PREVALENCE OF BETA THALASSAEMIA / BETA HAEMOGLOBIN VARIANT AMONG THALASSAEMIA SCREENING IN HOSPITAL TENGKU AMPUAN RAHIMAH (HTAR), KLANG

BY

DR NORLYIYANA BINTI MOHYEE

DISSERTATION SUBMITTED IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PATHOLOGY (HAEMATOLOGY)

SCHOOL OF MEDICAL SCIENCES

UNIVERSITI SAINS MALAYSIA

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

Symbols/Abbreviations	Meaning
%	Percentage
+	Positive or addition
0	Negative or minus
±	Plus-minus
<	Less than
>	Greater than
≤	Less than or equal to
>	Greater than or equal to
^{0}C	Degree Celcius
µ	Micro
CDC	Centers for Disease Control and Prevention
CE	Capillary electrophoresis
EDTA	Ethylenediaminetetraacetic acid
et al	Et alia (and others)
FBC	Full blood count
FBP	Full blood picture
fl	femtolitre
FSC	Forward scatter
Gln	Glutamine
Glu	Glutamic acid
Hb	Haemoglobin
Hct	Haematocrit

Symbols/Abbreviations	Meaning
HPLC	High performance liquid chromatography
HTAR	Hospital Tengku Ampuan Rahimah
HUSM	Hospital Universiti Sains Malaysia
i.e	id est (that is)
IEF	Isoelectric focusing
KKM	Kementerian Kesihatan Malaysia
Lye	Lysine
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
ml	Mililiter
NMRR	National Medical Research Registry
nm	Nanometer
NPV	Negative predictive value
OFT	Osmotic fragility test
pg	Pictogram
pH	Unit to measure acidity or alkalinity of a solution
PS	Power sample
RBC	Red blood cell
RDW	Red cell distribution width
RT	Retention time
SD	Standard deviation
SPSS	Standard Package for the Social Science
Val	Valine

Symbols/Abbreviations	Meaning
WBC	White blood cell
WHO	World Health Organization
Z	Zone
α	Alpha
β	Beta
γ	Gamma
δ	Delta
ε	Epsilon
ζ	Zeta

PREVALENS BETA-TALASEMIA / VARIAN BETA-HEMOGLOBIN DI KALANGAN SARINGAN TALASEMIA DI HOSPITAL TENGKU AMPUAN RAHIMAH, KLANG

ABSTRAK

Pengenalan: Talasemia adalah antara penyakit genetik yang paling kerap di temui di kalangan penduduk yang tinggal di Asia Tenggara. Memandangkan penyakit talasemia ini mempunyai spektrum klinikal yang luas, maka ia sukar disahkan, terutamanya di kalangan pembawa apabila diperiksa di klinik kesihatan awam. Di Malaysia, program penyaringan talasemia dilakukan pada wanita hamil, pasangan yang ingin berkahwin, pelajar tingkatan 4 dan ahli keluarga pesakit talasemia. Dalam kajian ini, kami berhasrat untuk mengkaji prevalens beta-talasemia dan varian beta-hemoglobin, seterusnya hubungan penyakit tersebut dengan parameter hematologi. Metodologi: Kajian keratan lintang melibatkan pengumpulan data sekunder sebanyak 4548 sampel penyaringan talasemia yang dihantar ke Hospital Tengku Ampuan Rahimah, Klang, Malaysia pada tahun 2014 hingga 2015. Sebahagian besar sampel telah diambil dari subjek yang melakukan pemeriksaan lanjut bagi anemia dan daripada Saringan Talasemia Pelajar Tingkatan 4 Peringkat Nasional. Parameter hematologi diperiksa menggunakan mesin automatik Sysmex XN 9000 dan analisa hemoglobin menggunakan sistem automatik CE (Capillarys 2; Sebia, Perancis) bersama sistem *High Performance Liquid Chromatography (HPLC)*. Diagnosis dibuat berdasarkan kriteria yang telah ditetapkan. Keputusan: Daripada 4548 subjek, 88.6% (4029) tergolong daripada subjek yang mempunyai nilai MCH $\leq$ 80 fl dan/atau MCH $\leq$ 27 pg. 27.4% daripada kumpulan ini disyaki mempunyai beta-talasemia atau varian beta-hemoglobin. Majoriti subjek adalah terdiri daripada wanita (72.6%) dan berasal

daripda kaum Melayu (86.5%). Penyakit yang paling kerap ditemui di dalam populasi saringan adalah beta-talasemia minor (11.0%) diikuti oleh heterozigot HbE (10.3%). Lain-lain jenis termasuk varian beta-talasemia yang tidak diketahui (1.5%), disyaki mempunyai paras HbA_2 pinggiran beta-talasemia (0.7%), Homozigot HbE (0.5%) dan talasemia HbE/beta (0.3%). Selebihnya adalah talasemia major: 2 subjek, heterozigot HbS: 2 subjek dan homozigot HbS: 1 subjek. Di dalam kajian ini kami mendapati purata (SD) parameter hematologi bagi pembawa genetik beta-talasemia adalah seperti berikut; RBC: 5.38 (0.84), Hb: 11.04 (1.55), MCV: 64.18 (5.81), MCH: 20.70 (2.04) dan RDW: 16.00 (2.19). Seterusnya, bagi heterozigot HbE pula adalah seperti berikut; RBC: 4.94 (0.65), Hb: 11.98 (1.54), MCV: 73.10 (5.56), MCH: 24.36 (2.17) dan RDW: 14.30 (2.10). Kesimpulan: Kajian ini menunjukkan prevalens yang tinggi di kalangan beta-talassemia / varian beta-hemoglobin dan kumpulan yang paling kerap ditemui adalah beta-talasemia minor dan heterozigot HbE. Kajian ini juga mendapati, program saringan talasemia di Malaysia adalah sangat bermanfaat dan harus diperbaiki dari semasa ke semasa. Maka, kesedaran mengenai penyakit ini di kalangan masyarakat mestilah terus dipertingkatkan.

