MOLECULAR ANALYSIS AND PROTEIN QUANTIFICATION OF Rh BLOOD GROUP SYSTEM AMONG BLOOD DONORS AT THE NATIONAL BLOOD CENTRE, MALAYSIA

BY

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DECLARATION

Here, I declare that this research work is forwarded to Universiti Sains Malaysia (USM) for the degree of Doctor of philosophy in Transfusion Medicine. It has not been sent to any other university. With that, this research may be used for consultation purpose and photocopied as reference.

ROZI HANISA MUSA

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ABBREVIATIONS

AIHA	Autoimmune Hemolytic Anemia
Anti	Antibody
CCD	Charge Coupled Device
cDNA	Complementary Deoxyribonucleic Acid
D+	RhD positive
D-	RhD negative
D^u	Weak D
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitiol
EDTA	Ethylenediaminetetraacetic Acid
FDA	Food and Drug Administration
G	G-Force
GS	Gel Station
HDFN	Haemolytic Disease of the Fetus and Newborn
HTR	Haemolytic Transfusion Reaction
IgG	Immunoglobulin G
IS	Immediate Spin
ISBT	International Society of Blood Transfusion
mRNA	Messenger Ribonucleic Acid
NBC	National Blood Centre

PDN	Pusat Darah Negara
PCR	Polymerase Chain Reaction
RB	Reducing Buffer
RBC	Red Blood Cell
RFLP	Restriction Fragment Length Polymorphism
Rh	Rhesus
RhAG	Rh Associated Glycoprotein
Rh-Hr	RhD modern nomenclature
RhD Var	RhD Variants
RhD	D antigen
RHD	D gene
RNA	Ribonucleic Acid
SDS-PAGE	Sodium Docecyl Sulfate Polyacrylamide Gel Electrophoresis
SPSS	Statistical Package for Social Sciences
PCR-SSP	Polymerase Chain Reaction, Sequence Specific Primers
UV	Ultraviolet
µl	Micro liter
ng/ µl	Nanogram per micro liter
nm	Nanometer
˚C	Degree Celsius

ANALISIS MOLEKULAR DAN KUANTIFIKASI PROTIN UNTUK SISTEM KUMPULAN DARAH Rh DI KALANGAN PENDERMA-PENDERMA DARAH DI PUSAT DARAH NEGARA, MALAYSIA

ABSTRAK

Rh adalah sistem kumpulan darah manusia yang paling polimorfik dan imunogenik, dengan lebih 50 antigen kini dikenal pasti. Antigen Rh terletak di atas dua protein, RhD dan RhCE: yang pertama membawa antigen D, manakala yang kedua membawa antigen C, c, E dan e. Antigen-antigen ini boleh dibahagikan lagi kepada pelbagai genotip. Kepentingan klinikal antigen Rh adalah berkaitan dengan keupayaan D negatif atau beberapa jenis D varian untuk membentuk antibodi D jika terdedah kepada antigen D. Antibodi D adalah punca utama penyakit hemolitik bagi janin dan bayi yang baru lahir dan juga boleh menyebabkan hemolitik transfusi. Beberapa faktor boleh merumitkan penentuan status D iaitu termasuk penggunaan metodologi dan reagen yang berbeza di mana tindak balasnya juga berbeza dengan gen-gen RHD yang polimorfik. Aplikasi teknik molekular untuk mengesan antigen Rh amat berguna untuk mengurangkan risiko alloimmunisasi. Tujuan kajian ini adalah untuk mencirikan gen Rh dengan kuantifikasi protin antigen Rh yang sepadan di kalangan penderma darah di Pusat Darah Negara (PDN), Kuala Lumpur, Malaysia. Seramai 1014 penderma darah yang dikaji yang terdiri daripada 360 Melayu , 434 Cina , 164 India, dan 56 daripada kumpulan-kumpulan etnik minoriti yang lain. Sampel darah yang dikumpul telah difenotip secara serologi dengan menggunakan mesin automatik, Olympus PK7200. Kemudian sampel ini diteruskan

dengan ujian analisis molekular dengan menggunakan teknik Polymerase Chain Reaction, Rentetan Primers Khusus (PCR- SSP). Kuantifikasi protein dan penjujukan automatik telah dijalankan ke atas 120 sampel yang menunjukkan perbezaan keputusan di antara serologikal dan analisis molekular.

