Original Article

Evaluation of haemoglobin constant spring phenotypes and their haematological characteristics among high school students in Terengganu, Malaysia

A single - centred study

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ABSTRACT

الأهداف: تقييم انتشار HbCS في سكان ترينجانو، وتقييم المعايير الدموية للأفراد المصابين بـ HbCS المتغاير الزيجوت، و HbCS المتماثل الزيجوت، و HbCS المتغاير الزيجوت المركب، ومقارنة فعالية الكروماتوغرافيا السائلة عالية الأداء (HPLC) والتفريغ الكهربائي الشعري (CE) في الكشف عن HbCS.

المنهجية: أجريت دراسة مقطعية في مستشفى السلطانة نور زاهيرة (HSNZ) شملت طلاب المدارس الثانوية (الصف الرابع) من ترينجانو الذين شاركوا في برنامج فحص الثلاسيميا الذي أجرته وزارة الصحة من يناير 2019 إلى ديسمبر 2022. تم إجراء تحليل الهيموغلوبين باستخدام طريقتين مختلفتين: نظام Capillarys2 Flex-Piercing (صربيا) وكروماتوغرافيا السائل عالية الأداء (HPLC) باستخدام Biorad Variant II. تم إجراء دراسات جزيئية باستخدام تقنية PCR متعددة والمضاعفة باستخدام النظام المقاوم للطفرات (ARMS) للكشف عن الألفا-ثلاسيميا الحذفية وغير الحذفية.

النتائج: أظهر انتشار HbCS أن 92.2% من الحالات كانت متغايرة الزيجوت، 7.2% كانت متغايرة الزيجوت المركب، و 0.5% كانت متماثلة الزيجوت، مع قيم قمة المنطقة الثانية في CE بمعدل 0.7% و 1.2% و 4.5% على التوالي.

الخلاصة: تبرز الدراسة انتشارًا كبيرًا لـ HbCS بين سكان ترينجانو، مع كون الحالات المتغايرة الزيجوت هي الأكثر شيوعًا. كانت قيم القمة في المنطقة 2 من CE تختلف بشكل كبير بين الحالات المتغايرة الزيجوت، والمتغايرة الزيجوت المركب، والمتماثلة الزيجوت لـ HbCS، مما يشير إلى إمكانية استخدام هذه القياسات في تمييز الأنماط السريرية المختلفة.

Objectives: To assess the prevalence of hemoglobin constant spring (Hb CS) in the Terengganu population, to evaluate the haematological parameters of individuals with heterozygous Hb CS, homozygous Hb CS, and compound heterozygous Hb CS, and to compare the effectiveness of high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) in detecting Hb CS.

Methods: This study employed a cross-sectional design involving Form 4 secondary school students from Terengganu. Hemoglobin variants were analyzed using CE (CAPILLARYS2 Flex-Piercing System) and HPLC (VARIANT II). Molecular testing, including multiplex polymerase chain reaction (PCR) and amplification refractory mutation system-PCR techniques, was carried out to detect alpha thalassemia mutations.

Results: The prevalence of Hb CS revealed 92.2% heterozygous (mean zone 2 CE peak value of 0.7%), 7.2% compound heterozygous (mean zone 2 CE peak value of 1.2%), and 0.5% homozygous cases (mean zone 2 CE peak value of 4.5%).

Conclusion: The study highlights a significant prevalence of Hb CS among the Terengganu population, with heterozygous cases being the most common. The peak values in zone 2 CE varied significantly among the heterozygous, compound heterozygous, and homozygous HbCS cases, indicating the potential utility of these measurements in distinguishing between different clinical phenotypes.

