THE SIGNIFICANCE OF CAPILLARY ELECTROPHORESIS IN THE DETECTION OF HAEMOGLOBIN CONSTANT SPRING AND ITS RELATIONSHIP WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

By

DR NABILAH BINTI RAMELI

Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Pathology (Haematology)

SCHOOL OF MEDICAL SCIENCES
UNIVERSITI SAINS MALAYSIA

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LIST OF ABBREVIATIONS

ARMS	Multiplex Amplification Refractory Mutation System
CE	Capillary electrophoresis
CS	Constant Spring
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
et al	et alia (and others)
FBC	Full blood count
FBP	Full blood picture
fl	Femtolitre
FSC	Forward scattered light
Hb	Haemoglobin
Hct	Haematocrit
HPLC	High performance liquid chromatography
HRPZ II	Hospital Raja Perempuan Zainab II
HUSM	Hospital Universiti Sains Malaysia
i.e.	id est (that is)
IMR	Institute of medical Research
KKM	Kementerian Kesihatan Malaysia
MCH	Mean cell haemoglobin
MCHC	Mean cell haemoglobin concentration
MCV	Mean cell volume
ml	Millilitre
MPLA	Multiplex ligation-dependent probe amplification
mRNA	Messenger ribonucleic acid

OFT	Osmotic fragility test
PCR	Polymerase chain reaction
pg	Picogram
RBC	Red blood cell count
RDW	Red cell distribution width
RNA	Ribonucleic acid
SD	Standard deviation
SEA	South-East Asia
SFL	Side fluorescence light
SLS	Sodium lauryl sulphate
SPSS	Statistical Package for the Social Science
WHO	World Health Organization
α	Alpha
α^{+}	Alpha plus (reduced production of alpha chain from the affected gene)
α^{o}	Alpha zero (no alpha chains produced)
$\alpha\text{-}^{3.7}$	Alpha plus thalassaemia 3.7 deletion
$\alpha\text{-}^{4.2}$	Alpha plus thalassaemia 4.2 deletion
α^{CS}	Alpha Constant Spring
$\alpha^{T}\alpha$	α2 gene mutation
$\alpha\alpha^{T}$	α2 gene mutation
γ	Gamma
δ	Delta

KEPENTINGAN KAPILARI ELEKTROFORESIS (CE) DALAM MENGESAN KEWUJUDAN HAEMOGLOBIN CONSTANT SPRING DAN HUBUNGANNYA DENGAN KROMATOGRAFI CECAIR PRESTASI TINGGI (HPLC)

ABSTRAK

Pengenalan: Hemoglobin Constant Spring (Hb CS) ialah salah satu alpha (α) talasemia jenis bukan pemadaman yang yang biasa dijumpai di rantau Asia Tenggara. Sifat gen globin yang tidak normal ini adalah tidak stabil, labil dan hadir dalam kuantiti yang sangat sedikit di dalam darah. Oleh itu, ini boleh menyebabkan kesilapan dalam diagnosis penyakit. Kajian ini dijalankan untuk menentukan kekerapan Hb CS dalam kalangan rakyat negeri Kelantan dan membandingkan nilai puncak pada zon 2 kapilari elektroforesis bagi 3 kumpulan berbeza Hb CS (heterozigot Hb CS, homozigot Hb CS dan kompoun heterozigot Hb CS) dan untuk membandingkan parameter hematologi antara kumpulan ini. Penyelidikan juga bertujuan untuk mengkaji kaitan antara penemuan HPLC dan CE dalam mengesan Hb CS.

Metodologi: Kajian keratan rentas ini melibatkan pengumpulan data sekunder sebanyak 378 sampel yang menunjukkan kehadiran puncak pada zon 2 kapilari elektroforesis. Semua sampel diambil daripada ujian saringan Nasional Talasemia untuk pelajar Tingkatan 4 dari semua daerah di Kelantan. Indesis sel darah merah dianalisis menggunakan automasi Sysmex XN 3000. Analisis Hb dilakukan dengan menggunakan sistem automatik CE (CAPILLARYS2 Flex-Piercing System Sebia), HPLC Biorad Variant II dan analisis DNA dilakukan dengan menggunakan multiplex polymerase chain reaction (PCR) dan multiplex Amplification Refractory Mutation System (ARMS) untuk mengesan alfa-talasemia jenis delesi dan alfa-talasemia jenis bukan-delesi.

Keputusan: 376 sampel (99.5%) menunjukkan kehadiran puncak pada zon 2 kapilari elektroforesis disahkan mempunyai mutasi pada kodon penamatan menggunakan kajian molekul. Majoriti sampel yang diuji ialah jenis heterozigot Hb CS iaitu 344 sampel (91.5%), diikuti dengan kompaun heterozigot Hb CS iaitu 31 sampel (8.2%) dan hanya satu sampel (0.3%) dalam kumpulan homozigot Hb CS. Nilai min ± sisihan piawai bagi nilai puncak pada Zon 2 kapilari elektroforesis untuk heterozigot Hb CS ialah 0.61 ± 0.13 dan bagi kompaun heterozigot Hb CS ialah 0.77 ± 0.34. Satu sampel dalam kategori homozigot Hb CS menunjukkan nilai puncak pada Zon 2 kapilari elektroforesis iaitu 4.9%. Terdapat perbezaan parameter hematologi yang ketara di antara heterozigot Hb CS dan kompaun heterozigot Hb CS iaitu bagi paras hemoglobin, MCV, MCH dan MCHC. Kajian ini menunjukkan bahawa terdapat kolerasi linear yang baik antara penemuan puncak dalam kawasan C pada HPLC dan nilai puncak pada Zon 2 kapilari elektroforesis dalam mengesan Hb CS, r=0.73.

Kesimpulan: Oleh itu, dengan menggabungkan parameter hematologi dan keputusan kedua-dua ujian pelengkap CE dan HPLC, diagnosis Hb CS dapat dikesan sebelum ujian pengesahan oleh kajian DNA molekul yang jauh lebih mahal.