(377 perkataan)

PREVALENCE OF BETA THALASSAEMIA/ BETA HAEMOGLOBIN VARIANT AMONG THALASSAEMIA SCREENING IN HOSPITAL TENGKU AMPUAN RAHIMAH, KLANG.

ABSTRACT

Introduction: Thalassaemia is the most common genetic disorders among population living in the Southeast Asia. In view of complexity and heterogeneity of the disease, hence it is difficult to diagnose the disease, especially the carriers at the health clinics. In Malaysia, thalassaemia screening program has been carry out by screening the targeted population such as pregnant women, prenuptial couples, form 4 students and family members with thalassaemia patients. In this study, the aim is to evaluate the prevalence of beta thalassaemia/ beta haemoglobin variant and its association with haematological parameters. Methodology: A cross sectional study involving secondary data collection of 4548 samples of thalassaemia screening that sent to Hospital Tengku Ampuan Rahimah, Klang, Malaysia in year 2014 to 2015. Majority of the samples taken from the subjects investigated for anaemia and the National Thalassaemia screening for Form 4 students. The samples were analysed for haematological parameters using Sysmex XN 9000 automated blood cell analyser (Capillarys 2; Sebia, France) and further analysed for haemoglobin fraction using CE and/or HPLC. The diagnoses made using the established standard criteria. Result: Out of 4548, 88.6% (4029) subjects had MCV $\leq$ 80 fl and/or MCH $\leq$ 27 pg. From this subjects group, 27.4% were suspected to have beta thalassaemia or beta haemoglobin variant. Majority were from female subjects (72.6%). The percentage of subjects according to races were predominately by Malay 86.5%, followed by other ethnic

group. The most frequent type among screening populations was β thalassaemia traits (11.0%) followed by HbE traits (10.3%). Others include unknown variant (1.5%), suspected borderline HbA_2 β thalassaemia (0.7%), HbE homozygous (0.5%) and HbE/β thalassaemia (0.3%). The remaining are β thalassaemia major, HbS heterozygous and HbS homozygous (2, 2 and 1 subjects respectively). The mean (SD) haematological parameters for β thalassemia trait were RBC: 5.38 (0.84), Hb: 11.04 (1.55), MCV: 64.18 (5.81), MCH: 20.70 (2.04) and RDW: 16.00 (2.19). Meanwhile for HbE traits, the mean (SD) were RBC: 4.94 (0.65), Hb: 11.98 (1.54), MCV: 73.10 (5.56), MCH: 24.36 (2.17) and RDW: 14.30 (2.10). Conclusion: This study demonstrated high prevalence of β thalassaemia/ β haemoglobin variant, meanwhile the β thalassaemia trait and HbE trait were the commonest group. This data suggests, thalassaemia screening program in our country is very beneficial and have to be improved from time to time. The awareness of the disease among community must be equally highlighted.

(389 words)

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1: GENERAL INTRODUCTION

Haemoglobin disorders were originally endemic in 71% of 229 countries, potentially affecting 89% of births worldwide (Modell and Darlison, 2008). Inherited haemoglobin disorder (thalassaemia and haemoglobin variant) are the most common genetic disorders among the people living in Southeast Asia. Prevalence of haemoglobinopathy gene carriers was 5 to 40% and can up to 70% regionally (Kohne, 2011). Given the high prevalence of thalassaemia in this particular region, public health burden eventually will be increased (Fucharoen and Winichagoon, 2011).

Thalassaemia is a heterogeneous group of inherited autosomal recessive blood disorder result from the diminished rate of synthesis of one or more globin chains and consequently causing reduced rate of synthesis of haemoglobin. Thalassaemia is classified based on the type of haemoglobin affected, which the commonest one is α- and β- thalassaemia follow by δβ-, γδβ-, δ-, γ-, εγδβ-thalassaemia (Hoffbrand *et al*, 2016).

Meanwhile, the haemoglobin variant arises from the mutation of α or β globin gene that produce structurally abnormal globin proteins and have biochemically properties with physiological effects ranging from insignificant to severe manisfestation (Thom *et al.*, 2013). There are >1000 haemoglobin variants which most of the type are clinically silent and some can produce clinical manifestations of varying severity. Haemoglobin variants such as HbS, HbC, HbE, Hb CS and HbD are clinically significant. HbE and Hb CS are the most common type of haemoglobin variant found in South East Asia (Fucharoen and Winichagoon, 2011). In view of varieties of thalassaemia and haemoglobinopathies in the population, interactions between them

leading to complex syndromes are common and render their diagnosis difficult in routine practices (Srivorakun *et al.*, 2014).

Malaysia is the multi-ethnic country and generally divided into Bumiputera and non-Bumiputera. Bumiputera group consists of Malay, Peninsular Aborigines, Indigenous group of Sabah and Sarawak and as for non-Bumiputera, it consists of Chinese, Indian and others. According to the Department of Statistic Malaysia in 2017, the population in Malaysia estimated around 32.0 million with 28.7 million are citizens and 3.3 million are non-citizens. Majority of citizens population are from Bumiputera group (68.8%), followed by Chinese group (23.2%) Indian group (7.0%) and others (1.0%). State of Selangor record the highest population distribution which 19.9% from the total distribution (Department of Statistic, 2011). It makes the Selangor state a more suitable area for this prevalence study.

