alpha^{4.2}$; β/β^{E})] that showed level of 0.8% and 0.7% respectively.[16]

Interactions between the different determinants of thalassemia and Hb CS can produce a wide spectrum of clinical and hematological phenotypes, ranging from normal to intermediate conditions of thalassemia.[17] Association of compound heterozygous such as Hb CS with α^{0} thalassemia can lead to severe Hb H disease commonly encountered in China and Southeast Asia.[14] In a 1997 study by Styles *et al.* that compared the deletion forms of Hb H disease (--/-α) with HbH/HbCS (--/αCSα), the investigators reported that patients with the latter genotype were more likely to have splenomegaly or have undergone appendectomy, and to have received transfusions.[2] It appears that interactions of the non-deletion forms of α-thalassemia are associated with a more severe phenotype overall. This laboratory diagnosis is necessary for genetic counseling in regions with a high prevalence of Hb CS and alpha thalassemia as couples with homozygote of Hb CS and alpha thalassemia trait have a higher risk of conceiving foetuses with Hb H-CS disease than those with heterozygote of Hb CS.

In a 1977 study by Ganesan *et al.* on the interaction of Hemoglobin E (HbE) with α thalassemia and Hb CS in a Malay family. It was reported that Hb E, α^{0} thalassemia and Hb CS occur at significant frequencies in Malaysia, and is thus not surprising to find HbE, α^{0} thalassemia and Hb CS in combination.[18] It is important to be aware that this combination of disorders can cause moderately severe hemolytic anemia and to ensure correct diagnosis when such patients are encountered.

Among 344 samples confirmed as heterozygous Hb CS, 290 (84.3%) samples were able to be identified as Hb CS by HPLC. For compound heterozygous Hb CS, 28 (90.3%) out of 31 samples were able to be picked up by using HPLC. In this study, only 1 sample turned out to be homozygous Hb CS and it was able to be detected by both methods of haemoglobin analysis.

One study was conducted by Wisedpanichkij *et al*. in Thailand involving pregnant ladies attending antenatal clinic to evaluate the efficiency of HPLC in detecting Hb CS in peripheral blood sample. A small bump was present in the chromatogram at the retention time of 4-5 minutes, but it was unable to distinguish between three groups of Hb CS. All 7 (100%) samples of homozygous Hb CS were able to be detected by HPLC. However, only 59 (93.2%) samples of heterozygous group and 17 (94.1%) samples of compound heterozygous were able to be detected in chromatogram.[19] Their findings were similar to our study in which the presence of Hb CS in HPLC were either qualitatively identified and few were quantitatively measured. In general, HPLC may be able to detect homozygous Hb CS, but it may miss heterozygous Hb CS.[20] Thus, HPLC was not proposed as the main screening tools in thalassemia programme as it has low sensitivity in detecting the carrier of Hb CS.

Even though the HPLC cannot distinguish the genotypes of Hb CS between heterozygous Hb CS, homozygous Hb CS and compound heterozygous α-thal-2 with Hb CS, this method is still useful for the screening of Hb CS before DNA analysis. It is preferred as screening tools as it is cheaper and more suitable to be used in centres with limited budget.

In this study, also showed good to moderate correlation between HPLC and CE findings for Hb CS detection evidenced by good linear correlation using Pearson correlation coefficient test. We observed that the CE patterns were easier to read than HPLC patterns of Hb CS as there were peak with a value in Zone 2 of CE compared to small bump at C window in HPLC. Furthermore, most of the samples that were detected in HPLC were without the quantitative measurement. Another interesting finding showed that the value detected in CE is much higher than in HPLC.[13]

There were several limitations when conducting this study. These include (1) not all 835 samples that had peak value in Zone 2 CE were proceeded DNA molecular study so, this had significantly reduced the total number of samples studied, (2) larger sample size is needed especially for compound heterozygous and homozygous Hb CS for better evaluation of the difference in peak value on Zone 2 of CE.

Conclusion

In conclusion, automated HPLC can be used as a standard method for Hb CS identification. However, it may lead to some misdiagnosis of Hb CS. CE had a high efficacy for detecting and quantifying Hb CS, and it was superior to HPLC for detecting the heterozygous and compound heterozygous Hb CS. Integrating the results of both CE and HPLC, the diagnosis of Hb CS would likely be noticed. Hence, in a setting where DNA molecular analysis could not be carried out, the diagnosis of Hb CS can still be considered.

Conflict of interests

No potential conflict of interests to declare.

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