(393 patah perkataan)

THE SIGNIFICANCE OF CAPILLARY ELECTROPHORESIS IN DETECTION OF HAEMOGLOBIN CONSTANT SPRING AND ITS RELATIONSHIP WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

ABSTRACT

Introduction: Haemoglobin Constant Spring (Hb CS) is one of the most common non-deletional types of alpha (α) thalassaemia in Southeast Asia region. The nature of this abnormal globin gene is that it is unstable, labile and is present in minute amount in the peripheral blood. Thus, this may lead to underdiagnosis of the disease. This study was conducted to determine the proportion of Hb CS among the Kelantan population and to compare range of peak value in Zone 2 CE findings for 3 groups of Hb CS (heterozygous, homozygous, and compound heterozygous) and their haematological parameters. The study aimed to look at the findings of HPLC in relation to CE results in detecting Hb CS.

Study design and methods: This was a cross-sectional study involving secondary data collection from 378 samples which showed peak value on Zone 2 of CE. The samples were taken from the National Thalassemia screening of Form 4 students from all districts in Kelantan. The haematological parameters of red cells were analysed using Sysmex XN 3000 automated blood cell analyser, Hb analysis was performed using automated CE system (CAPILLARYS2 Flex-Piercing System Sebia), HPLC Biorad variant II, DNA analysed using multiplex polymerase chain reaction (PCR) and multiplex Amplification refractory mutation system (ARMS) to detect both deletional and non-deletional α-thalassaemia.

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 of Hb CS 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. The mean ± SD of peak value in Zone 2 of heterozygous Hb CS and compound heterozygous Hb CS were 0.61 ± 0.13 and 0.77 ± 0.34 respectively. The only sample of homozygous Hb CS showed the value of 4.9% of peak value in Zone 2 of CE. The significant differences of haematological parameters between heterozygous and compound heterozygous Hb CS were observed in haemoglobin level, MCV, MCH and MCHC. This study showed there was a good linear correlation between peak in C-window on HPLC and peak value in Zone 2 of CE in detecting Hb CS, r=0.73.

Conclusion: Thus, by combining the haematological parameters and complementary tests of both CE and HPLC, the diagnosis of Hb CS can be detected prior to confirmation by DNA molecular study that is far more expensive.

(399 words)

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CHAPTER 1

GENERAL

INTRODUCTION

CHAPTER 1: GENERAL INTRODUCTION

Alpha (α) thalassemia is one of the most common inherited haemoglobin disorders across the world, characterised by the absence or reduced α-globin gene due to mutation or deletion of α globin gene (Galanello and Cao, 2011). Southeast Asian countries including Malaysia have higher prevalence of α-thalassemia compared to β-thalassemia carrier which is 15.8% and 4.5% respectively. Thus, it is a public health concern in Malaysia (Wee *et al.*, 2005).

Haemoglobin (Hb) Constant Spring is one of the non-deletional α-thalassemia involving the α2 gene as the result of impaired RNA translation due to termination codon point mutation cause the defect (TAA>CAA) and resulted in the elongation of the α chain up to another 31 amino acid residues (Liao *et al.*, 2010).

The natural features of haemoglobin 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. However, the interaction of CS gene with deletional type of alpha-thalassemia is the main cause of non-deletional Hb H (β_4) (--/$\alpha^{cs}\alpha$). This non-deletional α thalassemia is more severe than deletional α-thalassemia, where some patients became transfusion-dependent (Pornprasert *et al.*, 2016) (Liao *et al.*, 2010).

There are several methods of haemoglobin analysis available worldwide. Capillary electrophoresis (CE) and High-Performance Liquid Chromatography (HPLC) are among the widely used method for the screening of abnormal haemoglobin. CE will

give a peak at the Zone 2 for Hb CS. Another common variant that also shares the same peak is, Hb Pakse (Singsanan *et al.*, 2007). In HPLC findings, Hb CS gives a very small peak at the C window with the retention time of 4.90 - 5.30 minute (Greene *et al.*, 2012).

The aim of this study is to determine the significant of Zone 2 peak on CE in diagnosing Hb CS and its relationship with HPLC findings. Hb CS is often goes undiagnosed due to almost normal red cells indices and is present at a low level in peripheral blood. Currently, the gold standard for diagnosis is still based on molecular results which is costly and tedious. In Malaysia, only several centres offer these molecular tests. Most of the health centres in Malaysia are still using HPLC as the screening tool and certain centres started to use CE as the main method in detecting haemoglobinopathies.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2: LITERATURE REVIEW

2.1 Haemoglobin genetics

A normal haemoglobin molecule is made up of haem and globin. Haemoglobin is a tetrameric structure comprised of two pairs of polypeptide chains consisting of one pair of α-globin chains and one pair of β-globin chains. The main role of haem is for oxygen transport. The globin is involved in the protection of haem from oxidation and is also involved in oxygen affinity (Bain, 2020).

The globin chain synthesis involves two phases of 'switch'. The first switch occurred as early as the fifth week of gestation and is completed by week 10 where the embryonic haemoglobin switches to fetal haemoglobin. The β-globin synthesis starts around week eight of gestation. However, the production is still low with a high upregulation just before birth, followed by a decrease in γ-globin expression. This signifies the switches from fetal haemoglobin to adult haemoglobin (Swee and David, 2016).

The main component of haemoglobin in an adult is haemoglobin A (Hb A) constitutes about 96-98% of total haemoglobin content while a lesser fraction is haemoglobin A_2 (Hb A_2). Haemoglobin F (Hb F) is found only in minimal amount (<1%) in adult, however plentiful in the neonatal period; is composed of 2 α-chains and 2-γ chains (Swee and David, 2016).

Globin genes which encode for globin chains are located in two different clusters: one on chromosome 16 and another on chromosome 11. The α-globin cluster is found near the telomere of chromosome 16 and includes a ζ and two α genes. The β cluster gene

is located on chromosome 11 includes ε gene, two γ genes, an δ gene and a β-gene (Bain, 2020).

