UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF REVERSE DOT BLOT HYBRIDISATION STRIP ASSAYS TO IDENTIFY COMMON BETA-THALASSAEMIA ALLELES IN MALAYS AND CHINESE IN MALAYSIA

TEH LAI KUAN

FPSK(M) 2010 6

DEVELOPMENT OF REVERSE DOT BLOT HYBRIDISATION STRIP ASSAYS TO IDENTIFY COMMON BETA-THALASSAEMIA ALLELES IN MALAYS AND CHINESE IN MALAYSIA

By

TEH LAI KUAN

© COPYRIGHT UPM

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

May 2010

Abstract of thesis presented to the Senate of University Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

DEVELOPMENT OF REVERSE DOT BLOT HYBRIDISATION STRIP ASSAYS TO IDENTIFY COMMON BETA-THALASSAEMIA ALLELES IN MALAYS AND CHINESE IN MALAYSIA

By

TEH LAI KUAN

May 2010

Chairman: Professor Dr. Elizabeth George

Faculty: Medicine and Health Sciences

Beta-thalassemia is the most common autosomal genetic disorder in Malaysia particularly among Malays and Chinese-Malaysians. The heterozygous carrier frequency of β-thalassaemia in Malaysia is estimated to be 4.5% from micro-mapping studies. The spectrum of β-thalassaemia mutations differs in each ethnic group in Malaysia. There are four to five common mutations responsible for more than 95% of the mutations seen in each ethnic group respectively.

The current diagnostic method in Malaysia, amplification refractory mutation system (ARMS-PCR), is only able to identify one mutation in each reaction. It is found labour intensive and time consuming when few mutations need to be identified. Therefore, there is a need to have an effective and accurate laboratory method that can identify common mutations simultaneously in each ethnic group.

© COPYRIGHT UPM

In this study, the reverse dot blot hybridization (RDBH) technique was used in development of strip assays for characterisation of the β-thalassaemia mutations. Two strip assays were designed specifically for Malays and Chinese-Malaysians respectively with each strip to identify six common mutations simultaneously. The mutations identified with the strip assays were validated with the gold standard method, ARMS-PCR.

A total of 177 patients (354 alleles) from University Malaya Medical Centre (UMMC) and the Institute of Medical Research (IMR) in Malaysia were studied. One hundred and thirty seven were Malays (274 alleles) and 40 were Chinese-Malaysians (80 alleles) respectively. One hundred and nineteen (86.9%) Malay patients consisting of 238 alleles were identified by the RDBH-Strip M(6). In the Malays, the most common β-thalassaemia mutations identified was CD 26, followed by IVS I-5, IVS I-1, CD 19 and the least with CD 8/9. In view of possible inter-marriage with Chinese, the RDBH-Strip C(6) was used to identify the 18 unidentified alleles in the Malays. The mutations identified were common Chinese mutations, CD 41/42 (5 heterozygous), CD 17 (2 heterozygous), -29 (2 heterozygous) and CD 71/72 (1 homozygous). Thus, a total of 129 (94.6%) Malay patients consisting of 258 alleles were identified using the RDBH-Strip Assays [RDBH-Strip M(6) and RDBH-Strip C(6)] . In the Chinese-Malaysians by the RDBH-Strip C(6), mutations were identified in 32 (80%) patients consisting of 64 alleles. IVS II-654 and CD 41/42 were the two most common β-thalassaemia mutations amongst Chinese-Malaysians, followed by CD17 and -28. In the Chinese-Malaysians, RDBH-Strip M(6) identified CD 26 (3 heterozygous) and IVS I-5(1 heterozygous). Thus, a total of 36 (90.0%) Chinese-Malaysians patients consisting of 72 alleles

© COPYRIGHT UPM

were identified using the RDBH-Strip Assays [RDBH-Strip C(6) and RDBH-Strip M(6)] . The RDBH-Strip Assays developed in this project study identified 93.2% of the mutations seen in the Malays and Chinese-Malaysians. There were remaining 11 heterozygous beta-thalassaemia carriers (eight Malays and four Chinese) whose mutations could not be identified. These unknown mutations require DNA sequencing for ultimate diagnosis.

ARMS-PCR was used to confirm and validate the presence of the six mutations used in the RDBH-Strip Assays. It amplified each mutation as a separate and distinct PCR product. The Strip Assays showed 100% sensitivity and specificity through validation by ARMS-PCR. Therefore, the Strip Assay can be defined as a reliable diagnostic tool for accurate beta-thalassaemia mutation identification in Malays and Chinese Malaysians.

In conclusion, the developed RDBH- Strip Assays [M(6) and C(6)] are accurate and rapid diagnostic tools for the identification of beta-thalassaemia mutations in the Malays and Chinese-Malaysians.

© COPYRIGHT UPM

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

PENCIPTAAN TES STRIP "REVERSE DOT BLOT HYRIDISATION" BAGI MENGENAL PASTI MUTASI YANG BIASA DIJUMPAI DALAM BETA-TALASEMIA BAGI ORANG MELAYU DAN CINA DI MALAYSIA

Oleh

TEH LAI KUAN

Mei 2010

Pengerusi: Profesor Dr. Elizabeth George

Fakulti: Perubatan dan Sains Kesihatan

Beta-Talasemia adalah penyakit gangguan genetik autosom yang paling umum di Malaysia khususnya di kalangan Melayu dan Cina. Frekuensi pembawa heterozigus β-Talasemia di Malaysia adalah dianggarkan sebanyak 4.5% melalui kajian mikro-pemetaan. Spektrum mutasi β-Talasemia adalah berbeza bagi setiap kumpulan etnik di Malaysia. Terdapat empat atau lima mutasi biasa yang bertanggungjawab lebih daripada 95% mutasi-mutasi bagi setiap kumpulan etnik.

Kaedah diagnostik di Malaysia sekarang, "amplification refractory mutation system" (ARMS-PCR), hanya berupaya mengenalpasti satu mutasi dalam setiap tindak balas. Ini didapati intensif buruh dan makan masa apabila beberapa mutasi hendak dikenalpasti. Oleh itu, satu kaedah makmal yang berkesan dan tepat adalah

© COPYRIGHT UPM

diperlukan untuk mengenalpasti mutasi-mutasi biasa secara serentak dalam kumpulan etnik masing-masing.

Dalam kajian ini, teknik "reverse dot blot hybridization" (RDBH) telah digunakan dalam pembangunan jalur cerakin dalam pencirian mutasi-mutasi bagi β-talasemia. Dua ujian jalur direka khususnya untuk orang Melayu dan orang Cina masing-masing. Setiap jalur dapat mengenal pasti enam mutasi yang biasa secara serentak dalam kumpulan etnik masing-masing. Mutasi-mutasi yang dikenalpasti dengan ujian jalur ini disahkan dengan kaedah piawai emas, ARMS-PCR.

Sejumlah 177 orang pesakit (354 alel) daripada University Malaya Medical Centre (UMMC) dan Institut Penyelidikan Perubatan (IMR) di Malaysia telah dipelajari. Ia terdiri daripada seratus tiga puluh tujuh orang Melayu (274 alel) dan 40 orang Cina (80 alel). Seratus sembilan belas (86.9%) pesakit Melayu yang mengandungi 238 alel dapat dikenalpasti dengan RDBH-Strip M(6). Bagi orang Melayu, mutasi β-talasemia yang paling lazim dikenalpasti ialah CD 26, diikuti dengan IVS I-5, IVS I-1, CD 19 dan yang paling sedikit ialah CD 8/9. Memandangkan kemungkinan kahwin campur dengan Cina, RDBH-Strip C(6) digunakan untuk mengenal pasti 18 pesakit Melayu yang mutasinya tidak dapat dikenalpasti. Mutasi-mutasi yang dikenalpasti ialah mutasi yang paling biasa dijumpai bagi orang Cina iaitu CD 41/42 (5 heterozigot), CD 17 (2 heterozigot), -29 (2 heterozigot) dan CD 71/72 (1 homozigot). Dengan itu, sejumlah 129 (94.6%) pesakit Melayu yang terdiri daripada 258 alel dapat dikenal pasti dengan menggunakan RDBH-Strip Assays [RDBH-Strip M(6) dan RDBH-Strip C(6)] . Dengan menggunakan RDBH-Strip C(6) bagi orang Cina, mutasi-mutasi bagi 32 (80%) pesakit yang terdiri daripada 64 alel dapat