Keputusan-keputusan analisis antigen Rh dan gen RH menunjukkan kepelbagaian dan taburan yang signifikan di antara semua kumpulan etnik ($p < 0.001$). Ia menunjukkan, CCDDee (R1R1) adalah paling tinggi pada etnik Melayu, ccDDEE (R2R2) etnik Cina dan ccee (rr) etnik India. Perbezaan di antara 120 keputusan analisa fenotip dan genotip telah diperhatikan. Keputusan percanggahan banyak berlaku di dalam alel D di kalangan penderma-penderma darah RhD negatif yang menunjukkan perkaitan yang signifikan di antara kumpulan-kumpulan etnik yang berbeza. Daripada keputusan yang diperolehi serta kajian-kajian lain, ia menunjukkan bahawa kelaziman dan asas molekul varian D di Asia adalah berbeza daripada orang-orang dalam populasi Eropah dan Afrika. Penemuan lain di dalam kajian ini yang signifikan adalah penemuan pelbagai mutasi novel (23) dan mutasi yang telah diterbitkan (5). Perkaitan yang signifikan antara keputusan percanggahan dan mutasi ditemui di alel D dan C/c, dan ia juga didapati mempunyai hubungan antara mutasi dan tahap pengurangan kepekatan protein RHD. Kepekatan protein antara 0 hingga 500 (ng/µl) adalah kepekatan biasa untuk protein RHD, protein RHCE dan glikoprotein RhAG. Sebagai kesimpulan, dengan menjalankan ujian analisis molekular RH bagi penderma darah, asas molekular yang dikaitkan dengan antigen kumpulan darah Rh dan fenotipnya dapat dijelaskan serta mewujudkan pangkalan data untuk genotip RH penderma darah dari kumpulan etnik utama di Malaysia.

MOLECULAR ANALYSIS AND PROTEIN QUANTIFICATION OF Rh BLOOD GROUP SYSTEM AMONG BLOOD DONORS AT THE NATIONAL BLOOD CENTRE, MALAYSIA

ABSTRACT

Rh is the most polymorphic and immunogenic human blood group system, with over 50 antigens now identified. The Rh antigens are located on two proteins, RhD and RhCE: the former carries the D antigen, whilst the latter carries the C, c, E and e antigens. These antigens can be further subdivided into various genotypes. The clinical significance of the Rh antigen is related to the ability of D negative or some D variant types to form anti-D if exposed to D antigens. Anti-D is a major cause of Haemolytic Disease of the Fetus and Newborn (HDFN) and also can cause Haemolytic Transfusion (HTR). Multiple factors can complicate the determination of the D status which include different methods and reagents used that can react differently with the polymorphic RHD genes. Thus, applications of molecular techniques for the detection of Rh antigens are useful for reducing the risk of alloimmunization. The aim of this study was to characterize the Rh genes and its corresponding Rh antigens protein quantification among blood donors at the National Blood Centre (NBC), Kuala Lumpur, Malaysia. The study subjects were 1014 blood donors comprising of 360 Malays, 434 Chinese, 164 Indians, and 56 from other minor ethnic groups. Blood samples collected were serologically phenotyped using automated machine, Olympus PK7200. Then these samples were subjected to molecular analysis by applying the PCR- Sequence Specific Primers (PCR-SSP)

technique. Protein quantification and automated sequencing were performed on 120 samples with results that showed discrepancies from the serological and molecular analysis.

Results of the analyses of Rh antigens and RH genes showed heterogeneity and there was significant distribution between all the ethnic groups ($p < 0.001$). Also, CCDDee (R1R1) was highest in Malays, ccDDEE (R2R2) in Chinese and ccee (rr) in Indians. Discrepancies in 120 results between phenotype and genotype analysis were observed. Most of the discrepancies were found in allele D among the RhD negative blood donors which showed significant association between the different ethnic groups.