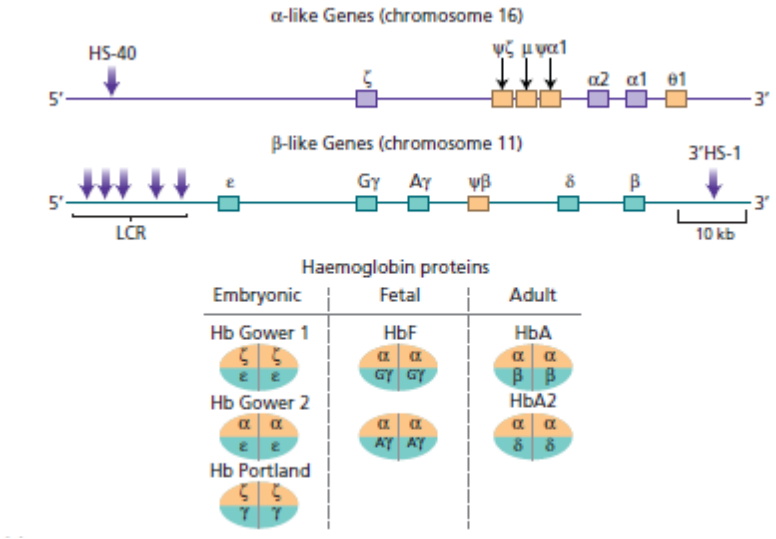


Figure 2.1: The genetic structure of α- and β-globin genes, which are situated on chromosome 16 and 11 respectively. (Adapted from Postgraduate Haematology 7th edition, 2016)

2.2 Introduction of thalassemia

2.2.1 Definition

Thalassemia is a heterogenous group of inherited autosomal recessive disorders of haemoglobin synthesis. It is characterised by quantitative defect of haemoglobin synthesis either due to the absence or reduced production of one or more globin chains of haemoglobin. Two major categories are α- and β-thalassemia and the rare forms include the ϒ, λ and εϒλβ (Bain, 2020).

Alpha (α) thalassemia is a group of disorder resulting from a reduced rate of synthesis of α-globin that consequently resulted in a variable degree of severity of the deficiency (Swee and David, 2016).

2.2.2 Background and incidence of α-thalassaemia

Alpha-thalassaemia is considered as a well-known public health problem in the world. It is a genetic blood disorder resulting from genetic defect either by deletion or mutation of one or more α-globin genes. Consequently, it leads to the absence or reduction in the production of α-globin polypeptides. In Malaysia, the gene frequency of α-thalassaemia was about 4.1%. It was comparable to other countries in Southeast Asia that had high prevalence of alpha-thalassaemia with the prevalence of 16-30% in Thailand, 5% in the Philippines, 2.6-11% in Indonesia, 4.3% in Brunei and 4.1% in Malaysia (Ahmad *et al.*, 2013).

The prevalence of thalassaemia trait in Malaysia is higher for alpha-thalassaemia trait with prevalence of as high as 15.8% compared to 4.5% for beta-thalassaemia trait (Raja Z.A. *et al.*, 2014). Alpha-thalassaemia can be manifested with wide spectrum of clinical phenotypes depending on the types of genetic abnormalities including the deletional or non-deletional type, with the former being more common than the latter (Ahmad *et al.*, 2013).

2.2.3 Genetic basis of α-thalassemia

Generally, α-thalassemia can be broadly divided into deletional and non-deletional thalassemia. Deletional α-thalassemia results in either alpha zero (α^0) or α^+-thalassemia depending on the length and nature of the deletion. Non-deletional α-thalassemia can also arise as a result of the mutation of the α2 gene ($\alpha^T\alpha$ thalassemia) or the α1 gene ($\alpha\alpha^T$ thalassemia) (Bain, 2020).

α-thalassemia is referred to the condition where there are no α-chains being produced from both linked pairs. If there are reduced production of α-chain from the affected chromosome, it is known as α^+. The α-thalassemia is caused by deletion of both α-globin genes. These deletions vary in size and different types of deletions are commonly associated with the geographical area (as shown in Figure 2.2).

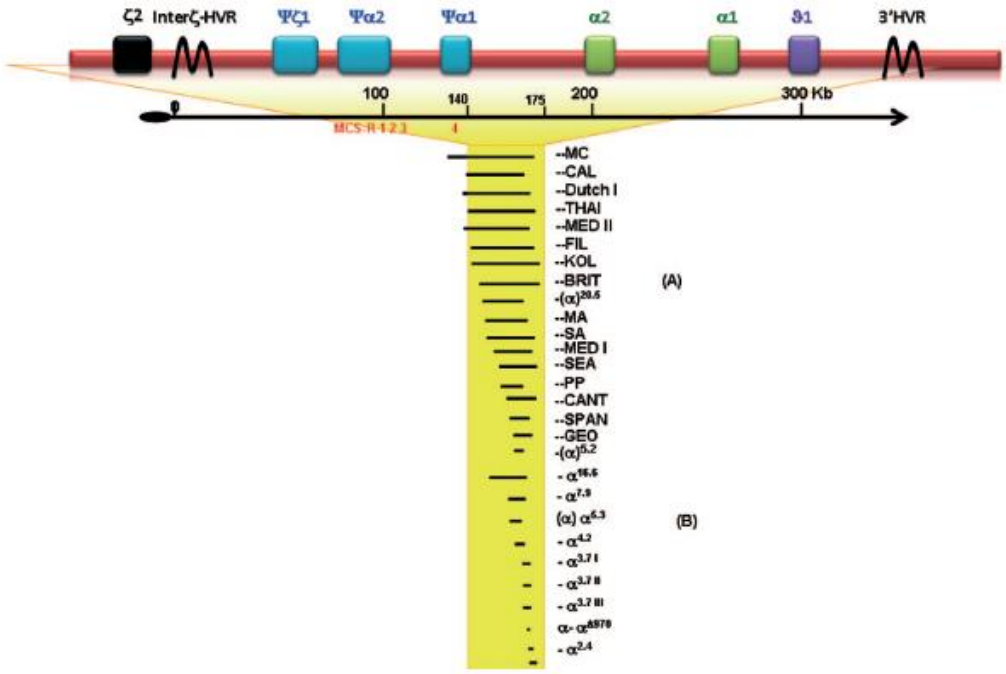


Figure 2.2: The α-globin gene cluster deletions that resulted in (A) α-thalassaemia and (B) α^+-thalassaemia. (Adapted from Alpha Thalassaemia: Genetest Review, 2011) (Galanello and Cao, 2011).

Deletion of one of the normal four α-globin genes will result in α^+-thalassemia (-α/αα). There are three homologous subsegments which are referred to as X, Y and Z (as shown in Figure 2.3). Z homology are the sites where the α1 (HBA1) and α2 (HBA2) genes are located. The most common α^+-thalassemia in Malaysia are α^+ thalassemia ($-\alpha^{3.7}$) and α^+ thalassemia 4.2 ($-\alpha^{4.2}$). $-\alpha^{3.7}$ is the result of rightward deletion in which there are misalignment and recombination between Z boxes that occurred during meiosis. The $-\alpha^{4.2}$ is leftward deletion in which there is crossover between X boxes which are 4.2kb apart (Swee and David, 2016).