© COPYRIGHT UPM

dikenal pasti. IVS II-654 dan CD 41/42 merupakan dua mutasi yang paling biasa dijumpai bagi orang Cina, diikuti dengan CD17 dan -28. Bagi orang Cina, RDBH-Strip M(6) mengenalpasti CD 26 (3 heterozigot) dan IVS I-5(1 heterozigot). Oleh itu, sejumlah 36 (90.0%) pesakit Cina yang mengandungi 72 alel dapat dikenalpasti dengan menggunakan RDBH-Strip Assays [RDBH-Strip C(6) dan RDBH-Strip M(6)]. RDBH-Strip Assays dibangunkan dalam kajian projek ini dapat mengenalpasti 93.2% mutasi-mutasi yang dijumpai dalam orang Melayu dan orang Cina. Mutasi-mutasi yang tidak diketahui memerlukan "DNA sequencing" sebagai diagnosis muktamad.

ARMS-PCR digunakan untuk mengesahkan enam mutasi seperti dalam RDBH-Strip Assays. Ia mengamplifikasikan mutasi masing-masing dengan produk PCR yang berbeza. Strip Assays menunjukkan 100% kepekaan dan ketentuan melalui pengesahan dengan menggunakan kaedah ARMS-PCR. Oleh itu, Strip Assays dapat ditakrifkan sebagai satu alat diagnostik yang boleh dipercayai dalam pengenalpastian mutasi beta-talasemia dengan tepat bagi orang Melayu dan Cina di Malaysia.

Sebagai kesimpulan, RDBH-Strip Assays [M(6) dan C(6)] adalah peralatan diagnostik yang tepat dan pesat dalam pengenalpastian mutasi-mutasi beta talasemia bagi orang Melayu dan orang Cina.

© COPYRIGHT UPM

ACKNOWLEDGEMENTS

First of all, I am truly indebted to Prof. Dr. Elizabeth George, my supervisor, for her consistent coaching, guidance, experience-sharing and funding. Her thoughtfulness towards the educational welfare of her students has inspired me tremendously. Moreover, her patience and countless contribution in finishing this project were greatly appreciated.

Secondly, my heartfelt thanks to my co-supervisor, Dr. Lai Mei I, for her assistance, her expertise and suggestions to improve in this research. Many thanks to Prof. Dr. Mary Anne Tan from UMMC, Dr Zubaidah Zakaria from IMR, Dr. Law Hai Yang from K.K. Women's and Children's Hospital, Singapore, for their generosity in providing me the patients' samples including positive control samples that ensured the research went smoothly. Their generosity will not be forgotten. Next, I would like to acknowledge the supporting staffs, Mr. Quek, Mr. Fahmi, Mrs. Amrina and others in Haematology Department, Faculty of Medicine and Health Sciences for assisting me a lot throughout this project.

My utmost gratitude to my colleague, Wai Feng, and also my seniors, Leslie, Choo and others for their knowledge and assistance which has been very helpful in completing this research. Not to be forgetten, I deeply acknowledged a bunch of my best friends, Wan-Fay, Heng Yaw, Yvonne, Hui Ceng and others for all your kind understandings, encouragements and assistance throughout my study. We had shared so many good and bad times; being there to support, comfort and cheer each other at times we were really stresses up throughout the study.

© COPYRIGHT UPM

And last but not least, there is no words can be expressing my deepest gratitude to my beloved parents and family members, because of you I am here today. Your endless supports, contributions and sacrifices would never be forgotten.

© COPYRIGHT UPM

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Elizabeth George, MD, MBBS, FRCPA, FRCPE.
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Lai Mei I, PhD
Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

_____

HASANAH MOHD GHAZALI, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 15 July 2010

© COPYRIGHT UPM

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

_____

TEH LAI KUAN

Date: 7 June 2010

© COPYRIGHT UPM

TABLE OF CONTENTS

© COPYRIGHT UPM

© COPYRIGHT UPM

© COPYRIGHT UPM

© COPYRIGHT UPM

LIST OF TABLES

© COPYRIGHT UPM

© COPYRIGHT UPM

© COPYRIGHT UPM

LIST OF FIGURES

© COPYRIGHT UPM

© COPYRIGHT UPM

© COPYRIGHT UPM

LIST OF ABBREVIATIONS

α	alpha
β	beta
δ	delta
ε	epsilon
γ	gamma
ζ	zeta
ψ	pseudo
μ	micro
μl	microlitre
μg	microgram
ml	mililitre
bp	base pair
kb	kilobase
DNA	deoxyribonucleic acid
TAE	tris-acetate- ethylenediaminetetraacetic acid

© COPYRIGHT UPM

CHAPTER 1

INTRODUCTION

1.1 HAEMOGLOBINOPATHIES

Haemoglobinopathies are inherited haematological disorders due to alteration in the globin gene expression. These abnormalities are characterised by both quantitative and qualitative defects in haemoglobin (Hb) synthesis (Daniel, 2004).

1.2 HAEMOGLOBIN SYNTHESIS

Human haemoglobin molecule carries and transports oxygen to all parts of the body. Haemoglobin binds oxygen at the iron porphyrin site due to its allosteric effect on oxygen binding. It is made up of two parts–heme and globin. Heme is a porphyrin containing iron. Globin has tetrameric structure of two globin chain pairs; two α- and two non α-chains. Alpha chain is encoded by two closely related genes, α_1 and α_2, on chromosome 16. The non-alpha chains-beta (β), gamma (γ) and delta (δ), are encoded by a cluster of genes on chromosome 11 (Figure 1.1) (Hartwell *et al.*, 2005; Shah, 2004; Bowden, 2001; Kulozik, 1992).

© COPYRIGHT UPM

Different globin chains are produced during embryonic, foetal, and postnatal/adult phases. Embryonic haemoglobins, Gower 1 ($\zeta_2\varepsilon_2$), Gower 2 ($\alpha_2\varepsilon_2$) and Portland ($\zeta_2\gamma_2$), are tetramers of α-like ζ-chains and β-like ε- or γ-chains. It is synthesised about 3 to 8 weeks of development. Foetal has a high amount of HbF ($\alpha_2\gamma_2$). A newborn has about 80% of HbF at the age of about one year old. Following that, the production of HbA ($\alpha_2\beta_2$) (96-98%) becomes dominant while HbA_2 ($\alpha_2\delta_2$) and HbF are only present in 2-3% and less than 1% respectively. The switch from HbF to HbA is due to the genetic control of γ-, α- and β-chains. After birth, the production of γ-chains slows down and β-chains increases correspondingly (Hartwell *et al.*, 2005; Shah, 2004; Bowden, 2001; Weatherall, 1999; Kulozik, 1992).

1.2.1 CLASSIFICATION OF HAEMOGLOBINOPATHIES

Haemoglobinopathies can be sub-divided into 3 major categories: structural haemoglobin variants, thalassaemias and hereditary persistence of foetal haemoglobin (HPFH) (Table 1.1) (Daniel, 2004).

Table 1.1: Subdivisions of haemoglobinopathies (*adapted* from Daniel, 2004)

Haemoglobinopathy	Type of defect	Causative factor	Example
Structural haemoglobin variants	Qualitative defect	• Synthesis of an abnormal globin chain synthesis • Substitution of single/ or more amino acid in globin chains	Sickle cell disease (Glu→Val)
Thalassaemias	Quantitative defect	• Reduced/imbalanced or absent synthesis of a normal globin chain	β-thalassaemia; α-thalassaemia
Hereditary persistence of foetal haemoglobin (HPFH)	Qualitative defect	• Genetic defects in the switch from foetal to adult Hb • Foetal haemoglobin remains high throughout life. • Benign condition	

© COPYRIGHT UPM

1.3 THALASSAEMIAS

Thalassaemia is the most common single gene disorder known. It is a heterogeneous group of genetic disorders which results from mutations that cause a diminished rate or total absence of synthesis of one or more globin chains. The imbalanced ratio of α- to non α-chains leads to a reduced synthesis of haemoglobin. Thalassaemias are classified either by the clinical manifestations or particular globin chain that is synthesized at a reduced rate; α-thalassaemia indicates a reduced rate of α-globin chain synthesis; similarly, β, δ, δβ and γδβ-thalassaemias indicate a reduced synthesis of the β, δ, δ+β and γ+δ+β chains, respectively (Daniel, 2004; Hoffbrand *et al.*, 2003; Weatherall, 1999).