Keywords: alpha thalassemia, haemoglobin constant spring, high performance liquid chromatography, capillary electrophoresis

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Thalassemia refers to a group of inherited blood disorders marked by the decreased or absent production of one or more globin chains, which are essential components of haemoglobin (Hb). This condition leads to anaemia, which can range from mild to severe.[1,2] Thalassemia is primarily classified into alpha (α) thalassemia and beta (β) thalassemia, depending on which globin chain is affected.[3] Alpha thalassemia is caused by either deletional or non-deletional mutations in the α-globin genes on chromosome 16. The severity of the disease is influenced by the number of affected α-globin genes and the type of mutation.[4,5]

Haemoglobin constant spring (Hb CS) is a non-deletional α-thalassemia variant, characterised by elongation of the α-globin chain, resulting in an unstable Hb molecule. Haemoglobin constant spring is most commonly found in populations in Southeast Asia, including Thailand, Malaysia, and parts of China. Studies have reported varying frequencies of the Hb CS allele in these regions. For instance, in Malaysia, the prevalence of Hb CS carriers can be as high as 3-4%.[6] Haemoglobin constant spring has significant clinical implications, especially when co-inherited with other α-thalassemia mutations.[7]

The clinical manifestations of Hb CS are significantly influenced by its zygosity and the presence if co-inherited α-thamassemia mutations.[8] In the heterozygous Hb CS (αα/αCSα), individuals are typically asymptomatic or exhibit mild microcytic hypochromic anaemia. Routine hematological parameters may demonstrate a slightly reduction in mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH). In contrast, homozygous Hb CS (αCSα/αCSα), which is uncommon, is generally associated with a more severe clinical phenotype characterized by moderate to severe anaemia.[8] Compound heterozygosity such as the HbCS/α-thalassemia-1 coexistence, may result in haemoglobin H disease, which is associated with moderate to severe hemolytic anaemia, splenomegaly, and other related complications necessitating ongoing medical management.[8,9]

The unstable messenger ribonucleic acid of Hb CS reduces α-globin chain production, making it often undetectable in routine laboratory tests, particularly in heterozygous individuals.[9,10] Diagnosis of Hb CS involves a combination of haematological tests and molecular techniques.[11,12] A complete blood count (CBC) often shows microcytic hypochromic anaemia. High-performance liquid chromatography (HPLC) can sometimes identify Hb CS, although its sensitivity varies.[13] Capillary electrophoresis (CE) is the method with the highest reported sensitivity for detecting Hb CS. However, the definitive approach to identifying the Hb CS mutation is molecular diagnosis through DNA analysis using methods such as polymerase chain reaction (PCR) and sequencing. This technique is crucial for prenatal diagnosis and genetic counselling. This study aimed to evaluate the prevalence of Hb CS in the Terengganu population and to compare the haematological parameters and CE findings across 3 distinct categories of Hb CS: heterozygous, homozygous, and compound heterozygous.

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Methods. This cross-sectional study was carried out among 16-year-old form 4 secondary school students in Terengganu, who participated in the national thalassemia screening programme from January 2019 to December 2022. Inclusion criteria mandated the presence of a distinct hemoglobin peak in zone 2 on CE and a small peak in the C window on HPLC. Participants were excluded if they had undergone blood transfusion within the preceding 3 months.

Peripheral blood samples were collected into ethylenediaminetetraacetic acid containers and subsequently subjected to hemoglobin analysis at Sultanah Nur Zahirah Hospital, Terengganu. Samples exhibiting a zone 2 peak on CE were further selected for advanced molecular diagnostics, culminating the analysis of approximately 389 samples. The study protocol received ethical approval by the national medical research registry (NMRR ID-23-00137-ACP) and Sultan Zainal Abidin University's human research ethics committee (UHREC, UniSZA/UHREC/2023/569). All procedures were carried out in accordance with the principles of the Declaration of Helsinki.