These findings, together with other studies, indicate that the prevalence and molecular basis of D variants in Asia are different from those in Caucasian and African populations. The other significant finding was the discovery of multiple novel mutations (23) and published mutations (5) in this study. Significant associations between discrepancies in results and mutations were found in allele D and C/c and it was also found to be correlated between mutation and degree of reduction of RHD protein concentration. The protein range of 0 to 500 (ng/µl) concentrations was common in the RHD protein, RHCE protein, and RhAG glycoprotein. In conclusion, performing RH molecular analysis in blood donors clarifies the molecular basis associated with Rh blood group antigens and phenotypes and provides database for RH genotypes of blood donors from major ethnic groups in Malaysia.

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Rh is the most important and complex human blood group system. The Rh blood group system was first described more than 70 years ago in 1940. In this report, a woman had a transfusion reaction when transfused with blood from her husband following the delivery of a stillborn child with erythroblastosis fetalis. Her serum agglutinated red blood cells (RBC) from her husband and from 80% of Caucasian ABO compatible blood donors with her blood. The woman's serum was found that it reacted with 77 percent of blood donors. She had been exposed to blood from her fetus and produced an antibody that reacted with it. The same antigen was present in the baby's father, explaining the woman's reaction to his blood (Avent & Reid, 2000).

A year later, Landsteiner and Weiner (1941) reported an antibody made by guinea pigs and rabbits when these animals were transfused with rhesus monkey red cells. This antibody, which agglutinated 85% of human red cells, was named “Rh” (Denise, 1999). If an individual’s RBC were clumped together by this antiserum, they were said to have the Rhesus factor on their RBC, thus termed RhD positive. If an individual’s RBC were not agglutinated by the antiserum, they were said to lack the Rhesus factor, termed RhD negative.

It is now known that the Rh system is very complex and the most polymorphic, with over 50 antigens identified (Peyrard et. al, 2009). The most common antigens of the

system are D, C, E, c and e. The Rh antigen is the most clinically significant antigen and can be further subdivided into various phenotypes (Mouro et al., 1993). The clinical significance of the Rh antigen is related to the ability of people who are Rh D negative or some D variant types to form anti-D if exposed to D antigens. Anti-D is a major cause of Haemolytic Disease of the Fetus and Newborn (HDFN) and can cause transfusion reactions (Brecher et al., 2005).

Serologic detection of polymorphic blood group antigens and of phenotypes provides a valuable source of appropriate blood samples for study at the molecular level. The ability to clone complementary Deoxyribonucleic Acid (DNA) and sequence genes encoding the Rh proteins have led to an understanding of the molecular basis associated with some of the Rh antigens.

1.1.1 Rh Blood Group System

The Rh system is the most important of the other commonly utilized blood grouping systems. It is known that the Rh system is very complex and our present understanding is based on the Fisher-Race System. There are three genes that make the Rh antigens: C, D, and E, found on chromosome 1 (Avent & Reid, 2000). There are two possible alleles at each locus: c or C; d or D; and e or E. One haplotype consisting of c/C, d/D, e/E is inherited from each parent, and the resulting Rh type of the individual depends on their inherited genotype (Westhoff, 2004). The RH genes are designated by capital letters, with or without italics, and include erythroid *RHD*, *RHCE* and *RHAG*, as well as the non-erythroid homologs expressed in other tissues. The different alleles of the *RHCE* gene are designated *RHce*, *RHCe*, and *RHcE*, according to which antigens they encode. The proteins are indicated as RhD and

RhCE or according to the specific antigens they carry Rhce, RhCe, or RhcE and include erythroid RhAG and those found in other tissues (Westhoff, 2007). The haplotypes are given a code as seen in Table 1.1.

Table 1.1: The Phenotypes and Genotypes of the Rh Blood Group System

Phenotypes expressed on cell	**Genotype expressed in DNA**	
	Fisher-Race	**Wiener**
D+ C+ E+ c+ e+ (RhD+)	Dce/DCE	RoRz
	Dce/dCE	Rory
	DCe/DcE	R1R2
	DCe/dcE	R1r
	DcE/dCe	R2r
	DCE/dce	Rzr
D+ C+ E+ c+ e- (RhD+)	DcE/DCE	R2Rz
	DcE/dCE	R2ry
	DCE/dcE	Rzr
D+ C+ E+ c- e+ (RhD+)	DCe/dCE	R1ry
	DCE/dCe	Rzr’
	DCe/DCE	R1Rz
D+ C+ E+ c- e- (RhD+)	DCE/DCE	RzRz
	DCE/dCE	Rzry
D+ C+ E- c+ e+ (RhD+)	Dce/dCe	Ror’
	DCe/dce	R1r
	DCe/Dce	R1Ro
D+ C+ E- c- e+ (RhD+)	DCe/DCe	R1R1
	DCe/dCe	R1r’
D+ C- E+ c+ e+ (RhD+)	DcE/Dce	R2Ro
	Dce/dcE	Ror”