1.4 ALPHA THALASSAEMIAS

Alpha thalassaemias result from a defect in α-globin chains synthesis either reduced or absence in production of α-globin chains. It is primarily caused by gene deletions or point mutations and is classified as α-thal 1 (--/αα) or α^{o}-thalassaemia and α-thal 2 (-α/αα) or α^{+}-thalassaemia. This is related to the number of genes which are non-functional and to the amount of α-chains produced (Daniel, 2004; Hoffbrand *et al.*, 2003; Weatherall, 1999).

1.5 BETA THALASSAEMIAS

Beta thalassaemias result from a defect in the β-globin chain synthesis either reduced or absence in production of β-globin chains. It is considered as one of the most

© COPYRIGHT UPM

common autosomal single-gene disorders worldwide and constitutes a major health problem (Bain, 2006; Hoffbrand *et al.,* 2003; Weatherall *et al,* 2001). Severe anaemia may be due to deficiency of normal HbA ($\alpha_2\beta_2$). The excess α-globin chains precipitates cause red blood cell membrane damage and early destruction of red blood cells (Suthat *et al.,* 1985).

1.6 BETA GLOBIN GENE CLUSTER

Beta globin and beta-like gene is a linked cluster on chromosome 11, which spread over approximately 60 kb. They are arranged in the order 5'-ε-^{G}γ-^{A}γ-ψβ-δ-β-3'. The ψβ gene is pseudogene. It has sequences that resemble the β-gene but contains mutations that prevent synthesis of any products. Beta-globin gene contains three exons with two introns (intervening sequences; IVS), between codons 30, 31 and 104, 105, respectively (Figure 1.1). Locus control region (LCR) is found at 20kb region upstream from the ε-globin gene. It contains several types of regulatory elements that promote erythroid specific gene expression and co-ordinate changes in globin gene activity during development (Weatherall, 1999).

© COPYRIGHT UPM

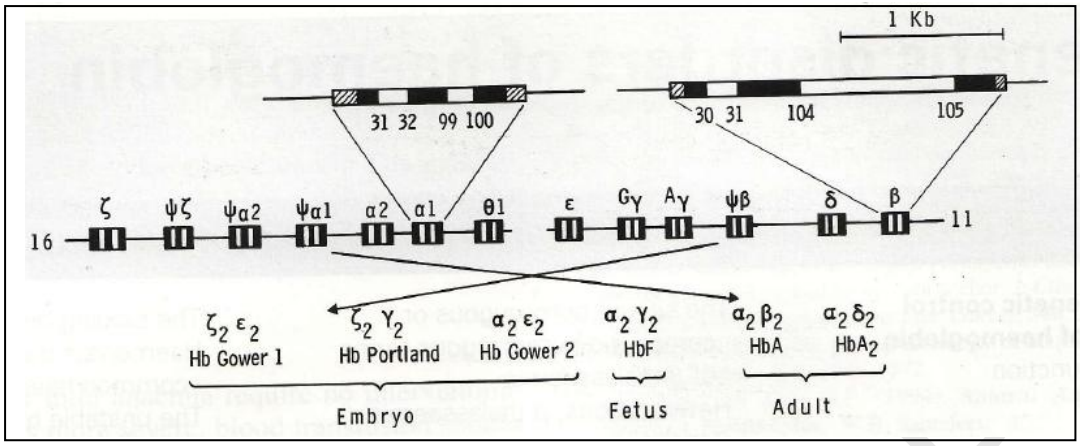

Figure 1.1: The genetic control of haemoglobin: Structural organization of the globin gene clusters (from Weatherall, 1999).

1.6.1 MOLECULAR BASIS OF BETA THALASSAEMIA

Beta thalassaemia represents a great heterogeneity as more than 200 different types of mutations has been identified, occurring in a wide range of ethnic groups on the β-globin gene in molecular level (Bain, 2006). However, each geographic population has its own unique and common mutations. These mutations may occur within the gene complex itself, promoter/enhancer regions, exons, introns or the exon-intron boundary of the β-globin gene in transcription. It causes abnormal RNA splicing, RNA modification and translational defects (Hoffbrand *et al.*, 2003; Suthat *et al.*, 1985). Majority of the genetic lesions are point mutations and a small proportion are frameshift or deletions at β-gene itself or controlling sequences 5' to the gene (upstream from the gene); abbreviated as β-LCR (Bain, 2006).

Beta thalassaemia mutations are divided into two broad categories**:** β^0-thalassaemia and β^+-thalassaemia (Weatherall and Clegg, 2001). In β^0-thalassaemia, there is either an abnormal gene or, less often, gene deletion and causes absence of HbA formation.

© COPYRIGHT UPM

In β^+-thalassaemia, there are some production of HbA by the abnormal gene. The extent of impairment of HbA production is one of the determinants of clinical severity in β-thalassaeamia; β^0-thalassaemia often has greater clinical impact than β^+-thalassaemia (Weatherall and Clegg, 2001).

1.6.2 CLINICAL MANIFESTATIONS IN BETA THALASSAEMIA

Beta thalassaemia can be divided into three general categories: β-thalassaemia trait, intermediate and major (Table 1.2) (Daniel, 2004; Hoffbrand *et al.*, 2003; Weatherall, 1999).

Beta thalassaemia trait or β-thalassaemia minor is characterised by heterozygosity of one deleted or mutated gene and one normal functioning gene. Individuals are usually asymptomatic (Table 1.2) (Daniel, 2004; Hoffbrand *et al.*, 2003; Weatherall, 1999).

Beta thalassaemia intermediate is referred to a clinical phenotype with diverse genetic explanations but not to the degree of chronic transfusion therapy. Individuals will have a homozygous or heterozygous β-globin mutation that causes a decrease in β-globin chain production. There are significant clinical problems in comparison with a typical patient with β-thalassaemia trait (Table 1.2) (Bain, 2006; Daniel, 2004; Hoffbrand *et al.*, 2003; Weatherall, 1999).

Beta thalassaemia major or Cooley's anaemia refers to patients with homozygosity or compound heterozygosity for β-thalassaemia. There is absence of β-globin chain

© COPYRIGHT UPM

and HbA production (Weatherall and Clegg, 2001). Affected individuals are transfusion dependent for life. The only possible cure is the transplantation of compatible stem cell from a human leucocytes antigen (HLA) matching donor to the patient (Hoffbrand *et al.*, 2003).

Table 1.2: Categorization of beta thalassaemia (*adapted* from Daniel, 2004; Hoffbrand *et al.*, 2003; Weatherall and Clegg, 2001; Weatherall, 1999)

Phenotype	β (%)	α (%)	Mutations	Clinical Manifestations
minor/ trait (β^+/β) (β^0/β)	>50	100	Only one β globin allele bears a mutation	• Asymptomatic • Mild microcytic anaemia
intermediate (β^+/β^+) (β^0/β^+)	10-50	100	One or both β globin allele bears a mutation	• Intermediate between the major and minor forms • May need occasional transfusions
major (β^0/β^0)	0	100	Both alleles bears mutations	• Severe microcytic, hypochromic anaemia • Transfusion dependent for life

1.7 BETA THALASSAEMIA IN MALAYSIA

Beta thalassaemia has emerged as one of the most common genetic disorders of haemoglobin synthesis. It is a public health problem in Malaysia, particularly among Malays and Chinese-Malaysians (Rozitah *et al.*, 2008; Tan *et al.*, 2004; George E., 2001). Previous studies reported about 4.5% of the population are heterozygous carriers for beta thalassaemia (Tan *et al.*, 2004; George, 2001). However, the mutations that caused beta thalassaemia are ethnicity dependent. Each ethnic group comprises of common and some rare mutations (George, 2001). A study by Tan *et al.* (2004) showed that CD41/42, IVSII-654, -28 and CD 17 comprise 92% of β-thalassaemia mutations in the Chinese population while CD 19, IVSI-5, poly A and

© COPYRIGHT UPM

Hb E caused 76% of β-thalassaemia in the Malay population. The Filipino 54-kb deletion with mutations at CD 41/42 encompasses 87% of β-thalassaemia in the indigenous group namely Kadazan, Bidayuh and Dusun people (Tan *et al.,* 2004).