All samples underwent a CBC using the automated 6-part haematology analyser, Sysmex XN-3000 (Sysmex Corporation, Kobe, Japan). Haemoglobin analysis was carried out using CE and HPLC methods. Samples were analysed within 24 hours, first with a CE system (CAPILLARYS2 Flex-Piercing System, Sebia, Lisses, France) and then with HPLC (VARIANT II, Bio-Rad Laboratories, Hercules, CA, USA), following the manufacturers' instructions. Capillary electrophoresis separates molecules based on their charge-to-mass

ratio under the influence of an electric field within a capillary tube filled with an electrolyte. Different types of Hb, because of their different charges, migrate at varying speeds and can be separated and identified by measuring absorbance at 415 nm at the cathodic end. In this study, the primary focus was the detection of Hb CS, which is found in zone 2. If a peak in zone 2 was observed on the CE electropherogram, HPLC was used as a supplementary method to examine the peak in the C window. High-performance liquid chromatography separates the components of a mixture based on their interactions with column and solvent, using their retention times. These retention times are then compared to those of known types of Hb, such as HbA, HbF and HbA2, as well as other variants. Quantification is carried out by integrating the area under each peak.

Samples showing a zone 2 peak on CE were referred for DNA analysis at a reference laboratory to confirm the presence of Hb CS. Non-deletional α-thalassemia mutations were detected using the multiplex amplification refractory mutation system-PCR.

Statistical analysis. Statistical analyses were carried out using the Statistical Package for the Social Sciences for Windows, version 18.0 (SPSS Inc., Chicago, Ill., USA). Descriptive statistics were employed for demographic data, with numerical data expressed as mean ± standard deviation (SD) and categorical data presented as frequency and percentage. Peak values in zone 2 were analysed using one-way ANOVA, while haematological parameters among the Hb CS groups were examined using an independent t-test. A *p*-value of <0.05 was considered significant.

Results. A total of 10,630 samples were analysed during this study period, and 584 samples exhibited a peak in zone 2 CE. However, due to budget constraints, only 389 of the 584 samples presumed to have Hb CS were randomly selected for molecular studies. Of these 389 samples, 140 (36.0%) were male, and 249 (64.0%) were female students. The study predominantly included Malays due to Terengganu's demographics, with 388 (99.7%) participants being Malay and one (0.3%) participant being Chinese. The prevalence of Hb CS among the 10,630 samples analyzed was 3.6%, based on molecular confirmation of 389 cases. Among these, 359 (92.2%) samples were heterozygous Hb CS, 28 (7.2%) samples were compound heterozygous Hb CS and 2 (0.5%) samples were homozygous Hb CS (Table 1). The compound heterozygous Hb CS cases were further analysed and categorised based on their genotypes and co-inheritance as either α- or β-thalassemia. Among these cases, the most common co-inheritance with α-thalassemia was Hb CS with -α3.7 (4.3%), followed by Hb CS with -α4.2 (0.7%), and Hb CS with Hb Adana (0.5%). Additionally, there was one case each of Hb CS co-inherited with β-thalassemia, including Hb Malay, Hb D Punjab, and β-thalassemia (Table 1).

Table 1 - Genotypes of hemoglobin constant spring (N=389).

Types and coinheritance	n (%)
Heterozygous Hb CS	359 (92.2)
Homozygous Hb CS	2 (0.5)
Compound heterozygous Hb CS	
Heterozygous alpha 3.7	17 (4.3)
Heterozygous alpha 4.2	3 (0.7)
Haemoglobin adana	2 (0.5)
Heterozygous IVS-1	1 (0.3)
Heterozygous alpha SEA	1 (0.3)
Haemoglobin Quang Sze	1 (0.3)
Haemoglobin Malay	1 (0.3)
Beta thalassemia trait	1 (0.3)
Haemoglobin D Punjab	1 (0.3)

Values are presented as numbers and percentages (%).
Hb CS: hemoglobin constant spring, IVS-1: intervening sequence-1, SEA: Southeast Asian

Using the CE method and confirmed by molecular studies, 359 samples showed Hb CS with a mean peak value of 0.7% in zone 2, indicating a diagnosis of heterozygous Hb CS. Two samples with a mean peak of 4.5% in zone 2 were confirmed to be homozygous Hb CS. Additionally, approximately 28 samples with a mean peak value of 1.2% in zone 2 were identified as compound heterozygous Hb CS (Table 2).