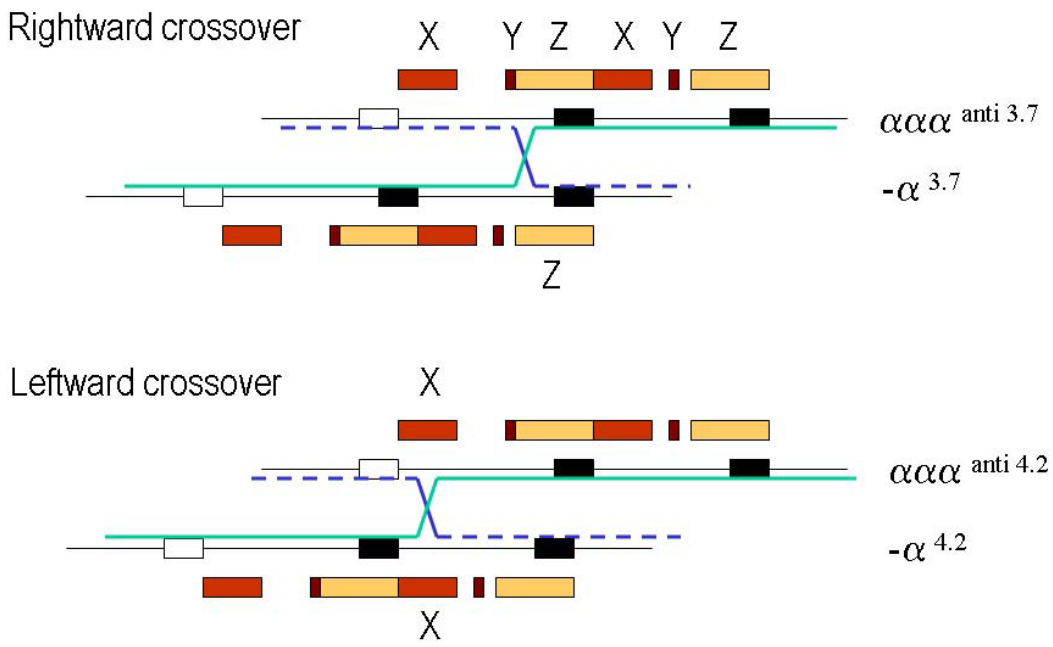


Figure 2.3: The molecular mechanism of deletional alpha-thalassaemia.
(Adapted from Reviewed Article α thalassaemia) (Harteveld and Higgs, 2010)

2.2.4 Pathophysiology of α-thalassemia

Generally, α-thalassemia can be categorised clinically into four syndromes depending on the numbers of functional α-globin gene affected (as shown in figure 2.4).

Hb Barts hydrops fetalis which is incompatible with extrauterine life happens when all four α-globin genes are deleted (--/--). Alpha-thalassaemia intermedia or Hb H disease occurs when only one α-globin gene is left. Patient with this type of thalassemia may experience varying severity associated with chronic haemolytic anaemia and they can be either transfusion-dependent or non-transfusion dependent. However, patient with non-deletional alpha-thalassemia Hb H disease typically demonstrates a much more severe form of clinical presentation as compared to Hb H type of deletional alpha-thalassaemia (Traivaree *et al.*, 2018).

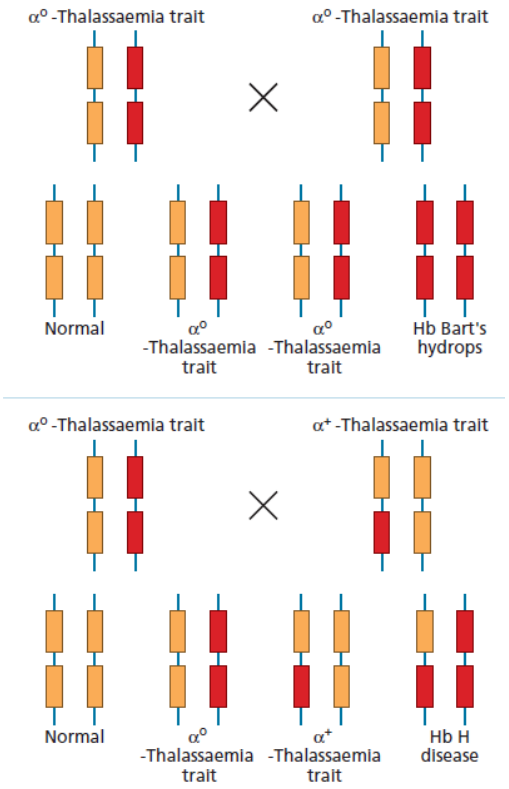


Figure 2.4: α-thalassemia and its genetics
(Adapted from Postgraduate Haematology 7th edition, 2016).

Alpha-thalassaemia trait is characterised by the presence of only 2 functioning α-globin genes. The heterozygous state for α^0-thalassaemia (--/αα) or homozygous state for α^+-thalassaemia (-α/-α) may occur as a result of either *cis*- or *trans*- positional loss of α-globin gene (Ahmad *et al.*, 2013). These types of people with alpha-thalassemia trait usually display mild hypochromic microcytic anaemia. An individual with 3 functioning α-globin genes (-α/αα) or also known as αthalassemia carrier is often clinically asymptomatic with normal to mild decrease in red cell indices (Singer, 2009).

2.3 Haemoglobin Constant Spring

2.3.1 Introduction and background of Haemoglobin Constant Spring

Hb CS is the most prevalent non-deletional alpha thalassemia in South East Asia. Most of non-deletional alpha thalassemia involve α2 gene compared to α1 gene with the ratio of around 3:1. The patient with α2 gene mutation has more severe phenotype compared to those with α2 gene deletion. A mutation at the termination codon at the α2 gene (TAA>CAA) leads to the formation of abnormal haemoglobin known as Hb CS. This mutation leads to the addition of 31 amino acids to the normal α-globin sequence and results in the production of unstable mRNA. The mRNA of this mutant gene is very unstable and produce an α-thalassemia 2-like effect (Fucharoen and Winichagoon, 2011).