The World Health Organization has highlighted the importance of ways for community control of β-thalassaemia (Weatherall and Clegg, 2001). Therefore, the accuracy in newborn screening, prenatal diagnosis and mutation diagnosis is essential to manipulate the incidence of β-thalassaemias.

In Malaysia, screening tests (full blood count, full blood picture, haemoglobin analysis and haemoglobin electrophoresis) are done for presumptive identification of β-thalassaemia but DNA studies are necessary for definitive diagnosis in thalassaemia (George, 2001). It is essential to implement a sensitive and reliable diagnostic method in the detection and diagnosis of β-thalassaemia disorders.

1.8 BETA THALASSAEMIA DIAGNOSIS

Currently, strategies in thalassaemia definitive diagnosis are mainly DNA-based diagnostic techniques using PCR-based protocols. In Greece, Kanavakis *et al.* (1997) characterized β-thalassaemia mutations with denaturing gradient gel electrophoresis (DGGE) and amplification refractory mutation system (ARMS-PCR) as primary and principal method while restriction endonuclease (RE) analysis of PCR fragments, oligonucloetide hybridization and "gap" PCR as secondary techniques for thalassaemia diagnosis (Kanavakis *et al.,* 1997).

© COPYRIGHT UPM

According to Kanavakis *et al., (*1997), the DGGE method was a technically challenging method. It requires sophisticated equipment, experience in handling and interpretation. It is subject to many causes for methodological failure including chemical components. Therefore, DGGE pattern alone can never be considered as definitive diagnosis (Kanavakis *et al.*, 1997).

Polymerase chain reaction (PCR) based methods, ARMS-PCR and gap-PCR, are the methods used for thalassaemia diagnosis in Malaysia (Tan *et al.*, 2004). These molecular techniques are direct mutation detection and rapid in producing results within a few hours of PCR-set-up (Kanavakisi *et al.*, 1994). However, each PCR can address only single known mutation or two/three mutations in a single reaction and is time consuming to screen for different polymorphisms (Tan *et al.*, 2001; Tan *et al.*, 2004). Thalassaemia molecular diagnosis is challenged by existence of a great number of different β-thalassaemia mutations even within a defined ethnic group (Sutcharitchan *et al.,* 1995*).* Moreover, it is found difficult in differentiation between heterozygous and homozygous genotypes for single pathological mutations (Kanavakis *et al.*, 1997). Two reactions need to be carried out in order to check for heterozygous or homozygous condition of patients. These techniques are labour-intensive, costly and time consuming (Sutcharitchan *et al.*, 1995; Hossein *et al.*, 2001).

Restriction enzymes (RE) analysis is also widely used in Malaysia as shown by Rozitah *et al.* (2008). It is a direct mutation analysis but limited to specific mutations only. Analyses by both ARMS-PCR and RE methods require electrophoresis of

© COPYRIGHT UPM

ethidium bromide stained agarose gel which is carcinogenic to the body (Kanavakis *et al.,* 1997).

Reverse dot-blot hybridization (RDBH), a PCR-based technique has emerged as a beneficial tool to scientists in the globin field, who have been engaged in the diagnosis of thalassaemia. This technology combined with a hybridization technique of specific oligonucleotide probes and primers can detect mutations in an individual. It offers the possibility of screening several mutations with a single hybridization reaction which is less labourious when compared to the PCR-based method of ARMS (Hossein *et al.,* 2001; Lappin *et al.,* 2001; Saiki *et al.,* 1989; Sutcharitchan *et al.,* 1995; Tuzmen and Schechter, 2001).

RDBH technique is convenient and easy as well. It enables the process of differentiation the heterozygous or homozygous condition in a single hybridization reaction. The result with appearance of blue dots is easy to interpret without any highly-skilled technician. RDBH allows accurate distinction of mutant alleles and reduces false-negative results. It showed higher reliability compared to ARMS-PCR (Hossein *et al.*, 2001).

© COPYRIGHT UPM

1.9 SIGNIFICANCE OF STUDY

There have not been studies done in Malaysia to develop a strip assay utilising RDBH technique to identify common β-thalassaemia mutations in the various ethnic groups of Malaysia.

The developed strip assay will contain probes for detection of the most common β-thalassaemia mutations in Malays and Chinese-Malaysians in Malaysia. This diagnostic approach has potential for routine clinical, neonatal and prenatal diagnosis for both local and regional use in the identification of β-thalassaemia mutations. The development of a strip assay to identify β-thalassaemia in different ethnic groups in Malaysia will help to improve the efficiency of our health care system with minimal costs.

© COPYRIGHT UPM

1.10 GENERAL OBJECTIVE

The general objective of this study is:

- To develop a molecular tool to identify common beta thalassaemia mutations in Malays and Chinese-Malaysians in Malaysia.

1.11 SPECIFIC OBJECTIVES

This study aims to satisfy the following specific objectives:

- To develop a reverse dot blot hybridisation (RDBH) strip assay to identify common beta-thalassaemia alleles in Malays and Chinese-Malaysians in a single hybridization reaction.
- To compare the RDBH-strip assays with the amplification refractory mutations system (ARMS) in current use in the identification of the beta thalassaemia alleles.
- To validate the mutations identified in RDBH-strip assays using the ARMS- polymerase chain reactions.
- To compare the RDBH-strip assays with the commercial beta globin strip assay from Viennalab in the identification of the beta thalassaemia alleles.

© COPYRIGHT UPM

REFERENCES

Ahmed, S., Petrou, M. and Saleem, M., (1996) Molecular genetics of β-thalassaemia in PakistanL a basis for prenatal diagnosis. In *British journal of Haematology* 94: 476-482.

Amselem, S., Nunes, V., Estivill, X., Wong, C., d'Auriol, L., Vidaud, D., Galibert, F., Baiget, M., and Goossens, M., (1988). Determination of the spectrum of β-thalassaemia genes in Spain by use of Dot-Blot analysis of amplified β-globin DNA. *Am. J.Hum.Genet.* 43: 95-100.

Altman, D.G. and Bland, J.M. (1994). Diagnostic test I: Sensitivity and specificity. In *BMJ*, 308: 1552.

Ausebel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Sediman, J.G., Smith, J.A., Struhl, K. (1992). Current protocols in molecular biology. *New York : Greene publishing Association, Wiley Interscience.* 1.:11-17.

Background Note*: Malaysia*. People History Government Political Conditions Economy Foreign Relations U.S. Retrieved 8th December 2009 from http://www.state.gov/r/pa/ei/bgn/2777.htm

Baig, S.M., Rabbi, F., Hameed, U., Qureshi, J.A., Mahmood, Z., Bokhari, S.H., Kiani, A., Hassan, H., Baig, J.M., Azhar, A. and Zaman, T., (2005) Molecular characterization of mutations causing β-thalassemia in Faisalabad Pakistan using the amplification refractory mutation system (ARMS-PCR). In *Indian Journal of human genetics* 11:80-83.

Bain, B.J., (2006). The α, β, δ and γ thalassaemias and related condition. In *Haemoglobinopathy diagnosis*, vol. 2: pp 63-138. London: Blackwell publishing Ltd.

Bain, B.J., (2006). Haemoglobins and their structure and function. In *Haemoglobinopathy diagnosis.* pp 1-19. London: Blackwell publishing Ltd.

Bain, B.J., (2006). Organization of a haemoglobinopathy service. In *Haemoglobinopathy diagnosis.* pp 204- 215. London: Blackwell publishing Ltd.