Generally, the objective in this research is to study the prevalence and haematological parameters of beta thalassaemia and beta haemoglobin variant by using the haemoglobin separation and quantitation such as capillary electrophoresis (CE), High performance liquid chromatography (HPLC) and gel electrophoresis. The β thalassaemia trait is identified by increase $HbA_2 \geq 4\%$ but less than 10% and normal or slightly raise HbF with no other abnormal peak seen in other zone or window. Meanwhile, for β thalassaemia major, the majority haemoglobin fraction is from HbF, slightly raised HbA_2 (<10%) with markedly reduced HbA depending on genotype involved (George *et al.*, 2001). If the HbA_2 level were ranging from 3.0% to 3.9% with relatively low haemoglobin level, high RBC count and low MCV and/or MCH, the samples were categorised under unable to exclude borderline HbA_2 β thalassaemia (Rosnah *et al.*, 2017).

As for the common type of β haemoglobin variants such as HbE trait, the HbE level was ranged from 21% to 30% with slightly raised HbA_2 and HbF by CE (Bain, 2008). As for the pure homozygous HbE, the abnormal haemoglobin level (HbE) was more than 80% with no HbA production. Meanwhile, if the CE demonstrate HbE level around 40%-60%, HbF 60% - 40% and increased HbA_2 with a marked range of amount HbA (if associated with β^+-thalassaemia), the samples were categorised under HbE/β thalassaemia (Vichinsky, 2007). In heterozygous HbS, the variant Hb falls in zone 5 at CE and S-window with RT 4.3 to 4.5 min and ranging from 40% to 50%. As for the homozygous state, the variant Hb account 90-95% with no HbA production (Bain, 2008; Shrivastav *et al.*, 2013). However, in some other haemoglobin variants, they may co-elute with each other and not well differentiated between them even with the help of other supplementary tests such as HPLC or gel electrophoresis. With this problem, the result will be interpreted as unknown variant.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2: LITERATURE REVIEW

2.1 Structure and genetic of haemoglobin

Haemoglobin molecule is an important structure inside of mature red blood cells and is essential for human life as it is crucial in oxygen transportation to the tissue. It is composed of two dissimilar pairs of polypeptide chains α-like (α or ζ) and β-like (ε, γ, δ or β) globin chains, each pair link to iron-containing porphyrin and term as haem. Each of the α-like and β-like globin chains is encoded by genetically distinct gene clusters located at chromosome 16p and chromosome 11p15.5, respectively. The combination of this polypeptide chains will give rise to different type of haemoglobin and expressed at different stages of development. During embryo stage, the main haemoglobin types are Hb Portland (ζ2 γ2), Hb Gower I (ζ2ε2), and Hb Gower II (α2ε2), later switch to HbF (α2γ2) as early as 5 weeks of gestation and increasing in trend through the development of the foetus. β globin expression starts as early as 8 weeks of gestation in low amount and then increased up to 10% at 30-35 weeks of gestation. The increasing production of β globin proportionate to the declining of γ globin expression. At birth, HbF is the main haemoglobin (60-80%) of total haemoglobin and reducing to approximately around 5% at the age of 6 months and eventually reaching adult level around 1% at the age of 2 years old. This HbF slowly being replaced by HbA ($\alpha_2\beta_2$) and become the most common type of haemoglobin (>95%) with a minor component of HbA_2 ($\alpha_2\delta_2$) in the adult red blood cells. However, the switch of adult haemoglobin production is not total, low amount of γ globin production persists throughout adult life with the residual amount of HbF around 0.2% to 7% and calls as F cells (Porwit *et al.*, 2011).

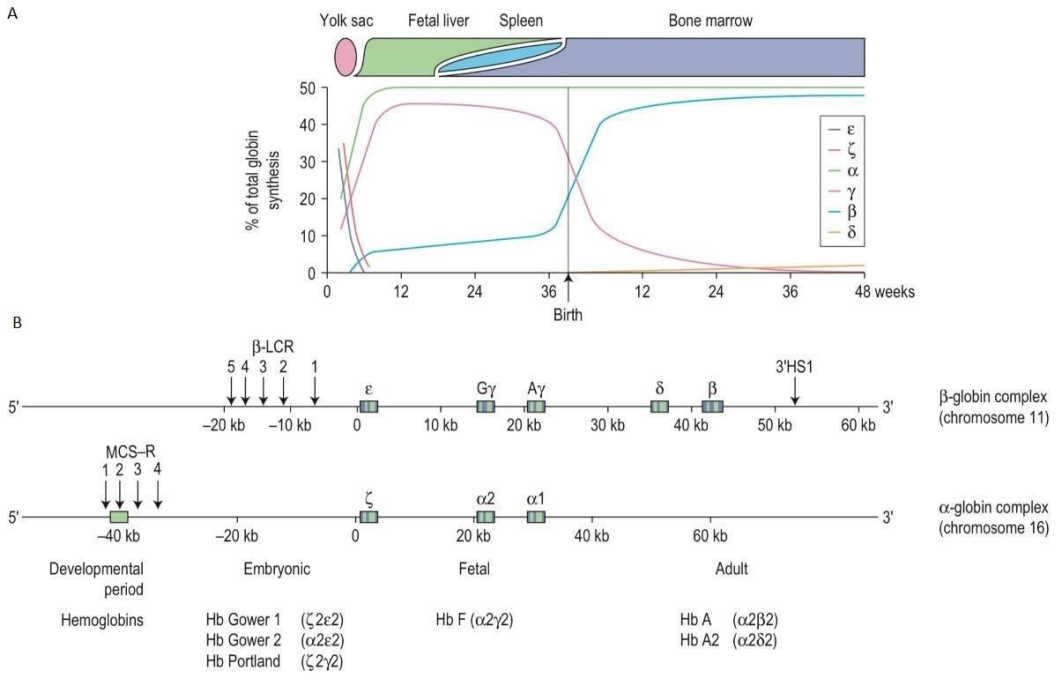

Figure 2-1: (A) The timeline for expression of globin gene from intrauterine life to within the first year of life (B) The genetic structure of α and β globin genes, which situated on chromosome 16 and chromosome 11, respectively (Adapted from Blood and Bone Marrow Pathology 2nd edition, 2011).