A research conducted by Schrier *et al.*, in 1997 found that there was disturbance in volume regulation and cell hydration that occurred early in erythroid precursors and was fully expressed at the reticulocytes stage. The unstable α^{CS}-globin chain precipitated in erythroblasts and led to an increase in membrane rigidity and mechanical stability in RBCs containing Hb CS in comparison to Hb H and α-thalassemia 1 trait erythrocytes. Therefore, α^{cs}-chains may have lethal impacts on the cellular and membrane properties of red blood cells. These changes may lead to an increase in the rate of haemolysis (Schrier *et al.*, 1997).

2.3.2 Prevalence and incidence of Haemoglobin Constant Spring

The prevalence of Hb CS in our neighbouring country, Thailand varied from 1 to 8% (Pornprasert and Punyamung, 2015).

In Malaysia, thalassemia is one of the public health's burdens. The data collected from the Institute of Medical Research (IMR) on the distribution of α-thalassemia gene variants in diverse ethnic populations in Malaysia showed that there are three common non-deletional mutations; Hb Adana, Hb Quang Sze and Hb CS. Hb CS is more common compared to the other two types of mutational α-thalassemia. Among Malays and Sabahans, Hb CS was the third most common thalassemia determinants. Fascinatingly, the highest incidence was recorded among Orang Asli (11.5%) and the mutation was not detected among Malaysian Indians and Sarawakians (Ahmad *et al.*, 2013).

2.3.3 Features of three different types of Hb CS (heterozygous, homozygous, and compound heterozygous)

Hb CS can be categorised into heterozygous, homozygous, or compound with other thalassemia variants. The heterozygotes Hb CS are clinically asymptomatic and haematologically normal. However, the homozygotes can resemble thalassemia intermedia with mild anaemia and hepatosplenomegaly. Co-inheritance of Hb CS with α-thalassemia 1 can lead to Hb H-CS disease (-/$\alpha^{cs}\alpha$). This group of individuals may have phenotypically β-like thalassemia major and can become transfusion-dependant (Pornprasert and Punyamung, 2015).

A study conducted in Thailand by Pornprasert *et al.*, comparing the levels of Hb CS using CE in three different groups of Hb CS involving 40 samples revealed that 45% were heterozygous, 30% were homozygous Hb CS and 25% were compound heterozygous Hb CS. The results from their study showed the mean ± standard deviation (SD) of Hb CS level of the homozygous was significantly higher than that

of the heterozygous (1.9 ± 1.8 vs 0.4 ± 0.2, $p = 0.007$). However, the mean ± standard deviation (SD) of HB CS level between compound heterozygous group with homozygous Hb CS showed no significant difference 1.9 ± 1.8 vs 2.8 ± 1.3, $p = 0.13$) (Pornprasert *et al.*, 2012).

Other parameter that they studied was MCV level between these 3 groups. The MCV between heterozygous and homozygous groups showed no significant difference (76.9 ± 5.1 vs 79.8 ± 4.4, $p = 0.08$). However, there was statistically a significant difference between compound heterozygous and heterozygous (79.8 ± 4.4 vs 66.3 ± 5.7, $P < 0.001$) as well as between compound and homozygous group (76.9 ± 5.1 vs 66.3 ± 5.7, $p < 0.001$) (Pornprasert *et al.*, 2012).

The previous study conducted by Liao *et al*., concluded that CE was the preferable technique to be used as the screening tool for haemoglobin analysis as it detected the level of Hb CS as low as 0.1% in heterozygous Hb CS that ranged from 0.1% to 1.0%, with an average of 0.6 ± 0.1 (Liao *et al.*, 2010).

2.4 Laboratory investigations of haemoglobinopathies and thalassemia

In order to arrive at the right diagnosis of thalassemia and haemoglobinopathies, a combination of laboratory tests with a minimum of two techniques of haemoglobin analysis should be carried out. The request for haemoglobin analysis must be done together with complete identification details including name, age, ethnicity, clinical history and also family history (Bain, 2020).

The complete assessment for the diagnosis of thalassaemia/haemoglobinopathy should be integrated with the history, physical examinations and the suggested laboratory workup. The laboratory investigations include the assessment of haematological parameters comprising of red cell indices (FBC) and morphology (FBP), followed by separation (haemoglobin electrophoresis by acid gel) and quantitation of Hb fraction (CE and HPLC), others complementary tests (ie Hb H inclusion, sickling test) and eventually, a complete diagnosis by molecular analysis (Brancaleoni *et al.*, 2016).

2.4.1 Full blood count (FBC) and peripheral blood picture

Based on the guideline used for thalassemia screening programme in Malaysia by the Ministry of Health Malaysia, FBC is the initial screening tool to identify the carriers of thalassemia or haemoglobinopathies. The individuals with mean cell haemoglobin (MCH) of less than 27 pg will be identified to proceed with further tests such as haemoglobin analysis (Ministry Of Health Malaysia, 2016).

Red cell indices such as MCV, MCH and red cell distribution width (RDW) can help in identifying types of anaemia. Haematological parameters are used in combinations, to give a clue in characterising the features of either thalassemia or iron deficiency anaemia (Bain, 2020).

Thalassemia patients usually have high red blood cell (RBC) count with coexisting microcytosis and hypochromia. In contrast, patients with iron deficiency anaemia or anaemia of chronic disease, the reduction in RBC is seen as proportional to the decline of haemoglobin level. RDW is a measure of the degree of variation in red cell size. In iron deficiency anaemia, there is an increase in RDW, evidenced by

anisopoikilocytosis seen in FBP. In contrast, in thalassemia, it produces a more uniform microcytic red cell. Thus, there is no increase in RDW. These parameters may offer beneficial information to support the diagnosis, but it cannot be used as a single indicator for diagnosis (Clarke, 2000).

MCH is much more reliable than MCV and is preferably used as a cut-off point for thalassemia screening because MCV is more unstable due to the tendency for red cells to increase in size over time due to degradation over time. In other words, MCV is more susceptible to storage changes (Howard *et al.*, 2005).