Battistella, S., Damin, F., Chiari, M., Delgrosso, K., Surrey, S., Fortina, P., Ferrari,M., and Cremonesi, L. (2008). Genotyping β-Globin Gene Mutations on Copolymer-Coated Glass Slides with the Ligation Detection Reaction. *Clinical Chemistry*. 54(10): 1657–1663.

Bhardwaj, U., Zhang, Y., Lorey, F., McCabe Linda, L., and McCabe Edward, R.B., (2005). Molecular genetic confirmatory testing from newborn screening samples

© COPYRIGHT UPM

for the common African-American, Asian Indian, Southeast Asian, and Chinese β-thalassaemia mutations. *American Journal of Haematology*.78: 249-255.

Bowden, D.K., (2001) Abnormal laboratory results: Screening for thalassaemia. *Austratlian Prescriber* 24 (5): 120- 123.

Bridges, K.R. and Pearson, H.A. (2007) Thalassemia. In *Anemias and other red cell disorders,* pp. 278- 301. MacGraw-Hill Companies, Inc.

Cai, S.P., Chang, C.A., Zhang, J.Z., Saiki, R.K., Erlich, H.A. and Kan, Y.W., (1989). Rapid prenatal diagnosis of β-thalassaemia using DNA amplification and nonradioactive probes. *Blood* 73.(2): 372-374.

Centre for genetics education, 2007. Retrieved 8th September 2009 from *http://www.genetics.com.au/factsheet/fs34.html.*

Chang, J.G.,Chen, P.H., Chiou, S.S., Lee, L.S., Perng, L.I., and Liu, T.C., (1992). Rapid Diagnosis of P-Thalassemia Mutations in Chinese by Naturally and Amplified Created Restriction Sites. *Blood*. 80(8): 2092-2096.

Cheng, J., Zhang, Y. and Li, Q., (2004). Real-time PCR genotyping using displacing probes. *Nucleic acids research,* 32(7): 1-10.

Colah, R., Gorakshakar, A., Pahasgaonkar, S., Surve, R., Sawant, P, Mohant, D. and Ghosh, K., (2009). Regional heterogeneity of β-thalassaemia mutations in the multi ethnic Indian population. *Elseveir, blood cells, molecules and diseases,* 42: 241-246.

Colah, R., Nadkarni, A., Gorakshakar, A., Phanasgaonkar, S., Surve, R., Subramaniam, P.G., Bondge,N., Pujari, K., Ghosh ,K. and Mohanty, D., (2004). Impact of β globin gene mutations on the clinical phenotype of β-thalassemia in India., *Blood cells, Molecules and Diseases, Elsevier* 33: 153-157.

Colah, R.B., Surve, R., Sawant, P., D'Souza, E. , Italia, K., Phanasgaonkar, S. , Nadkarni, A.H. and Gorakshakar, A.C. (2007) HPLC Studies in Hemoglobinopathies. *Indian Journal of Pediatrics,* 74: 61-66.

Colosimo, A., Novelli, G., Cavicchini, A., Dallapiccola, B., (1996). Detection of eight thalassemia mutations using a DNA enzyme immunoassay.*lnt J Clin Lab Res*. 26: 136-139.

Daniel, Y. (2004).Haemoglobinopathy diagnostic tests: blood counts, sickle solubility test, haemoglobin electrophoresis and high-performance liquid chromatography. In *Practical Management of haemoglobinopathies,* ed Okpala, I., pp10-19. Blackwell publishing.

Dean, F.B., Seiyu, H., Fang, L., Wu, x., Faruqi, F., Bray-Ward, P., Sun, Z., Zong, Q., Du, Y., Du., J., Driscoll, M., Song, W., Kingsmore, S.F., Egholm, M., and Lasken, R.S., (2002). Comprehensive human genome amplification using multiple displacement amplification. *PNAS* 99(8): 5261-5266.

© COPYRIGHT UPM

Dongzhi, L. , Can, L., Jian, L., Xingmei, X., Yining, H., Huizhu, Z. and Jiaxue, W., (2006). Prenatal diagnosis of β-thalassemia in Southern China. *European Journal of Obstetrics & Gynecology and Reproductive Biology* 128: 81–85.

Fakher, R., Bijan, K. and Taghi, A.M., (2007). Application of diagnostic methods and molecular diagnosis of haemoglobin disorders in Khuzestan province of Iran. *Indian journal of human genetics* 13(1): 5-15.

Feriotto, G., Breveglieri, G., Finotti, A., Gardenghi, S. and Gambari, R., (2004). Real-time multiplex analysis of four beta-thalassaemia mutations employing surface plasmon resonance and biosensor technology. *Laboratory investigation, USCAP*, 84: 796-803.

Foglieni, B., Cremonesi, L., Travi, M., Ravani, A., Giambona, A., Rosatelli, M.C., Perra, C., Fortina, P. and Ferrari, M., (2004). β-thalassemia microelectronic chip: A fast and accurate method for mutation detection. *Clinical Chemistr, molecular diagnostics and genetics* 50(1): 73-79.

Fortina, P., Dotti, G., Conant, R., Monokian, G., Parrella, T., Hitchcock,W., Rappaport, E., Schwartz, E. and Surrey, S., (1992).Detection of the most common mutations causing β-thalassemia in Mediterraneans using a Multiplex Amplification Refractory Mutation System (MARMS). *Laboratory Press.*2: 163-166.

Fucharoen, S., Winichagoon, P., Wisedpanichkij, R., Sae-Ngow, B., Sriphanich, R., Oncoung, W., Muangsapaya, W., Chowthaworn, J., Kanokpongsakdi, S., Bunyaratvej, A., Piankijagum, A. and Dewaele, C. (1998). Prenatal and postnatal diagnosis of thalassaemias and haemoglobinopathies by HPLC. *Clinical Chemistry.* 44(4): 740-748.

Galbiati, S., Foglieni, B., Travi, M., Curcio, C., Restagno, G., Sbaiz, L., Smid, M., Pasi, F., Ferrari, A., Ferrari, M. and Cremonesi, L., (2008). Peptide-nucleic acid-mediated enriched polymerase chain reaction as a key point for non-invasive prenatal diagnosis of β-thalassaemia. *Haematologica* 93(4): 610-614.

Galbiati, S., Chiari, M., Macellari, M., Ferrari, M., Cremonesi, L. and Cretich, M. (2007). High-throughput mutational screening for beta-thalassemia by single-nucleotide extension. *Electrophoresis, Wiley interscience.* 28: 4289-4294.

George, E. (1998). Thalassaemia carrier diagnosis in Malaysia. *In Thalassaemia Diagnostic Service (ThalDS).*

George, E. (2001). Beta thalassaemia major in Malaysia, an on-going public health problem. *Med. J. Malaysia* 56.(4)

George, E., George, R., Ariffin, W.A., Mokhtar, A.B., Azman, Z.A. and Sivagengei, K., (1993). Spectrum of beta-thalassaemia mutations in transfusion dependent thalassaemia patients: practical implications in prenatal diagnosis. *Med. J. Malaysia* 48(3): 325-329.

© COPYRIGHT UPM

George, E., Jamal, A.R., Khalid, F. and Osman, K.A. (2001). High performance liquid chromatography (HPLC) as a screening tool for classical beta-thalassaemia trait in Malaysia. *Malaysian Journal of Medical Science.* 8(2):40-46.

George, E., Khuzaiah, R. (1984) Malays with thalassaemia in West Malaysia. *J Trop Geog Med*. 36(123-125).

George, E., Li, H.J., Fei, Y.J., Reese, A.L., Baysal, E., Cepreganova, B., Wilson, J.B., Gu, L.H., Nechtman, J.F., Stoming, T.A., Liu, J.C., Codrington, J.F. and Huisman, T.H.J., (1992). Types of thalassameia among patients attending a large university clinic in Kuala Lumpur, Malaysia. *Hemoglobin* 16 (1 & 2): 51-66.

George, E., Yang, K.G., Huisman, T.H.J., (1990). Chinese in West Malaysia. The geography of beta-thalassaemia mutations. *Sing. Med. J.* 31: 374-377.