2.2 **Introduction of thalassaemia**

Thalassaemia is a heterogeneous group of inherited autosomal recessive blood disorder which resulted from the diminished rate of synthesis one or more globin chains and consequently cause the reduced rate of synthesis of haemoglobin. Thalassaemia is classified based on the type of globin affected, which the commonest one is α- and β- thalassaemia followed by δβ-, γδβ-, δ-, γ-, εγδβ-thalassaemia (Hoffbrand *et al.*, 2016). In thalassaemia, a significantly reduced rate synthesis of one type globin chain will lead to unbalanced globin chain quantity by an excess of other normal globin chains. This condition contributing to the pathological defects causing

either damage to erythroid precursors and ineffective erythropoiesis or damage to mature erythrocytes and haemolytic anaemia. Thalassaemia may result from the deletion of a large part or all of the gene or the small deletion or other mutation of a gene (Bain, 2008). Thalassaemia is the most common genetic disorders among the people living in Southeast Asia. In the area of a high prevalence of thalassaemia, it causes an increasingly severe public health burden (Fucharoen and Winichagoon, 2011).

Table 2-1: Classification of the thalassaemia (Adapted from Haemoglobinopathy Diagnosis, 2nd edition 2016).

Type of thalassaemia	Chain or chains synthesized at a reduced rate	Haemoglobin or haemoglobins synthesized at a reduced rate
Alpha: α^0 or α^+	α	A, A_2 and F
Beta: β^0 or β^+	β	A
Gamma: γ	γ	F
Delta: δ^0 or δ^+	δ	A_2
Delta beta: $\delta\beta^0$ or $\delta\beta^+$	δ and β	A and A_2
^{A}Gamma delta beta: $^A\gamma\delta\beta^0$	$^A\gamma$, δ and β	A and A_2
Epsilon gamma delta beta*: $\varepsilon^G\gamma^A\gamma\delta\beta^0$	ε, $^G\gamma$, $^A\gamma$, δ and β	A, A_2 and F*
Haemoglobin Lepore	δ and β	A and A_2†

*Often referred to as γδβ thalassaemia; in fetal life, there is decreased synthesis of haemoglobins Gower 1 and 2 and Portland 1.
†Haemoglobin Lepore is synthesized at a reduced rate in comparison with haemoglobin A, but at an increased rate in comparison with haemoglobin A_2

2.3 **β thalassaemia**

β thalassaemia is the group of inherited blood disorder resulting from the reduced or complete suppression of synthesis of β globin chain which can give rise to carrier state (generally asymptomatic) to thalassaemia major (severe anaemia) which will required frequent blood transfusion. In many countries in Southeast Asia, β thalassaemia carrier

ranging from 1% to 9% from the total populations (Fucharoen and Winichagoon, 2011). In Malaysia, β thalassaemia carriers are 4.5% among Malays and Chinese populations (Tan *et al.,* 2010).

A recent study conducted in Malaysia showed β thalassaemia trait is one of the commonest type and it carries 23.4% from total thalassaemia screening populations (Pauzy *et al.,* 2018). Meanwhile, data from Malaysia Thalassaemia Registry 2009 showed the β thalassaemia major patients is part of transfusion dependent group. Therefore, it is well known to be a major public health issue with a large cost burden to the health ministry as treatment involved repeated blood transfusion and long term monitoring for treatment side effect (Lan *et al.*, 2003). Heterozygous state of β thalassaemia usually having mild anaemia and microcytosis and will be categorised phenotypically as β thalassaemia carrier or traits. This β thalassaemia trait usually completely asymptomatic and can become symptomatic anaemia and required blood transfusion during haemopoietic stress such as pregnancy or intercurrent infection. Meanwhile, for homozygous or compound heterozygous β thalassaemia, it manifests in varying degree of a severe form of anaemia and categorized either β thalassaemia major or intermedia (Bain, 2008; Nienhuis and Nathan, 2012).

2.3.1 Genetic basic of β thalassaemia

The β gene is located at the chromosome 11p15.5 and in normal individual; there are two alleles of β gene. Many types of β thalassaemia mutations and compound heterozygosity may occur with the affected individual having two mutant genes and no normal β gene. Phenotypically β thalassaemia are divided into two broad categories, such as β^0 thalassaemia and β^+ thalassaemia. In β^0 thalassaemia, the genotypically it can occur either homozygous or compound heterozygous state. The condition is due

to a total lack of β globin chain production and a total failure to produce HbA. Meanwhile for homozygous β^+ thalassaemia, there is some amount of HbA were still present produced from the remaining abnormal β gene. There are numbers of β^+ thalassaemia mutation which consist of a variety of defect causing mild to severe production of β chain synthesis. Compound heterozygous for β thalassaemia can occur either in combination from two different β^0 thalasaemia gene, two different β^+ thalassaemia gene or combination of β^0 and β^+ thalassaemia genes. The β thalassaemia usually results from a mutation in or near the β gene and a small number of cases arise from deletion, which occurs in the β gene itself or at the controlling sequences 5' to the gene. The mutation of this β gene will produce abnormal structure of β globin by reducing β chain production rate, produced very unstable β chain structure or produce very unstable haemoglobin (Bain, 2008; Hoffbrand *et al.*, 2016; Porwit *et al.*, 2011).