The evaluation on the diagnosis of thalassemia and haemoglobinopathy may require the examination of a stained peripheral blood film. The most typical findings of peripheral blood smear in thalassemia are microcytosis, hypochromia and anisopoikilocytosis. Other less common findings are basophilic stippling and target cells. Nucleated red blood cells are indicative of bone marrow hyperactivity and mostly seen in thalassemia major ie homozygous β-thalassemia. Meanwhile, heterozygous Hb CS has normal red cells parameters and some with mild hypochromic microcytic anaemia (Bain, 2020).

2.4.2 Haemoglobin H inclusion test

Hb H is an insoluble Hb tetramer comprising of four β-globin chain (β4-tetramers). It is easily precipitated following the disproportion of αβ-globin ratio. These lead to an excess of β-chain. These β4-tetramers are unstable, consequently resulting in red cell membrane damage to induce haemolysis. Hb H inclusion is visualised microscopically following the staining of unfixed cells with New Methylene Blue or Brilliant Cresyl

Blue. Blood smear examination will demonstrate cells with typical ‘golf-ball’ appearance (Hartwell *et al.*, 2005).

2.4.3 Haemoglobin electrophoresis

Haemoglobin electrophoresis is the main laboratory method used for the diagnosis of thalassemia and haemoglobinopathy. Haemoglobin electrophoresis uses cellulose acetate membrane at alkaline pH of 8.4 to 8.6, is regarded as simple, rapid and consistent in detecting clinically significant haemoglobin variant (Wild and Bain, 2017).

The main principle of haemoglobin electrophoresis is the negatively charged haemoglobin that will migrate towards the anode (+) when exposed to charge gradient, then the haemoglobin will be separated from each other and the separation band can be visualized by staining with either haem or protein stain (Bain, 2020).

It should be performed using lysed packed red cells to ensure a consistent amount of haemoglobin used. Lysed packed red cells are preferred over whole blood (WB) because paraproteins or high concentrations of polyclonal immunoglobulin in WB may lead to a prominent band and be interpreted as haemoglobin variant. Alkaline haemoglobin electrophoresis is useful because it can detect haemoglobin A, F, S/G/D/Lepore, C/E/O-Arab H and a number of less common variant haemoglobins (Bain, 2020).

Haemoglobin CS is highly unstable and is present in a very low amount in peripheral blood. Therefore, no specific band can be seen on haemoglobin electrophoresis

because it can be present in any areas. Thus, other technique such as HPLC or CE need to be performed to identify the variant haemoglobin (Clarke, 2000).

2.4.4 High-Performance Liquid Chromatography (HPLC)

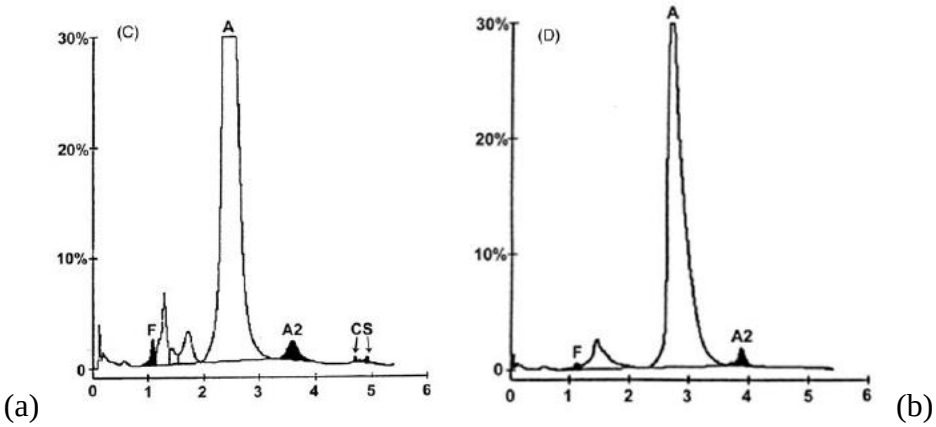
The principle used by HPLC is separation of molecules with net positive charges being separated into different fraction through adsorption onto a negatively charged static phase in a chromatography column. The elution process will follow this in a mobile phase with increasing concentrations of cations flowing in the column. Hence, the adsorbed positively charged haemoglobins are eluted from the column into the liquid phase at a rate signifying their affinity for the stationary phase. The haemoglobin molecules can be identified optically in the eluate, provisionally distinguished by their retention time and measured by area under the peak after being separated (Bain, 2020). Therefore, the normal and variant haemoglobin can be recognized based on the specific period of time or known as retention time (Brants, 2011).

The main advantage of HPLC compared to haemoglobin electrophoresis is that, it is automated, less laborious and moderately rapid with high precision. It is very useful in the case of β-thalassemia carrier since it can measure the percentage of Hb A_2 (George *et al.*, 2001).

The downside of HPLC is that some haemoglobin variants can overlap with each other. There are also some variant haemoglobins with the same retention times as normal haemoglobin. This may lead to problem in identifying the variant haemoglobins and difficulty to separate and quantify them accordingly. For example, Hb A_2 may overlap with Hb E, Hb D-Iran, Hb Lepore and so forth due to haemoglobin co-eluting with Hb

A_2. There are also several pre-analytical interferences such as bilirubin which may interfere with the result, leading to the presence of sharp peak at the same retention time as Hb H, Hb Bart and acetylated Hb F (Greene *et al.*, 2012).

Hb CS elute in the C window with the retention time of 4.90 - 5.30 minute, same as Hb O-Arab that also elute in the same window (refer Figure 2.5). Hb CS showed small peak at C-window and sometimes can be missed in HPLC (Waneesorn *et al.*, 2011; Greene *et al.*, 2012).



(a) (b)

HPLC showed (a) presence of a small peak at retention time 4.73-5.25 min for Hb CS (b) absence of Hb CS peak in the chromatogram (Wisedpanichkij *et al.*, 2015).

Figure 2.5: The chromatogram in HPLC in normal Hb analysis and Hb CS.

2.4.5 Capillary electrophoresis (CE)

2.4.5 (a) Introduction and principle

CE is a tool designed to separate human blood samples into Hb A, Hb A_2 and Hb F. It is useful for the identification of significant variant haemoglobin variants (Hb S, C, E and D) in the alkaline buffer. The Capillarys2 Flex Piercing is an automated analyser which executes a complete haemoglobin profile for the quantitative analysis of normal

haemoglobin fractions (Hb A, Hb A_2, Hb F) and for detection of major haemoglobin variants (Sebia, 2017).