George-Kodiseri, E., Yang, K.G., Kutlar, F., Wilson, J.B., Kutlar, A., Stoming, T.A., Gonzales-Redondo, J.M. and Huisman T.H.J. (1990). Chinese in West Malaysia: The Geography of Beta thalassaemia mutations. *Singapore Med. J.* , 31: 374-377.

Giambona, A., Passarello, C., Renda, D. and Maggio, A. (2009). The significance of the hemoglobin A_2 value in screening for hemoglobinopathies. *Clin. Biochem, Elseveir,* 10:1016.

Glynou, K., Kastanis, P., Boukouvala, S., Tsaoussis, V., Ioannou, P.C., Christopoulos, T.K., Traeger-Synodinos, J. and Kanavakis, E., (2007). High-throughput microtiter well-based chemiluminometric genotyping of 15 *HBB* gene mutations in a dry reagent format. *Clinical Chemistry,* 53(3): 384-391.

Gonzalez-Redondo, J.M., Stoming, T.A., Lanclos, K.D., Gu, Y.C., Kutlar, A., Kutlar, F. and Nakatsuji, T. (1988). Clinical and Genetic Heterogeneity in Black Patients With Homozygous β-Thalassemia From the Southeastern United States. *Blood* 72: 1007-1014.

Grey, V., Wilkinson, M., Phelan, L., Hughes, C. and Bain, B.J. (2007). Inaccuracy of high-performance liquid chromatography estimation of haemoglobin F in the presence of increased haemoglobin A_{1c}. *International journal of laboratory haematology.*29:42-44.

Gupta, A., Sarwai, S., Pathak, N. and Agarwal, S., (2008). Beta-globin gene mutations in India and their linkage to β-haplotypes. *Int. J. Hum Genet,* 8 (1-2): 237-241.

Hafeez, M., Aslam, M., Ali,A., Rashid, Y. and Jafri, H., (2007) Regional and ethnic distribution of beta thalassaemia mutations and effect of consanguinity in patients referred for prenatal diagnosis. *JCPSP* 17(3): 144-147.

© COPYRIGHT UPM

Harteveld, C.L., Kleanthous, M., Traeger-Synodinos, J., (2009). Prenatal diagnosis of hemoglobin disorders: Present and future strategies. *Clinical biochemistry.* 4C:1-13.

Hartwell, S.K., Srisawang, B., Kongtawelert, P., Christian, D. and Grudpan, K., (2005). Review on screening and analysis techniques for haemoglobin variants and thalassaemia. *Science direct.* Talanta 65.:1149-1161.

Haemoglobin Variants Database of huma haemoglobin Variants and thalassaemia mutations. Retrieved 2008 from http://globin.bx.psu.edu/hbvar.

Henderson, S., Timbs, A., McCarthy, J., Gallienne, A., Mourik, M.V., Masters, G., May, A., Khalil, M.S.M., Schuh, A. and Old, J., (2009). Incidence of haemoglobinopathies in various populations- the impact of immigration. *Clin Biochem, Elsevier,* 1-12.

Hillman, R.S., Ault, K.A., and Rinder, H.M., (2005). Thalassaemia . Haemoglobinopathies. In *Haematology in clinical practice,* pp 65-94. McGraw-Hill.

Hoffbrand, A.V., Pettit, J.E. and Moss, P.A.H., (2003). Genetic disorders of haemoglobin. In *Essential haematology*. Vol. 4 ed. pp 71-90. Blackwell science.

Hoffbrand, V., Catovsky, D. and Tuddenham, E.G.D. (2005). Postgraduate haematology. In *haemoglobin and the inherited disorders of globin synthesis. 5th ed.Weatherall, D.J.,* pp *85-103*.Blackwell publishing Ltd.

Hoisington, D., Khairallah, M. and Gonzalez-de-Leon, D. (1994). Laboratory Protocols: CIMMYT Applied Biotechnology Center. 2nd Edition, Mexico, D.F.: CIMMYT.

Hossein, N., Shahram, T., Talayeh, K., Maryam Neishabury, N., Farzin, P., Sayeh, J.N., Maryam, A., Christian, O. and Walter, K., (2001). Amplification refractory mutation system (ARMS) and reverse hybridization in the detection of beta-thalassemia mutations. *Archives of Iranian Medicine*.165-170.

Hung, C.C., Su, Y.N., Lin, C.Y., Chang, Y.F., Chang, C.H., Cheng, W. F., Chen, C.A., Lee, C.N. and Lin W.L., (2008). Comparision of the mismatch-specific endonuclease method and denaturing high-performance liquid chromatography for the identification of HBB gene mutations. *Biomed Central* (*BMC) biotechnology,* 8: 62.

Izadyar, M., Dastan, J., Sabokbar, T., Shoraka, S., Shojaei, A., Nasiri, H. and Ghaffari, S.R. (2007). Investigation of RBC indices and HbA_2 levels in parents of beta-thalassaemia patients: Impacts on premarital genetic counseling. *Journal of family and reproductive health.*1(2):93-95.

Kanavakis, E., Traeger-Synodinos, J., Vrettou, C., Maragoudaki, E., Tzetis, M. and Kattamis, C., (1997). Prenatal diagnosis of the thalassaemia syndromes by rapid DNA analytical methods. *Molecular Human Reproduction,* 3(6): 523–528.

© COPYRIGHT UPM

Kramvis, A., Bukofzer, S. and Kew, M.C., (1996). Comparison of Hepatitis B Virus DNA extractions from serum by the QIAamp blood kit, GeneReleaser, and the Phenol-Chloroform method. *Journal of Clinical Microbiology.* 34(11):2731-2733.

Kulozik, A.E. (1992). β-thalassaemia: molecular pathogenesis and clinical variability. In *European Journal of pediatrics, 151:* 78-84.

Lappin, S., Cahlik, J. and Bert, G. (2001). Robot Printing of Reverse Dot Blot Arrays for Human Mutation Detection. *In Journal of Molecular Diagnostics, 3(4):*178-188.

Li, D., Liao, C., Li, J., Xie, X., Huang, Y., Zhong, H. and Wei, J., (2006). Prenatal diagnosis of β-thalassaemia in Southern China. *European Journal of Obstetrics & Gynecology and reproductive biology.* 128: 81-85.

Li, W., Gao, F., Tang W., Zhang, X. and Xhang H., (2006). Detection of known thalassaemia point mutations by snapback single-strand conformation polymorphism: the feasibility analysis. *J. Clin. Biochem., Elseveir,* 39: 833-842.

Li, Q., Li, L.Y., Huang, S.W., Li, L., Chen, X. W., Zhou, W.J. and Xu, X.M., (2008). Rapid genotyping of known mutations and polymorphisms in β-globin gene based on the DHPLC profile patterns of homoduplexes and heteroduplexes. *J. Clin. Biochem, Elsevier,* 41:681-687.

Lubin, B.H., Witkowska, H.E. and Kleman, K. (1991). Laboratory diagnosis of haemoglobinopathies. *In Clin. Biochem,* 24:363-374.

Loong, T.W. (2003). Understanding sensitivity and specificity with the right side of the brain. BMJ, 327: 716-719.

Maggio, A., Giambona, A., Cai, S.P., Wall, J., Kan, Y.W. and Chehab, F.F., (1993). Rapid and Simultaneous Typing of Hemoglobin S, Hemoglobin C, and Seven Mediterranean 8-Thalassemia Mutations by Covalent Reverse Dot-Blot Analysis: Application to Prenatal Diagnosis in Sicily. *Blood,* 81(1):239-242.

Makhoul, N.J., Wells, R.S., Kaspar, H., Shbaklo, H., Taher, A., Chakar, N. and Zalloua, P.A., (2005) Genetic heterogeneity of beta thalassameia in Lebanon reflects historic and recent population migration. *Annals of Human Genetics.* 69:55-66.

Maslow, W.C., Beutler, E., Bell, C.A., Hougie, C. and Kjeldsberg, C.R. (1980). Practical Diagnosis: Haematologic disease. In *Disorders of haemoglobin synthesis,* pp126-153. Houghton Mifflin Professional Publishers.