	DcE/dce	R2r
D+ C- E+ c+ e- (RhD+)	DcE/DcE	R2R2
	DcE/dcE	R2r”
D+ C- E- c+ e+ (RhD+)	Dce/Dce	RoRo
	Dce/dce	Ror
D- C+ E+ c+ e+ (RhD-)	dce/dCE	rry
	dCe/dcE	r’r”
D- C+ E+ c+ e- (RhD-)	dcE/dCE	r”ry
D- C+ E+ c- e+ (RhD-)	dCe/dCE	r’ry
D- C+ E+ c- e- (RhD-)	dCE/dCE	ryry
D-C+ E- c+ e+ (RhD-)	dce/dCe	rr’
D- C+ E- c- e+ (RhD-)	dCe/dCe	r’r’
D- C- E+ c+ e+ (RhD-)	dce/dcE	rr”
D- C- E+ c+ e- (RhD-)	dcE/dcE	r”r”
D- C- E- c+ e+ (RhD-)	dce/dce	rr

Note:
An uppercase R is used to describe haplotypes that produce D antigen and a lowercase r is used when D is absent. The C or c and E or e Rh antigens carried with D are represented by 1 for Ce (R1), 2 for cE (R2), o for ce (Ro), and z for CE(Rz). The symbols prime (’) and double prime (”) are used with r to designate the CcEe antigens; for example, prime is used for Ce (r’), doubleprime (”) for cE (r”), and “y” for CE (ry). The R versus r terminology allows one to convey the common Rh antigens present on one chromosome in a single term (a phenotype).

(Adapted from www.wikipedia.org/wiki/Rh_blood_group_system)

If an individual's Rh genotype contains at least one of the C, D, or E antigens, he or she is considered as RhD positive individual. Only individuals with the genotype dce/dce (rr) are RhD negative. For the purpose of the blood transfusion Rh types, r'r and r"r is classified as RhD positive donors. Recipients of blood transfusions with Rh types r' and r" should receive RhD negative (rr) blood. This is to prevent sensitization to Rh antigens and subsequent Rh antibody formation. The most common Rh antibody is anti-D, but it is possible to form antibodies to c, C, e and E as well, and to form combinations of antibodies. There is no anti-d due to the complete absence of D antigen (Brecher et al., 2005).

1.1.2 The Rh Genes and Rh Proteins

Two genes (*RHD*, *RHCE*) in close proximity on chromosome 1 encode the erythrocyte Rh proteins, RhD and RhCE; one carries the D antigen and the other carries CE antigens in various combinations (ce, Ce, cE, or CE) (Figure 1.1/A). The genes each have ten exons and are 97% identical (Westhoff, 2007).

RhD and RhCE proteins differ by 32-35 out of 416 amino acids (Figure 1.1/B). Individuals who lack the RhD protein most often have a complete deletion of the *RHD* gene (Figure 1.1/A). An important consideration in the immunogenenicity of a protein is the degree of foreignness to the host. The large number of amino acid changes explains why exposure to RhD antigen can results in a potent immune response in a D-negative individual (Westhoff, 2007).

RHCE, expressed in all but rare D-- individuals, encodes both C/c and E/e antigens on a single protein. C and c antigens differ by four amino acids, but only the amino

acid change Ser103Pro is extracellular (Figure 1.1/B). The E and e antigens differ by one amino acid, Pro226Ala, located on the fourth extracellular loop of the protein (Avent & Reid, 2000).