2.4 **Introduction to haemoglobin variant**

Haemoglobin variants or abnormal haemoglobins are inherited mutation disorders of globin gene, which form the abnormal structure of the haemoglobin molecule. Most of the haemoglobin variants are non-clinical significant. However, some could produce clinically relevant phenotypes when found in homozygous state, association with thalassaemia or other haemoglobinopathies (Srivorakun *et al.*, 2014). The majority of recognised haemoglobin variants are usually arisen from α and β chain variants. An α chain variant leads to formation variants of HbA, HbA_2 and HbF. Meanwhile β chain variant leads to a variant of HbA. Therefore, a β variant is expected to comprise of 50% of the total haemoglobin. However, if the variant β chain is synthesised at a reduced rate, the potential of combination with abnormal α chain is

possible. Among the β chain variant, β^E variant is one of the variant that have reduce in haemoglobin proportion and result in less than 50% of variant haemoglobin production. Other than α and β chain variant, δ and γ chain variants may also occur but less in frequency. Clinically insignificant haemoglobin variant can be confused with clinically significant variant because of the similar findings on haemoglobin analysis investigation (Bain, 2008). The haemoglobin variants vary among countries to countries depending on the ethnic distributions. In Thailand, the prevalence of haemoglobin variant among thalassaemia screening was 2.4% (Srivorakun *et al.*, 2014) and for India, the prevalence of haemoglobin variant range from 0.15% to 2% depending on the variant type (Warghade *et al.*, 2018). As for Malaysia, the prevalence of haemoglobin variants was 2.3% almost similar to the nearest countries (Sabariah *et al.*, 2013).

2.4.1 Haemoglobin E (HbE)

HbE is a β chain variant (β^E) and among the commonest structural haemoglobinopathy, especially in the north-eastern part of Thailand, Cambodia and Laos. It was also found to be highest in other Southeast Asia countries such as Malaysia, Philippines, Indonesia and Vietnam (Bain, 2008). In Malaysia, the prevalence of HbE varies from area to area of study, which carries the range from 11.25% to 28.0% (Pauzy *et al.*, 2018; Rong *et al.*, 2015; Rosline *et al.*, 2006). HbE occurs from a mutation at amino acid position 26 (Glu>Lys) β globin chain and this abnormality also results in reduced amount of β^E mRNA and subsequently lead to reduced β^E globin chains (Vichinsky, 2007). Therefore, the amount of HbE percentage is less than expected compared to other types of variant β globin chains and it is regarded as thalassaemia haemoglobinopathy. HbE also weakened $\alpha_1\beta_1$ contacts, leading to instability towards

oxidative stress. HbE can be inherited as heterozygous (also called as trait or carrier), homozygous (HbE disease) or compound heterozygous. Heterozygous and homozygous HbE usually asymptomatic and no clinical features but will cause haemolysis if exposed to oxidant stress. However, it will manifest a wide spectrum of severity when it occurs in the compound heterozygous form such as HbE/β thalassaemia and may require treatment influence by genetic and environmental factors. The genetic modifiers include different interaction of β thalassaemia alleles, co-inheritance of α thalassaemia alleles and relative level of persistent foetal haemoglobin synthesis (Fucharoen and Winichagoon, 2011).

2.4.2 Other β haemoglobin variants

Naturally, haemoglobin variants will cause a range of biochemical abnormality and some produces clinically significant manifestation. Other than HbE, there are many other form of β chain variant, which have clinical significant such as Haemoglobin S (HbS, β^S), Haemoglobin C (HbC, β^C), Haemoglobin D-Punjab (HbD-Punjab) and Haemoglobin O-Arab (HbO-Arab) (Thom *et al.*, 2013). The prevalence of this other β variant haemoglobinopathy such as HbS (also known as Sickle cell thalassaemia) or HbC are more common in Africa, America and some Middle East countries. They are considered as rare in other countries such as Southeast Asia. However, in view of migration of some population, the disease becomes more sporadic and high prevalence in other countries. The HbS occur when presence of mutation at amino acid position 6 (Glu>Val) β globin chain, meanwhile for HbC mutation is also occur at a position 6, however Glutamic acid (Glu) was substituted to Lysine (Lys). The mutation for HbD-Punjab and HbO-Arab occur at amino acid position 121 by changing amino acid from Glu>Gln and Glu>Lys respectively. In India, the prevalence of HbS is 3.6% (Bain,

2008; Warghade *et al.*, 2018), however in Thailand and Malaysia the prevalence is much lower which is 0.2% and 0.253% respectively (Sabariah *et al.*, 2013; Srivorakun *et al.*, 2014). Generally, the clinical manifestation for this other variant varies. For example, Sickle cell thalassaemia has more potent clinical features such as thrombosis when exposed to hypoxia condition. This is due to during deoxygenation condition the HbS reduce solubility and form polymerization. Subsequently distort the red cell into a crescent shape that hinders the blood flow through capillaries and cause endothelial cells injury, which further leads to thrombosis (Hoffbrand *et al.*, 2016).

2.5 **Investigation modalities for thalassaemia and haemoglobinopathy**

The diagnosis of thalassaemia and haemoglobinopathy, a combination of laboratory investigation is required with minimum of at least two different methods to evaluate the parameters and reliability of each test. For clinical purposes, the specific laboratory investigation together with the clinical features, ethnic origin of the subject and blood count and film is adequate for presumptive identification (Bain, 2008). It is clinically important to differentiate between provisional diagnosis of microcytic anaemia because each has an entirely different cause, treatment and prognosis (Eldibany *et al.*, 1999; Vehapoglu *et al.*, 2014).

The 1975 International Committee for Standardization in Haematology expert panel on abnormal haemoglobins and thalassaemia recommended the diagnostic laboratory investigations of these conditions to include full blood count, gel electrophoresis at pH 9.2, test for solubility and sickling and quantification of HbA_2 and Hb F (Clarke and Higgins, 2000). In the diagnosis of iron deficiency anaemia, the more accurate diagnostic tools are serum ferritin, transferrin saturation, serum soluble transferrin

receptors, and the serum soluble transferrin receptors–ferritin index (Lopez *et al.*, 2016).

Laboratory investigations for thalassaemia and hemoglobinopathies were perform most conveniently on the venous blood sampling with ethylene diamine tetra-acetic acid (EDTA) as anticoagulant. The sample should be stored at 4^0C and ideally, the test should be tested within a week because of as longer storage leads to denaturation of haemoglobin (Bain, 2008).