Michlitsch, J., Azimi, M., Hoppe, C., Walters, M.C., Lubin, B., Lorey, F. and Vinchinsky, E., (2009). Newborn screening of hemoglobinopathies in California.*Pediatr. Blood Cancer, Wiley Interscience,* 52: 486-490.

© COPYRIGHT UPM

Minunni, M., Tombelli, S., Scielzi, R., Manneli, I., Mascini, M. and Gaudiano, C., (2003). Detection of β-thalassemia by a DNA piezoelectric biosensor coupled with polymerase chain reaction. *Analytica Chimica Acta, Elsevier,* 481:55-64.

Mirasena, S., Shimbhu, D., Sanguansermsri, M. and Sanguansermsri, T., (2007). The spectrum of β-thalassaemia mutations in Phitsanulok Province: Development of multiplex ARMS for mutation detection. *In Naresuan University Journal.* 15 (1): 43-53.

Moorsel, C.H.M., Wijngaarden, E.E.V., Fokkema, I.F.A.C., Dunnen, J.T.D., Roos, D., Zwieten, R.V., Giordano, P.C. and Harteveld, C.L.,(2004). β-Globin mutation detection by tagged single-base extension and hybridization to universal glass and flow-through microarrays. *European Journal of human genetics.* 12:567-573.

Mosca, A., Paleari, R., Leone, D. and Ivaldi, G. (2009) The relevance of hemoglobin F measurement in the diagnosis of thalassemias and related hemoglobinopathies. *Clinical Biochemistry.* 42: 1797-1801.

Nadine, S. (2005) Recipes for buffers and other laboratory solutions used in electrophoresis, PCR and DNA extraction. Lougheed Laboratory Manual.1-11.

Nal, N. , Manguoglu, A.E., Sargin, C.F., Keser, I., Kupesizt, A. and Luleci, G., (2005). Two rare mutations in Turkey: IVS I.130(G–C) and IVS II.848(C–A) *In Clin. Lab. Haem.,* 27:274–277.

Newton, C.R., Graham, A., Heptinstall, L.E., Powell, S.J., Summers, C., Kalsheker, N., Smith, J.C. and Markham, A.F., (1989). Analysis of any point mutation in DNA. The amplification refractory mutation system. *Nucleic Acids Research,* 17(7): 2503-2516.

Nopparatana, C. (1998). Molecular diagnosis of thalassemias. *Songklanakarin Medical Journal*, 16:145-149.

Nuntakarn, L., Fucharoen, S., Fucharoen, G., Sanchaisuriya, K., Jetsrisuparb, A. and Wiangnon, S. (2009). Molecular, hematological and clinical aspects of thalassemia major and thalassemia intermedia associated with Hb E-β-thalassaemia in Northeast Thailand. *Blood cells, molecules, and diseases, Elseveir*. 32-35.

Old, J.M., Varawalla, N.Y. and Weatherall, D.J., (1990) Rapid detection and prenatal diagnosis of β-thalassaemia: studies in Indian and Cypriot populations in the UK. *Lancet.* 336: 834-837.

Orkin, S.H., Markham, A.F. and Kazazian, H.H., (1983). An alternative approach for prenatal diagnosis: Detection of the Common Mediterranean β-Thalassemia Gene with Synthetic DNA Probes. *J. Clin. Invest*. 71: 775-779.

© COPYRIGHT UPM

Panigrahi, I., Agarwal, S., Gupta, T., Singhai, P. and Pradhan, M., (2005). Hemoglobin E-beta thalassaemia: factors affecting phenotype. *Indian pediatrics.* 42:357-362.

Pavlovic, S., Urosevic, J., Dureinovic, T., Janic, D. and Krivokapic-Dokmanovic, L. (2002). Rapid characterization of β-thalassaemia mutations by reverse dot blot and allele-specific PCR analysis. *Jugloslov. Med. Biochem.* 21(3):283-286.

Quek, D.L., Ng, Y., Wang, W., Tan, A.S.C., Tang-Lim, G., Ng, I.S.L., Law, H. and Chong, S.S., (2007). Rapid carrier screening for β-thalassaemia by single-step allele-specific PCR and detection. *Science Direct.* 40:427-430.

Rahim, F., Keikhaei, B., Aberumand, M., (2007). Prenatal diagnosis (PND) of β-thalassaemia in the Khuzestan Province, Iran. *Journal of Clinical and Diagnostic Research.* 1(6):454-459.

Rahimi, Z., Raygani, A.V., Merat, A., Haghshenass, M., Gerard, N., Nagel, R.L. and Krishnamoorthy, R., (2006). Thalassemic mutations in Southern Iran. *Iran J. Med. Sci.* 31(2): 70-73.

Rozitah, R., Nizam, M. Z., Nur Shafawati, A. R., Nor Atifah, M. A., Dewi, M., Kannan, T. P., Ariffin, N., Norsarwany, M., Setianingsih, I., Harahap, A., Zilfalil, B. A. (2008). Detection of beta-globin gene mutations among Kelantan Malay thalassaemia patients by polymerase chain reaction restriction fragment length polymorphism.*Singapore Med. J.* 49(12): 1046-1049.

Roudknar, M.H., Najmabadi, H., Derakhshandeh, P. and Farhud, D.D., (2003). Detection of rare and unknown mutations in β-thalassaemia traits in Iran. *Iranian J.Publ. Health* 32(1): 11-14.

Saiki, R.K., Walsh, P.S., Levenson, C.H. and Erlich, H.A., (1989). Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. Proc Natl Acad Sci USA 86:6230.

Salk, J.J., Sanchez, J.A., Pierce, K.E., Rice, J.E., Soares, K.C. and Wangh, L.J., (2006). Direct amplification of single-stranded DNA for pyrosequencing using linear-after-the exponential (LATE)-PCR. *Analytical Biochemistry,Elsevier* 353 :124-132.

Sanchaisuriya, K., Fucharoen, S., Fucharoen, G., Ratanasiri, T., Sanchaisuriya, P., Changtrakul, Y., Ukosanakarn, U., Ussawaphark, W. and Schelp, F.P., (2005). A reliable screening protocol for Thalassaemia and Haemoglobinopathies in pregnancy. *Am J. Clin. Pathol.* 123:113-118.

Seiyu, H., Faruqi, A.F., Dean, F.B., Du, Y., Sun, Z., Wu, X., Du, J., Kingsmore, S.F., Egholm, M. and Lasken, R.S., (2003). Unbiased Whole-genome amplification directly from clinical samples. *Cold Spring Harbor Laboratory Press* 13: 954-964.

© COPYRIGHT UPM

Setianingsih, I. , Williamson, R., Marzuk, S., Harahap, A., Tamam, M. and Forrest, S. (1998). Molecular basis of β-thalassaemia in Indonesia: Application to prenatal diagnosis. *Molecular Diagnosis* 3(1): 11-20.

Shah, A., (2004). Thalassaemia syndromes. *Indian J. Med. Sci.* 58(10): 445-449.

Sheth, J.J., Sheth, F.J., Pandaya, P., Priya, R., Davla, S., Thakur, C. and Flavin, V., (2008). β-thalassemia mutations in Western India. *Indian Journal of Pediatrics*, 75: 567-570.

Shih, H.C., Er, T.Z., Chang, T.J., Chang, Y.S., Liu, T.C. and Chang, J.G., (2009). Rapid identification of *HBB* gene mutations by high-resolution melting analysis. *Clinical Biochemistry, Elseveir Inc.* 42: 1667-1676.

Simmons A.,(1997). Blood collection. In *hematology: A combined theoretical and technical approach*. Second edition. Butterworth- Heinemann. pp 251-254.

Sripichai, O., Munkongdee, T., Kumkhaek, C., Svasti, S., Winichagoon, P.,and Fucharoen, S. (2008). Coinheritance of the different copy numbers of α-globin gene modifies severity of β-thalassemia/Hb E disease. Ann Hematol. 87: 375-379.