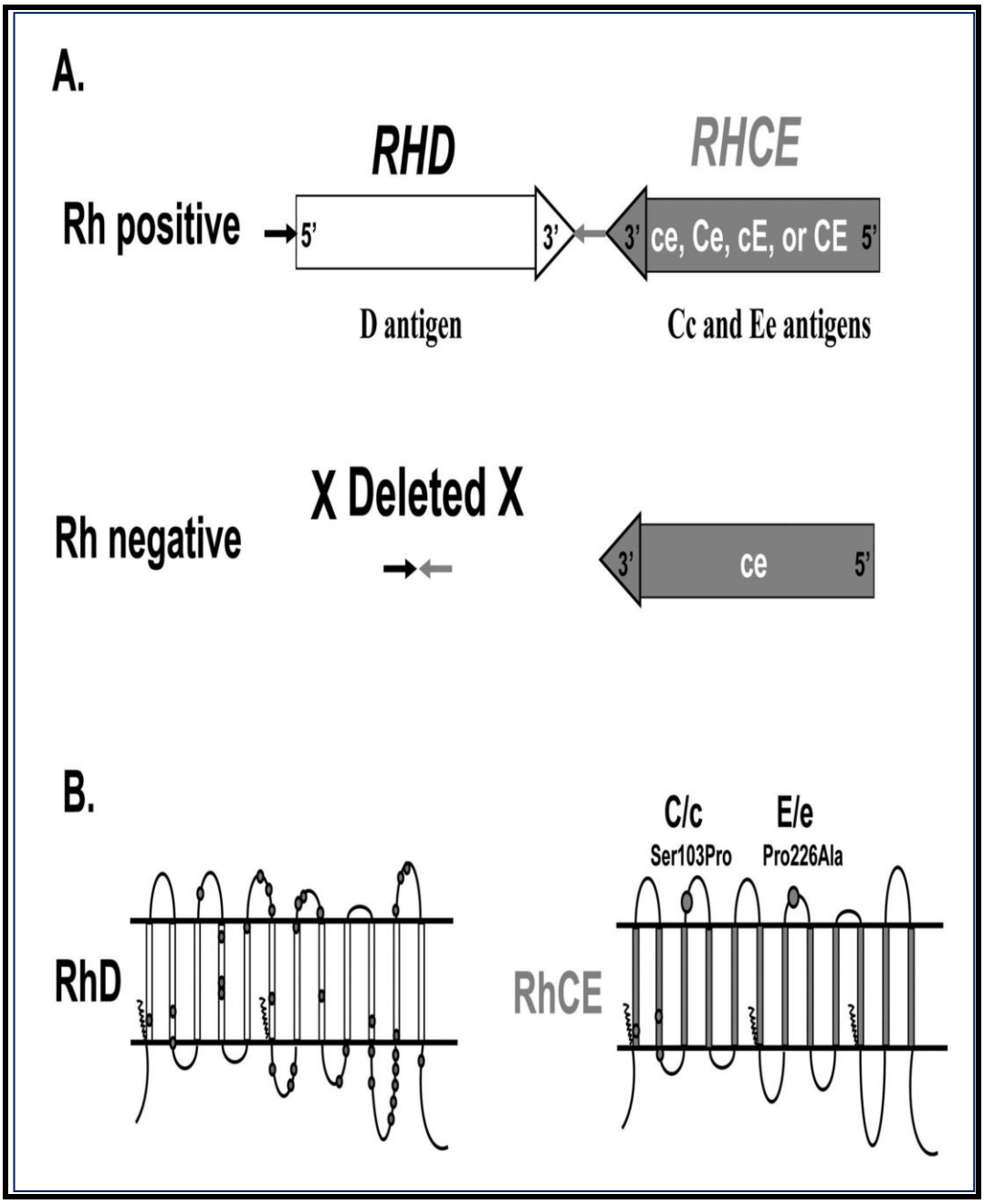

Figure 1.1: Diagram of the *RHD* and *RHCE* Locus and Rh Proteins in RBC Membrane.

A) The two RH genes have opposite orientation, with the 3' ends facing each other. Rh negative Caucasians individuals have a complete deletion of RHD.

B). The RhD and RhCE proteins are predicted to have twelve transmembrane domains. Amino acids positions that differ between RhD and RhCE are shown as dark circles on RhD. The amino acids changes responsible for C/c and E/e polymorphisms are shown on RhCE.