The fresh sample is collected in EDTA bottle and the assay is executed on the haemolysate of whole blood samples. The sample can be stored up only for 1 week at the temperature of 2-8℃ because progressive degradation of haemoglobin might occur and interfere with the results (Sebia, 2017).

To arrive at the diagnosis of thalassemia/haemoglobinopathy, two diagnostic methods are required to mutually complement and improve the accuracy and precision of the diagnosis (Bain, 2020). It is suggested that these methods (ie, CE and HPLC) should be performed together. The result of haemoglobin analysis is essential to decide whether there is a need to proceed with molecular test for final diagnosis of Hb variants especially in the centre with limited financial budget (Fucharoen and Winichagoon, 2011).

The CE instrument consists of components that incorporate the capillaries which work simultaneously in parallel allowing 8 synchronized analysis for haemoglobin quantification. The separation process of normal and haemoglobin variants in CE instrument is similar to the separation process in cellulose acetate electrophoresis, but it uses higher voltage and a different pH. Successively, the resulting electropherograms are set to be detected and evaluated for pattern abnormalities (Sebia, 2017).

A study conducted in Thailand by Suwannakan *et al.* in 2011 involving four different reference laboratories had issued a multicentre validation of fully automated CE for

the diagnosis of thalassemia and haemoglobinopathies. They analysed the performance characteristics including precision and accuracy, then compared it with current validated HPLC with automated cation exchange low-pressure liquid chromatography (LPLC). The comparison between these three methods was done to evaluate the accuracy of Hb A_2 and Hb F which revealed good linear correlation. Thus, it was proven that the CE system's quantification of Hb A_2 and Hb F was correlated well with both systems. In fact, the narrow scatter of the results had confirmed that the assay is reproducible (Sangkitporn *et al.*, 2011).

There are several advantages of automated CE including shortened turnaround time, high throughput, less sample employment, the requirement of small sample volume and most importantly, it is cost-effective (Sangkitporn *et al.*, 2011). It is stress free and less laborious for medical laboratory technicians to operate. In Malaysia, the estimated cost for a CE sample is about RM22 which is cost effective (Hafiza Alauddin *et al.*, 2017). One of major advantages of CE is the capability to separate Hb Bart's from early eluting interferences such as bilirubin (Greene *et al.*, 2012).

2.4.5 (b) Interpretation of results

The electropherogram in CE is reflected by the migration zones of different haemoglobin in zone 1 to zone 15. Normal blood sample will reveal normal limit of haemoglobin fractions with HbA2 is identified in Zone 3 and Hb A is seen in Zone 9. There are five main haemoglobin variants with clinical significance and can be seen at different migration sites: Hb S, C, E and O-Arab. Hb CS peak is detected in Zone 2 which is also known as zone C (refer Figure 2.6). Other haemoglobin variants such as

Hb C, Hb Paksê, Hb A2 variant and other types of rare haemoglobin variants share the same peak in Zone 2 (Sebia, 2017).

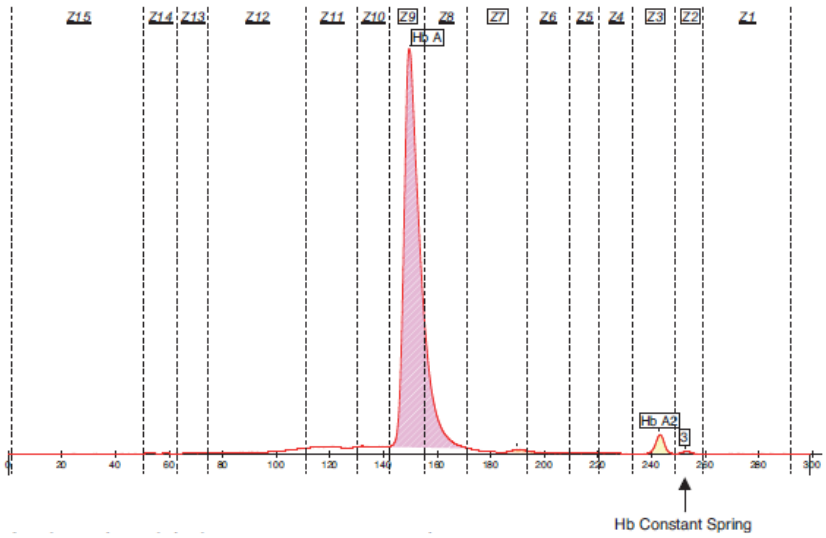


Figure 2.6: Electropherogram of Hb CS at Zone 2 of CE. Adapted from Manual of CE Using Capillarys 2 Flex-Piercing Instrument, 2017.

2.4.6 Molecular analysis

Molecular analysis is impressively accurate and precise. This molecular study is very crucial to confirm the presence of mutation or deletion after presumptive identification of thalassemia or haemoglobinopathy has been made. The results of molecular analysis are vital for genetic counselling (Clarke, 2000). Particularly in Malaysia, DNA tests are required in cases where there is inability to reach the final diagnosis of haemoglobinopathy by haematological tests. Besides, it is also for genetic counselling and prenatal diagnosis of haemoglobinopathy disorder (Hafiza Alauddin *et al.*, 2017).

Recently, most of DNA analysis of haemoglobinopathies used are based on polymerase chain reaction (PCR). For the detection of globin gene mutations, there are several different PCR-based techniques such as dot blot analysis, reverse dot blot

analysis, Amplification Refractory Mutation System (ARMS), restriction endonuclease analysis, gap-PCR and multiplex ligation-dependent probe amplification (MLPA). Different methods have their own advantages and disadvantages. Hence, the factors that affect the decision on which particular methods to be chosen depend on technical expertise available and also based on the variety of mutations that are commonly encountered among the target populations being tested (Old *et al.*, 2013).