Srivorakun, H., Fucharoen, G., Sae-Ung, N.., Sanchaisuriya, K., Ratanasiri, T. and Fucharoen, S. (2009) Analysis of fetal blood using capillary electrophoresis system: a simple method for prenatal diagnosis of severe thalassaemia disease. *European Journal of Haematology* 83: 57-65.

Sutcharitchan, P., Saiki, R., Huisrnan, T.H.J., Kutlar, A., McKie, V., Erlich, H., and Ernbury, S.H., (1995). Reverse Dot-Blot Detection of the African-American β-Thalassemia Mutations. *Blood* 86(4): 1580-1585.

Sutcharitchan, P., Saiki, R., Fucharoen, S., Winichagoon, P., Erlich, H. and Embury, S.H., (1995). Reverse dot-blot detection of Thai β-thalassaemia mutations. *British Journal of haematology* 90:809-816.

Suthat, F., Peter, T.R., Natalie, W. P., Florence, D. and Suzanne, G., (1985). β-thalassemia syndromes. *In thalassemia: pathophysiology and management. Proceedings of the International Conference on Thalassemia held in Bangkok.* pp 61-69.

Tan, J.A.M.A, George, E., Tan, K.L., Chow, T., Tan, P.C., Hassan, J., Chia, P., Subramanium, R., Chandran, R. and Yap, S.F. (2004). Molecular defects in the β-globin gene identified in different ethnic groups/ populations during prenatal diagnosis for β-thalassaemia: a Malaysian experience. *Clin. Exp. Med.* 4: 142-147.

Tan, J.A.M.A., Tan, K.L., Omar, K.Z., Chan, L.L., Wee, Y.C., George, E. (2009). Interaction of Hb South Florida (codon 1; GTG→ATG) and Hb E, with β-thalassaemia (IVS I-1; G→A): expression of different clinical phenotypes. *Eur J. Pediatr.* 168:1049-1054.

© COPYRIGHT UPM

Tan, K.L., Tan, J.A.M.A., Wong, Y.C., Wee, Y.C., Thong, M.K. and Yap, S.F., (2001). Combine ARMS: A rapid and cost-effective protocol for molecular characterization of β-thalassaemia in Malaysia. *Genetic testing, Mary Ann Liebert* 5(1): 17-22.

Thein, S.L. (2005). Pathophysiology of β-thalassaemia- a guide to molecular therapies. *Hematology:* 31-37.

Thein, S.L. (2004). The genetics and multiple phenotypes of beta thalassaemia. In *Practical management of haemoglobinopathies.* ed Okpala, I., pp 26 -37. Blackwell publishing.

Thein, S.L., Hesketh, C., Wallace, R.B. and Weatherall, D.J., (1988). The molecular basis of thalassaemia major and thalassaemia intermedia in Asian Indians: application to prenatal diagnosis. *British Journal of Haematology.* 70:225-231.

Thong, M.K., Tan, J.A.M.A., Tan, K.L. and Yap, S.F. (2005). Characterisation of β-globin gene mutations in Malaysian Children: A strategy for the control of β-thalassaemia in a developing country. *Journal of Tropical Paediatrics.* 51(6): 328-333.

Trent, R.J.A. (2006). Diagnosis of the haemoglobinopathies. *Clin. Biochem Rev.* 27, 27-38.

Tuzmen, S., and Schechter, A.N.. (2001) Genetic diseases of hemoglobin: diagnostic methods for elucidating β-thalassaemia mutations. *Harcout Publishers Ltd,* Blood reviews 15: 19-29.

Vaught, J.B., (2006). Blood collection, shipment, processing and storage. *Cancer Epidemiol Biomarkers Prev.* 15(9): 1582-1584.

Vetter, B., Schwarz, E., Kohne, E., and Kulozik, A.E., (1997). Beta-thalassaemia in the immigrant and non-immigrant German population. *British journal of haematology.* 97: 266-272.

Vrettou, C., Traeger-Synodinos, J., Tzetis, M., Malamis, G. and Kanavakis, E., (2003). Rapid screening of multiplex β-globin gene mutations by real-time PCR on the LightCycler: application to carrier screening and prenatal diagnosis of thalassaemia syndroms.*Clin. Chemistry, Hematology* 49(5): 769-776.

Wang, W., Kham, S.K.Y., Yeo, G., Quah, T. and Chong, S.S., (2003). Multiplex Minisequencing Screen for Common Southeast Asian and Indian β-Thalassemia Mutations. *Clinical Chemistry* 49(2): 209-218.

Waters, H.M., Howarth, J.E., Hyde, K., Goldstone, S., Cinkotai, K.I.,Manizheh, Elyaderani, K., and Richards, J.T. (1998) An evaluation of the Bio-Rad Variant Haemoglobin testing system for the detection of haemoglobinopathies. *Clin. Lab. Haem.*20: 31-40.

© COPYRIGHT UPM

Weatherall, D.J. and Clegg J.B., (2001). The Thalassemia Syndromes, Blackwell Science, Oxford, pp. 550– 572.

Weatherall, D.J. and Clegg, J.B. (2001). Inherited haemoglobin disorders: an increasing global health problem. *Bulletin of the World Health Organization* 79(8): 704-712.

Weatherall D.J. (1999). Genetic disorders of haemolgobin. In *Postgraduate Haematology*. ed. Tuddenham, A.V., Lewis, S.M. and Hoffbrand A.V. , pp91-119, Butterworth-Heinemann.

Wiedorn, K.H., Goldmann, T., Henne, C., Kühl, H. and Vollmer, E., (2001). EnVision+, a New Dextran Polymer-based signal enhancement technique for in-situ hybridization (ISH). *The journal of Histochemistry & Cytochemistry* 49(9): 1067-1071.

Wild, B. and Bain, B.J. (2007). Investigation of abnormal haemoglobins and thalassaemia. In *Practical Haematology*. ed. Lewis, S.M., Bain, B.J. and Bates, I., Vol. 10: pp 272-310.

Win, N., (2004). Blood transfusion therapy for haemoglobinopathis. In *Practical management of haemoglobinopathies.* ed Okpala, I., pp 99 -104. Blackwell publishing.

Winichagoon, P., Saechan, V., Sripanich, R., Nopparatana, C., Kanokpongsakdi, S., Maggio, A. and Fucharoen, S. (1999). Prenatal diagnosis of β-thalassaemia by reverse dot-blot hybridization. *Prenat. Diagn.* 19: 428-435.

Winichagoon, P., Fucharoen, S., Wilairat, P., Chihara, K., Fukumaki, Y. and Wasi, P., (1992). Identification of five rare mutations including a novel frameshift mutation causing β^0-thalassemia in Thai patients with β^0-thalassemia/ hemoglobin E disease. *Biochimica et. Biophysica Acta, Elseveir* 1139: 280-286.

Wong , C., Antonarakis, S.E., Goff, S.C., Orkin, S.H., Boehm, C.D. and Kazazian, H.H. (1986) On the origin and spread of β-thalassemia: Recurrent observation of four mutations in different ethnic groups. *Proc. Natl. Acad. Sci.* 83: 6529-6532.

Wong, H.B. (1984) Thalassemia in Singapore: problems and solutions. *Ann Acad Med Singapore*,;13:473–486.

Xu, X., Liao, C., Liu, Z., Huang, Y., Zhang, J., Li, J., Peng, Z., Qiu, L. and Xu, Q., (1996). Antenatal screening and fetal diagnosis of β-thalassaemia in a Chinese population: prevalence of the β-thalassaemia trait in the Guangzhou area of China. *Hum. Genet.* 98: 199-202.

Yi, P., Li, L., Yao, H., Deng, B., Chen Z.Q., Zhou, Y.G., (2005). A new technique of detecting β-thalassaemia mutations from a single cell. *Journal of US-China Medical Science* 2(1): 71-77.

© COPYRIGHT UPM

Zhang, Y., Coyne, M.Y., Will, S.G., Levenson, C.H. and Kawasaki, E.S., (1991). Sing-base mutational analysis of cancer and genetic diseases using membrane bound modified oligonucleotides. *Nucleic Acids Research* 19(14):3929-3933.

© COPYRIGHT UPM