(Adapted from Westhoff Connie M., 2007)

The *RH* genes and proteins detailed in Figure 1.1 and Figure 1.2(A) are typical for the majority of individuals, and commercial antibody reagents detect expression of these conventional D, C, c, E and e antigen shown. The proximity of the two *RH* genes and their inverted orientation augments opportunity for genetic exchange (Figure 1.1A). Many *RH* genes carry point mutations, or have rearrangements and exchanges between *RHD* and *RHCE* that result from gene conversion events (Yves et al., 1991). The latter encode hybrid proteins that have RHCE-specific amino acids in RhD or RhD-specific residues in RhCE. These can generate new antigens in the Rh blood group system and alter or weaken expressions of the conventional antigens; see Figure 1.2B (Westhoff, 2007).

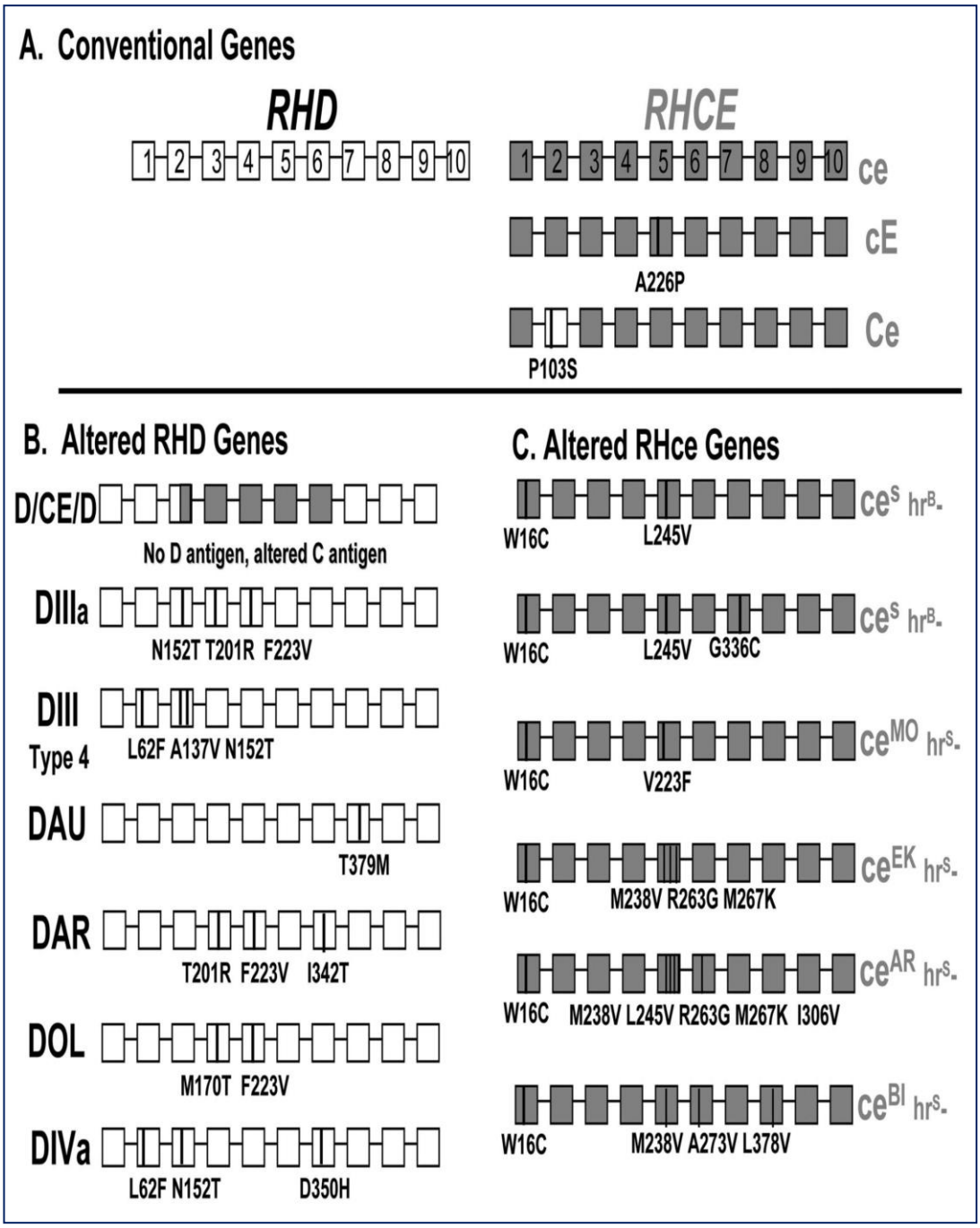

Figure 1.2: Diagram of the *RHD* and *RHCE* genes.
The ten RHD exons are shown as white boxes, and the RHCE as grey.
A) *RHD* and *RHCE* genes responsible for the common D, C, c, E and e antigen polymorphism.
B) Altered *RHD*; C) Altered *RHCE* genes indicating the changes often found in African-Americans that complicate transfusion, especially for sickle cell patients (Adapted from Westhoff Connie M., 2007)