2.5.1 Full blood count (FBC) and Full blood picture (FBP)

Commonly, thalassaemia and haemoglobinopathy are known to have hypochromic and microcytosis with or without anaemia, which requires a differential diagnosis to exclude iron deficiency anaemia (Brancaleoni *et al.*, 2016). In Malaysia, the prevalence of iron deficiency is high as 5.2% detected among children 7 to 12 years old (Nik Shanita *et al.*, 2018). Because iron deficiency remains the most common cause of anaemia, the differentiation between these two differential diagnoses is important (Eldibany *et al.*, 1999). The most widely used cut off values of mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) for indicating thalassaemia are 79 fl and 27 pg (Brancaleoni *et al.*, 2016). As screening for thalassaemia or haemoglobinopathy, it is recommended to use the MCH of less than 27 pg and MCV less than 80 fl during initial step (George *et al.*, 2005; Ministry of Health Malaysia, 2009).

Red cell indices such as red blood cell (RBC), MCV, MCH and red cells distribution width (RDW) have offered a basic identifying type of anaemia and facilitate in the diagnosis of thalassaemia or haemoglobinopathy when combine with other various tests (Briggs and Bain, 2016). In comparison to iron deficiency anaemia, thalassaemia

carrier have relatively high RBC, normal or slightly low haemoglobin level, disproportionate to reduced MCV and MCH, and having within or very close to the reference interval of RDW.

Peripheral blood film was made upon request by attending clinician or less frequently initiated by the laboratory for assessment of abnormal findings from automated count or patient clinical information that may help in diagnosis and/or treatment. There was a various clinical indication for peripheral blood film analysis and the commonest include investigation for unexplained cytopenia, anaemia, leucopenia or thrombocytopenia, unexplained leucocytosis, unexplained jaundice or haemolysis and screening tool for congenital red blood cells abnormality (Adewoyin, 2014). Morphology changes of red blood cells detected in most of the thalassaemia cases and usually manifested as microcytosis, hypochromia and anisopoikilocytosis. Less common findings are basophilic stippling and the presence of some target cells (Brancaleoni *et al.*, 2016).

2.5.2 Laboratory investigation for detection of different type of haemoglobin

In screening and diagnosing an individual with thalassaemia or haemoglobinopathy, apart from the laboratory investigation, the clinical information, sociodemographic background, family history and some significant physical examination findings are important. Together with the significant sociodemographic data, relevant clinical information and investigation findings from full blood count and blood film, haemoglobin analysis is the next step of making the diagnosis. There are many laboratory methods that commonly being employed for detection of abnormal haemoglobin (Bain, 2008).

Traditionally, gel electrophoresis is the method of choice for identification and quantification of haemoglobin variants. However, in recent years with more sophisticated technology, there is a variety of new technology available for making the diagnoses more comprehensive and extensive, which makes it complement each other. Other laboratories investigation that has been used now days are high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and isoelectric focusing (IEF) (Clarke and Higgins, 2000).

The first line diagnostic method for laboratory should be able provisionally identify all the relatively common, diagnostically important, normal and variant haemoglobin specifically HbA, HbA_2, HbF, HbS, HbC, HbD-Punjab, HbE and HbH. The laboratory also requires other specific tests, for example sickle solubility test, HbH preparation and test for haemoglobin instability as part of the basic requirement (Borbely *et al.*, 2013).

2.5.2 (a) Haemoglobin gel electrophoresis

Haemoglobin electrophoresis is still the most common technique for the initial detection and characterization of haemoglobin variants. It depends on the principle, which when protein applied to a membrane that exposed to a charge gradient, it will be separate from each other and can be visualized by either protein stain or a haem stain. Haemoglobin analysis can be carried out on the filter paper; a cellulose acetate membrane, a starch gel, a citrate agar gel or an agarose gel (Bain, 2008).

Cellulose acetate electrophoresis at alkaline pH (pH 8.4 to pH 8.6) is a simple, reliable and rapid method, which is the most useful technique that has been used. It is satisfactory for the detection of the most common, clinically important haemoglobin variants. The principle of this method used is when negatively charged haemoglobin

exposed to electrical current it will be migrate toward the anode (+). Haemoglobin variants that have a change in the charge on the surface at alkaline pH will be separated from HbA (Wild and Bain, 2017). Each haemoglobin variants carries a different net charge so that it will migrate at varying speeds (CDC, 2015).

Haemoglobin electrophoresis best done on the lysed packed red blood cells so that it will be consistent with the amount of haemoglobin applied with no interference by the plasma protein. The interference may lead to additional bands and can be mistaken for haemoglobin variant (Bain, 2008).

This method produced the separation of HbC, HbS, HbF, HbA and HbJ, as well as other haemoglobin variants with minimal preparation time. For example, the HbS, HbD, HbG and HbLepore co-migrate together and same as HbC, HbA_2, HbO-Arab and HbE (Wild and Bain, 2017). In view of this limitation, other approaches must be incorporated into the screening to visualize the different type of haemoglobin variants (CDC, 2015).

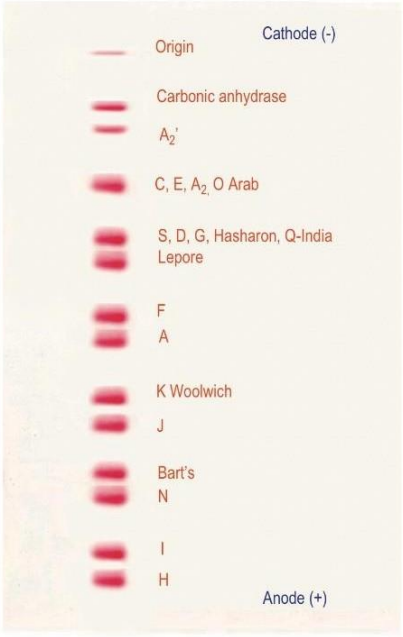

Figure 2-2: Schematic representation of relative mobility of some abnormal haemoglobin on cellulose acetate electrophoresis pH 8.5 (Adapted from Practical Haematology, Twelve edition, 2017).