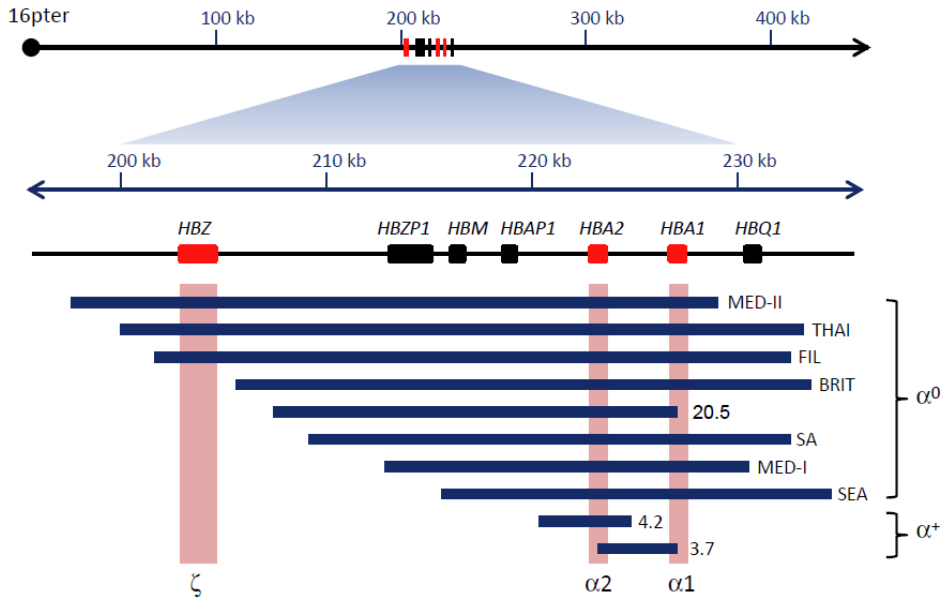
Multiplex GAP polymerase chain reaction (PCR) is used as the detection tool of deletions/mutations in α-globin gene in Malaysia, while multiplex ARMS PCR is for the detection of non-deletional type of alpha-thalassemia. Hb CS is detected using real-time PCR using allele-specific fluorescence (Ahmad *et al.*, 2013).

2.4.6 (a) Multiplex gap polymerase chain reaction (gap-PCR)

Multiplex Gap PCR test method is the technique chosen for the detection of deletional alpha-thalassemia in molecular laboratory in Malaysia. It is performed to detect single gene deletion ($\alpha\text{-}^{3.7}$, $-\alpha^{4.2}$), two gene deletion α-zero thalassemia South-East Asian-type deletion ($--^{SEA}$), α-zero thalassemia Filipino-type deletion ($--^{FIL}$) and α-zero thalassemia Thailand-type deletion ($--^{THAI}$) and α-zero thalassemia Mediterranean-type deletion ($--^{MED}$) and ($--^{20.5}$) (as shown in Figure 2.7). The mutations are chosen for the molecular study of deletional α-thalassemia as they are among the common and highly prevalent type of deletional alpha-thalassemia in this region (Ahmad *et al.*, 2013).

Multiplex Gap-PCR can detect large deletions with known endpoints. Primers that are located 5’ and 3’ near to the deletion breakpoints are used to amplify a small fragment

to make the fragments. The test can run the sample individually or on multiplex panels. This is the most popular multiplex assay in Malaysia and it covers seven deletions as previously mentioned (Bain, 2020).



The first eight deletions involve the inactivation of both α-globin genes (α⁰-thalassemia) whereas the 3.7 and 4.2 involve one gene deletion (α^{+}-thalassemia). Adapted from Waye *et al*, Diagnostic testing of α-globin gene disorders in heterogenous North American populations, 2012 (Waye and Eng, 2013).

Figure 2.7: The mutation on α-globin gene cluster sited on the distal short arm region of chromosome 16.

2.4.6 (b) Multiplex Amplification Refractory Mutations Systems (ARMS) PCR

Allele-specific assay is the choice of molecular analysis for the detection of non-deletional alpha-thalassemia. There are several types of allele-specific assays that include reverse dot blot hybridisation, ARMS, restriction endonuclease cleavage, pyrosequencing and quantitative real-time PCR. These allele-specific methods are unable to detect rare mutations, but it can identify common or known point mutations in the populations (Old *et al*, 2013).

This technique that has been used in molecular laboratory in Malaysia is aimed to detect point mutations 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) (Ahmad *et al.*, 2013).

Table 2. 1: Multiplex ARMS assay detecting allele-specific primer sequences.

Allele detected	*Primer sequences*	*Fragment*
Normal	(forward) ENG12 5′-GGATCCACGCAGTGCTAGAAG-3′ (reverse) ENG21 5′-GTAGAGATGGTGTTTTGCCATGT-3′	930 bp[a]
Initiation codon (ATG→A–G)	(forward) ARMSINC 5′-CACAGACTCAGAGAGAACCCAGCAG-3′ (reverse) BE17 5′-CCATTGTTGGCACATTCCGGGA-3′	869 bp
Codon 30 (ΔGAG)	(forward) ARMSC30 5′-GTATGGTGCGGAGGCCCTGAG-3′ (reverse) BE17	772 bp
Codon 35 (TCC→CCC)	(forward) ARMSC35 5′-TCTCCCCGCAGGATGTTCGTGC-3′ (reverse) BE17	645 bp
Codon 59 (GGC→GAC)	(forward) ARMSC59 5′-CTCTGCCCAGGTTAAGGGCCAAGA-3′ (reverse) BE17	574 bp
Codon 125 (CTG→CCG) Hb Quang Sze	(forward) ARMSC59 5′-CACCCCTGCGGTGCACGCCTCACC-3′ (reverse) BE17	234 bp
Termination codon (TAA→CAA) Hb Constant Spring	(forward) ARMSC59 5′-CCGTGCTGACCTCCAAATACGGTC-3′ (reverse) BE17	184 bp

Non-deletional α-thalassemia mutations detection using a single-tube Multiplex ARMS assay. Adapted from Eng *et al.*, 2001.

It is developed based on the ARMS system that allows direct analysis of any locus of interest using sequence-specific PCR primers. It permits the amplification of test DNA only when the target allele is present within the sample. This assay is a simple, rapid technique and has proven to provide accurate results to detect those common non-deletional α-thalassemia (Eng *et al.*, 2001).

2.4.6 (c) Multiplex ligation-dependent probe amplification assay (MLPA)

The uncommon α^{0}- and α^{+}-thalassemia cannot be detected by gap-PCR as their breakpoint sequences have not yet been discovered. MLPA is a molecular technique used for the discovery of gene deletions, duplications and gene arrangements (Wild and Bain, 2017). It is suggested to use MPLA technique as a complementary investigations for patients with high possibility of having α-thalassemia but showed