1.1.3 Molecular Basis of Rh Antigens

Previously, it was only possible to determine the Rh phenotype by serologic typing of RBC. This serologic approach can be inconclusive, for instance in Rh phenotyping of fetuses, in patients who have recently been transfused, and those harboring large quantity of donor red blood cells. In all these cases, Rh genotyping is an option (Flegel et al, 2009). Serologic detection of polymorphic blood group antigens and phenotypes provide valuable sources of appropriate blood samples for molecular studies (Anstee, 2009). Haemolytic Disease of the Fetus and Newborn (HDFN), Alloimmune and transfusion reactions are not only due to anti Rh-D antibodies but also sometimes to anti-Rh E/e or anti-RH C/c antibodies in such cases (Brecher et al., 2005).

Numerous reports investigating molecular testing for blood groups in transfusions have focused on RhD. RBC serologic typing for D can be challenging in the approximately 2% of samples with a weak D or partial D antigens. Individuals with partial D, as well as some weak D types, lack anti-D when exposed to conventional D. Serologic reagents cannot distinguish the majority of these RBCs from those with conventional or normal D. This is due to the different monoclonal antibodies used that can affect the level of expression the D antigens and their epitopes. The molecular *RHD* characterization methods can be a complement for serologic method to solve those problems (Westhoff, 2007).

Molecular investigation of D variants has revealed that there are numerous different phenotypes. Nucleotide mutations that encode amino acid changes in the D protein are a common cause of variant phenotypes. The position of the substitution is

thought to be important in determining the D epitope and hence whether the variant can make anti-D (Huang & Ye, 2010). At least 21 different genetic types of weak D antigen have now been found. However, the exact mechanism for low expression of D antigen is not clear. The mutations could interfere with membrane integration of D protein, possibly by influencing the interaction of RhD protein with RhAG glycoprotein (Flegel et al., 2002).

Other mutations are predicted to be in the extracellular region of the D protein. It is speculated that these mutations result in part of the D antigen mosaic being missing and thus affect the D epitope resulting in a qualitative change (partial D phenotype). This means that these individuals have the ability to form anti-D if exposed to part of the mosaic that they lack. Partial Ds of this type include DNB, where there is a single G to A nucleotide exchange at position 1063, which leads to glycine to serine amino acid substitution at codon 355 in extracellular loop 6 (Flegel et al., 2002).

Other partial D phenotypes are due to the substitution of a part of the *RHD* gene with part of the *RHCE* gene and this produces hybrid genes composed of exons from both *RHD* and *RHCE*. Category VI type II of partial D is caused by this type of hybrid gene. In these types of exons, 1 to 3 and 7 to 9 are from *RHD,* whereas exons 3 to 6 are from *RHCE* (Figure 1.3). Such recombination is relatively common in the Rh system. This is because the genes lie in close proximity to each other, are highly homologous, and also have numerous repetitive elements which may serve as 'hot spots' for recombination (Brecher et al., 2005).