2.5.2 (b) High performance liquid chromatography (HLPC)

High performance liquid chromatography (HPLC) is a process in which a mixture of molecules such as normal and variant haemoglobin with as net positive charge separated into its components by their absorption onto a negatively charged stationary phase in a chromatography column, followed by their elution by mobile phase. Then it will be detected optically, identified based on retention time and quantified by measuring the area under the corresponding peak in the elution profile. There is some correlation between HPLC retention time and mobility of band on cellulose acetate electrophoresis at alkaline pH. The more positively charged haemoglobin has a longer retention time in HPLC correlating with slower mobility on the alkaline pH electrophoresis (Bain, 2008).

HPLC is one of the reliable tests that been used for quantification of HbA, HbA_2, and HbF. Besides that, it also been used to detect, provisional identified and quantified of haemoglobin variant. Quantification of HbA_2 is the most valuable test for the detection and diagnosis of β thalassaemia. Generally, the normal range for HbA2 in a normal adult is between 2.0% to 3.2% (Menzel *et al.*, 2013). Meanwhile, for classical β thalassemia traits, the HbA_2 is more than 4.0% (George *et al.*, 2001), whereas for values of 3.3% to 3.9% are considered as borderline and they need further investigation, especially in young individual or couple at risk (Ou *et al.*, 2011).

In the HPLC, the HbE, abnormal haemoglobin is interpreted as the same retention as HbA_2, however it can be distinguished by the level of concentration. If the HbA_2/E peak is greater than 3.5% but less than 10%, the patient is only a β thalassemia carrier without the involvement of HbE. However, if the A_2/E peak is greater than 10% and HbA is present, the patient can be HbE trait or compound heterozygous HbE/β^+ thalassaemia. In this case, the HbA percentage will be less than HbE. If the HbA_2/E peak is greater than 10% and the HbA is not present, the patient classified as homozygous HbE or compound heterozygous HbE/β^0 thalassaemia. Homozygous HbE that usually asymptomatic with normal or slightly increased in HbF, whereas for the compound heterozygous HbE/β^0 thalassaemia, it has high HbF and usually presented clinically or phenotypically as thalassaemia intermedia or major (Sharma *et al.*, 2013).

The automated HPLC shown to have excellent resolution, reproducibility and able to quantify several normal and abnormal haemoglobin fractions that will allow accurate diagnosis of thalassaemia or haemoglobinopathy in addition to electrophoresis. For example, HbS was eluted at the S-window with the retention time of 4.3 to 4.7 min and the percentage of 40-50 (heterozygous HbS) or 90-95 (Homozygous HbS).

Meanwhile, for HbC, it eluted at the 4.9 to 5.3 min with an amount of 32% to 44% (heterozygous) and HbA slightly more than 50%. As for the HbC homozygous most of the haemoglobin was HbC with no HbA, HbF less than 3% and normal HbA_2 level (Bain, 2008).

Therefore, the retention time and percentage of haemoglobin variants can provide an important clues in differentiating variant haemoglobins that eluting in the same window (Khera *et al.*, 2015). However, HPLC is not without intrinsic interpretation problems. Some variant may co-elute at the same retention time and leads to difficulty in identifying and quantifying it. For instance, the HbA_2 can co-eluted with HbE (as mention above), HbD-Iran, Hb Lepore and others. Therefore the additional method is necessary to help in recognizing the haemoglobin variant that presents (Clarke and Higgins, 2000).

Peak name	Calibrated area %	Area %	Retention time (min)	Peak area
P1	– – –	0.2	0.81	3314
F	23.8*	– – –	1.12	397 418
P2	– – –	3.1	1.33	53 378
P3	– – –	2.6	1.71	44 107
Ao	– – –	39.7	2.48	683 561
A2	1.8*	– – –	3.61	34 884
S – window	– – –	15.5	4.41	267 188
C – window	– – –	13.9	5.11	239 583

Total area: 1 723 434

F Concentration = 23.8*%

A2 Concentration = 1.8*%

*Values outside of expected ranges

Analysis comments:

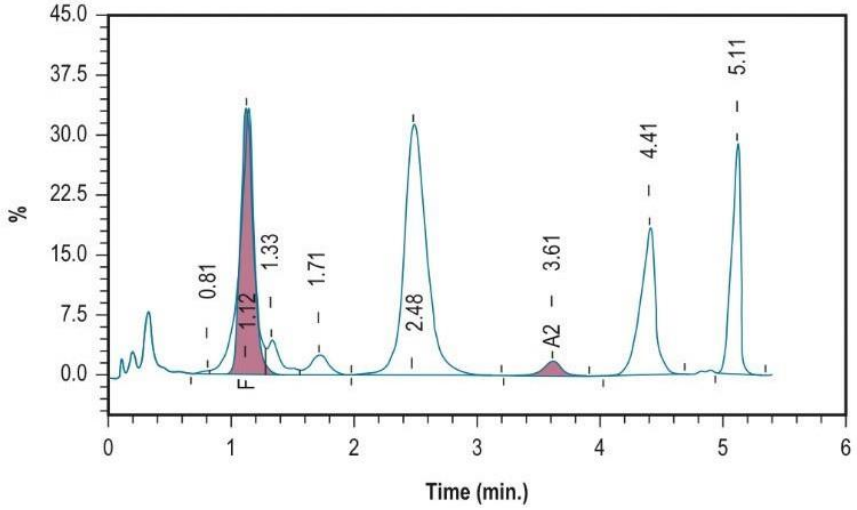

Figure 2-3: A mixture of haemoglobin separation by HPLC (Adapted from Practical Haematology, Twelve edition, 2017.