The CBC parameters for Hb CS, including Hb, MCV, and MCH levels, differed significantly between the 3 groups ($p<0.001$). However, no significant differences were observed between these 3 groups in terms of red blood cell count. The haematological parameters for the 28 samples with compound heterozygous Hb CS were further described based on their co-inheritance patterns, as summarised in Table 3.

Out of 359 heterozygous Hb CS cases identified by CE, only 173 cases were detected by HPLC. For compound heterozygous Hb CS, CE identified all 28 cases, while HPLC detected 25 cases. Both homozygous cases were detected by both CE and HPLC (Table 4).

Discussion. Alpha-thalassemia is highly prevalent in Malaysia and neighboring Southeast Asian countries. The prevalence in Malaysia stands at 4.1%, compared to 16% in Southern Thailand, 5% in the

Table 2 - The mean peak values in Zone 2 capillary electrophoresis findings and hematological parameters for heterozygous, homozygous, and compound heterozygous hemoglobin constant spring (N=389).

Haematological parameters	Types of Hb CS			*P*-values
	Heterozygous Hb CS (n=359)	Homozygous Hb CS (n=2)	Compound heterozygous Hb CS (n=28)	
Zone 2 peak on CE	0.7±0.4	4.5±1.1	1.2±0.8	<0.001
Hb (g/dL)	12.8±20.1	10.9±29.0	11.1±26.8	<0.001
RBC (10^{12}/L)	5.2±0.6	4.7±2.2	4.9±1.2	0.2
MCV (fl)	77.2±3.9	81.5±16.8	73.2±8.2	<0.001
MCH (pg)	25.0±1.5	24.4±5.3	22.7±2.0	<0.001

Values are presented as means ± standard abbreviations (SDs). All pairs of mean scores are significantly different by post hoc test (Tukey test; $p<0.001$).
Hb CS: hemoglobin constant spring, CE: capillary electrophoresis, Hb: hemoglobin, RBC: red blood cell, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin

Table 3 - Haematological parameters of compound heterozygous hemoglobin constant spring (n=28).

Coinheritance	n (%)	Haematological parameters				
		Hb (g/dL)	RBC (10^{12}/L)	MCV (fl)	MCH (pg)	Hb CS level (%)
Het alpha 3.7	17 (60.7)	11.9±18.2	5.2±0.9	72.8±3.6	22.9±1.4	1.1 (0.2)
Het alpha 4.2	3 (10.6)	11.4±19.4	5.4±0.7	67.3±3.1	21.1±0.9	0.9 (0.2)
Hb Adana	2 (7.1)	4.1±25.5	1.8±1.3	89.2±28.4	24.1±3.7	3.2 (2.3)
Beta thal trait*	1 (3.6)	-	-	-	-	-
Hb Quang Sze*	1 (3.6)	-	-	-	-	-
Het IVS-1*	1 (3.6)	-	-	-	-	-
Hb Malay*	1 (3.6)	-	-	-	-	-
Het alpha SEA*	1 (3.6)	-	-	-	-	-
Hb D punjab*	1 (3.6)	-	-	-	-	-

Values are presented as numbers and percentages (%) and means ± standard deviations (SDs). *No mean and SD value due to only one sample involved. Hb: hemoglobin, RBC: red blood cell, MCV: mean corpuscular volume,
MCH: mean corpuscular hemoglobin, Hb CS: hemoglobin constant spring, Het: heterozygous, IVS-1: Intervening Sequence-1, SEA: Southeast Asian

Table 4 - Detection of hemoglobin constant spring by capillary electrophoresis and high-performance liquid chromatography samples (N=389).

Genotypes of Hb CS	CE	HPLC	
	Zone 2	No peak	Small peak
Heterozygous Hb CS	359 (92.3)	186 (51.8)	173 (48.2)
Homozygous Hb CS	2 (0.5)	0 (0.0)	2 (100)
Compound heterozygous Hb CS	28 (7.2)	3 (10.7)	25 (89.3)

Values are presented as numbers and percentages (%). Hb CS: hemoglobin constant spring,
CE: capillary electrophoresis, HPLC: high-performance liquid chromatography

Philippines, and approximately 4.3% in Brunei.[6,14] Regional variations in the frequencies and the types of α-thalassemia mutatuins have been well-documented.[15] Hemoglobin constant spring, a common non-deletional α-thalassemia variant, is frequently encountered in the Southeast Asian population and often coexists with other thalassemia types. Screening for this abnormal Hb in Malaysian is imperative, as prior literatures have indentified Hb CS as the most prevalent non-deletional α-thalassemia in Southeast Asia.

In this study, the occurrence of Hb CS was determined to be 3.6%, based on the analysis of 10,630 samples. The prevalence observed in this study was marginally higher than the 3.2% reported by Nahanthiran et al[6] among individuals with α-thalassemia. Ethnicity-specific findings revealed the majority of Malays (99%), with only 1% Chinese, and no cases were found among Indians or other ethnic groups. These findings align with data from the Institute for Medical Research, which reported a prevalence of 4.3% among Malays,

0.7% among Chinese, and no cases identified among Indians.[6]

Of the Hb CS cases that were identified through molecular studies, 92.2% were heterozygous, 7.2% were compound heterozygous, and 0.5% were homozygous for Hb CS. The majority of compound heterozygous Hb CS cases had co-inheritance with the -α3.7 deletion. This finding is consistent with the results reported by Ramli et al,[8] who also identified co-inheritance with the -α3.7 deletion as the most common thalassemia in Malaysia. Among Malaysians, the most common α-thalassemia detected is the -α3.7 deletion, contributing to the higher incidence of co-inheritance with Hb CS.

In the current study, significant variations in hematological parameters, including zone 2 peak values on CE, Hb, MCV, and MCH, were observed across homozygous, heterozygous and compound heterozygous Hb CS cases. The mean Hb CS peak values in zone 2, as measured by CE, showed that homozygous Hb CS cases exhibited the highest mean peaks at 4.5%, followed by compound heterozygous Hb CS at 1.2%, and heterozygous Hb CS at 0.7%. Comparable findings were reported in a similar study by Waneesom et al,[16] which analyzed 102 Vietnamese individuals. Their results indicated that heterozygous Hb CS had peak values ranging from 0.1-0.8%, while homozygous Hb CS exhibited higher values, from 0.2-4.6%, and in compound heterozygous Hb CS, the zone 2 peak values ranged from 2.0-5.0%. These findings align with other studies, which consistently report significantly higher zone 2 peak values in homozygous cases compared to heterozygous cases.[8,16] Additionally, Liao et al[17] demonstrated that CE is capable of detecting Hb CS levels in heterozygotes as low as 0.1%. In contrast, the current study identified a minimum detectable Hb CS level of 0.2%, underscoring the sensitivity of CE technique in quantifying Hb CS.

In α-thalassemia, Hb levels typically correlate with the number of dysfunctional α-globin genes. This study found that students with homozygous Hb CS exhibited a low mean Hb level of 10.9 g/dL, indicative of mild anemia, while those with heterozygous Hb CS had Hb levels within the normal value. These results are consistent with those of previous researches, such as a study carried out in Vietnam, which reported that heterozygous Hb CS individuals demonstrated normal Hb levels of 12.0±1.3 g/dL, whereas homozygous Hb CS individuals presented with mild anaemia, with Hb levels averaging 9.9±0.8 g/dL.[18] In this study, Hb levels in individuals with compound heterozygous Hb CS ranged from 4.1-11.9 g/dL. Notably, 2 cases involving co-inheritance of Hb CS and Hb Adana demonstrated significantly reduced Hb levels, averaging 4.1 g/dL. Both Hb CS and Hb Adana are highly unstable Hb variants, and their co-inheritance often results in a more severe clinical phenotype or thalassemia intermedia. This condition is frequently associated with extramedullary hematopoiesis and the need for regular transfusions. A case series by Alauddin et al[19] similarly reported that co-inheritance of Hb Adana and Hb CS leads to markedly reduced Hb levels and more severe clinical manifestations compared to individuals with heterozygous Hb Adana alone. This highlights the compounded clinical impact of these 2 unstable Hb variants.