Table 2-2: Retention time of common normal and abnormal haemoglobin on Bio-Rad Variant II system compared with other haemoglobins that may have overlapping retention times (Adapted from Haemoglobinopathy Diagnosis, 2006).

'Window'	Retention time (min)	Window	Haemoglobins that may overlap
F	1.10	0.98–1.22	Okayama
'P2'*	0.11	1.28–1.50	Beckman, Geelong, **glycosylated A**, Hope, I-Philadelphia, K-Woolwich
'P3'†	1.70	1.50–1.90	Buffalo, Camden, Fannin-Lubbock, Grady, J-Bangkok, J-Meerut, J-Baltimore, J-Norfolk, N-Baltimore
A	2.50	1.90–3.10	**A**, glycosylated S, New York, Köln (when not denatured)
A_2	3.60	3.30–3.90	Deer Lodge, D-Ouled Rabah, D-Iran, **E**, G-Copenhagen, G-Coushatta, G-Ferrara, G-Honolulu, Kenya, Korle Bu, **Lepore**, M-Saskatoon‡, Osu-Cristiansborg, Spanish Town, Zurich
D	4.10	3.90–4.30	Alabama, **D-Punjab**, G-Norfolk, **G-Philadelphia**, Kempsey, Osler
S	4.50	4.30–4.70	Q-Thailand (Mahidol), A_2', Manitoba
C	5.10	4.90–5.30	Agenogi, **C**, Siriraj, **Constant Spring**

* A glycosylated fraction of haemoglobin A.
† A minor peak representing modified haemoglobin A.
‡ Plus a second peak in the C window.

2.5.2 (c) Capillary electrophoresis (CE)

- **Introduction of capillary electrophoresis**

Capillary electrophoresis (CE) is first described by Hjertén in 1967 by experiments of electrophoresis in the free solution carried out using a tube of 3mm in diameter and required about 50 years to develop commercial apparatus for it which is same as high performance liquid chromatography (HPLC) (Castagnola *et al.*, 1995). Over the past many years, the diagnosis of thalassaemia and haemoglobinopathy has dominated by the cellulose acetate electrophoresis and later has been replaced by the using high performance liquid chromatography (HPLC) (Bain *et al.*, 2016). In order to diagnose an individual with thalassaemia or haemoglobinopathy, two different diagnostic

methods must be use to quantify the HbA_2 and it also must be able to distinguished all the relatively common as well as diagnostically significant both normal and variant haemoglobin. The two diagnostic methods should be able to complement and enhance the accuracy and the precision of the diagnosis (Bain, 2008).

CE is a laboratory method that is able to perform a complete haemoglobin profile for the quantitative analysis of the normal haemoglobin fraction HbA, HbA_2, and HbF and the detection of major haemoglobin variants such as HbS, HbC, HbE and HbD (Sebia, 2013). In 2007, USA food and Drug Administration approved CE as one method for evaluation of thalassaemia or haemoglobinopathies (Keren *et al.*, 2008).

- **Principle of capillary electrophoresis and its usage**

Capillary electrophoresis (CE) is a method used to separate haemoglobin based on changed of electrophoretic mobility of an analyte as a result of specific interaction with a substance in the electrophoretic buffer (Castagnola *et al.*, 1995). This instrument principally using capillary electrophoresis in free solution. The charged haemoglobin molecules within a capillary tube in which made from fused silica were separated by the electrophoretic mobility in an alkaline buffer with a specific pH. The separation also occurs according to the electrolyte pH and electroosmotic flow (Sebia, 2013).

The CE instrument is a fully a straightforward automated analyser, in which the assay is performed on the haemolysate of whole blood samples collected in a tube containing EDTA as an anticoagulant. The fresh anticoagulated whole blood samples collected containing K_2-EDTA or K_3-EDTA as an anticoagulant is recommended for analysis with a minimum volume of 1 mL and can be stored up to 7 days if kept between 2^0C to 8^0C (Sebia, 2013). The degraded HbA is appeared by day 10 if the specimen were kept at 4^0C and by day 7 at room temperature. Meanwhile the HbA_2 percentage was

stable up to 16 days both at 4^0C and at room temperature. Therefore, the manufacturer was recommended maximum storage of 7 days at 4^0C (Borbely *et al.*, 2013).

This instrument has capillaries functioning in parallel allowing 8 simultaneously analysis for haemoglobin quantification from the whole blood sample. A sample dilution with a haemolysing solution was prepared and injected by aspiration at the anodic end of the capillary. Subsequently, a high voltage protein separation was then performed and direct detection of the haemoglobin made at an absorbance wavelength 415nm at the cathodic end of the capillary. Before each run, the capillaries is washed with a wash solution for preparation of the next analysis. Then, the electrophoregram is evaluated visually for pattern abnormalities (Sebia, 2013). The migration position for each haemoglobin is standardised relative to the position of HbA and HbA_2 bands and was measured between 0 to 300 in arbitrary unit (x value). Distinct peaks in the migration images were assigned to 15 zones and quantified as a percentage. HbA, HbA_2, HbF and HbC are provisionally identified and colour coded (Borbely *et al.*, 2013). The separation process of normal haemoglobin and haemoglobin variant in CE instrument are mostly similar to those separation process for cellulose acetate electrophoresis except using the higher voltage and marginally different pH (Wild and Bain, 2017).

This CE method provides accurate and precise detection of haemoglobin fraction including detection and recognition of haemoglobin variants such as separation of HbE from HbA_2 or HbC and HbS from HbD-Punjab. Currently, the CE system has be utilised for detection of various haemoglobinopathy, because of the capability of separation common and clinically significant haemoglobin variant from normal haemoglobin, such as HbE, HbS, HbD-Punjab, Hb Constant Spring (Hb CS), HbC, HbH and HbBart's (Borbely *et al.*, 2013). The high prevalence of HbE in this region