In addition to Hb levels, MCH and MCV can provide insights into the subtype of α-thalassemia. Typically, individuals with only one functional α-globin gene have lower MCV and MCH values compared to those with 2 functioning α-globin genes. In the current study, the mean MCV for individuals with compound heterozygous Hb CS and Hb Adana was normal at 89.20 fL, whereas those with compound heterozygous Hb CS of -α3.7 had lower MCV value of 72.8 fL and those of -α4.2 had lower MCV value of 62.2 fL. This suggests that the Hb CS variant with Hb Adana maintains an almost normal MCV, which aligns with findings from a previous study.[19] In a review article by Chui et al,[20] it was observed that the MCV in non-deletional α-thalassemia was higher compared to deletional α-thalassemia. However, it was noted that anemia was more severe in non-deletional α-thalassemia, leading to increased reticulocyte count, which might have contributed to the higher MCV values. Additionally, the mean MCH was significantly different across all compounds heterozygous Hb CS with α-thalassemia types, consistent with earlier research.[8] This indicates that co-inheritance of α-thalassemia can lead to reduced MCH levels.[21]

The current study compared the effectiveness of the CE and HPLC methods in detecting and quantifying Hb CS. According to the HPLC analysis, the Hb CS chromatogram displayed at least a minor peak in the C window, with a retention time between 4.90-5.30 minutes. This peak was observed in 2 individuals with homozygous Hb CS, 25 (89.3%) individuals with heterozygous Hb CS, and 173 (48.2%) individuals with compound heterozygous Hb CS. When analysed by CE, 359 (92.2%) out of 389 samples showed a peak in zone 2 and were identified as heterozygous Hb CS. Of the remaining samples,

2 were identified as homozygous Hb CS, and 28 (0.7%) were identified as compound heterozygous Hb CS. In this study, CE accurately identified all Hb CS cases, highlighting its superiority over HPLC in detecting the Hb CS variant, which is consistent with findings from other studies.[22,23] Capillary electrophoresis offers results that are comparable to those obtained with established HPLC and electrophoretic methods.[17,24]

Study limitations. Firstly, due to budget constraints, only 389 of the 584 samples with presumed Hb CS identified through CE were subjected to molecular studies. This may have introduced a selection bias and limited the generalizability of the prevalence data to the entire population. Secondly, the study was carried out within a single center and focused exclusively on high school students in Terengganu, which may not fully represent the broader demographic or ethnic diversity of Malaysia. Additionally, while molecular studies were used to confirm the presence of Hb CS, the analysis was limited to the most common α-thalassemia mutations and did not include a broader spectrum of rare genetic variants. Finally, the study relied on cross-sectional data, which provides a snapshot of the prevalence and haematological characteristics but does not capture longitudinal trends or potential clinical outcomes over time. Future studies should aim to address these limitations by expanding the sample size, including multiple centers, and incorporating a wider range of molecular analyses.

In conclusion, this study provides valuable insights into the prevalence and the haematological characteristics of Hb CS in the Malaysian population. By analyzing haematological parameters and employing electrophoresis techniques, including CE and HPLC, we demonstrated that a diagnosis of Hb CS can be reliably carried out without waiting for DNA molecular analysis. Our findings indicate that Hb CS exhibits distinct electrophoresis peaks and haematological profiles that correlate with its clinical phenotypes. These results underscore the importance of integrating multiple diagnostic approaches to accurately identify Hb CS and highlight the need for continued awareness and monitoring in clinical settings. This approach enhances diagnostic accuracy and contributes to better patient management and understanding of Hb CS in the Malaysian context.

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