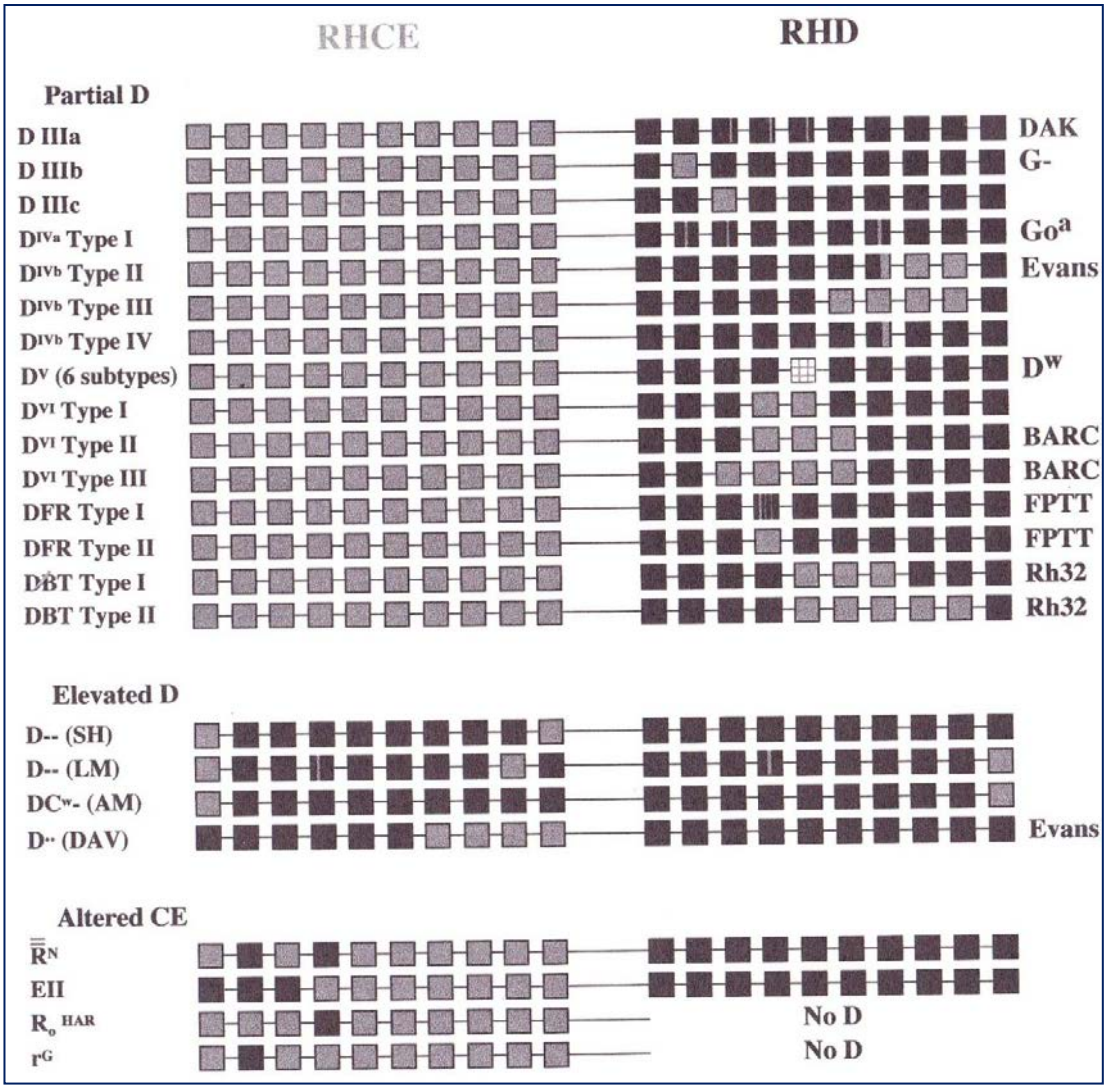

Figure 1.3: Diagram types of Partial D, Elevated D and Altered D
Most partial D phenotypes result from replacement of portion of *RHD* by *RHCE*, which cause elevated D phenotype with concurrent loss of the CE antigen or altered CE expression
(Adapted from Westhoff Connie M., 2007)

The ability to make anti-D is important in terms of developing a transfusion strategy for D variant patients. Researchers in Germany have suggested that the common European weak D types namely weak D types 1, 2 and 3 cannot make anti-D and therefore can and should be transfused with D positive units to save the D negative stocks. However, this and any other policy depends on the testing strategy that can detect and identify D variant phenotypes (Bretcher et al., 2005).

1.1.4 RhD Typing Discrepancies

Multiple factors can complicate the determination of the D status. These include the different methods used in various laboratories, the different monoclonal antibodies in FDA-licensed reagents that can react differently with variant D antigens. The large number of different *RHD* genes, which can affect both the level of expression and, potentially, the structure of the molecule and D-epitopes (Westhoff, 2007).

1.1.5 Justification of this Study

The Rh system is significant due to several factors. Firstly, Rh antigens are highly immunogenic and are of great importance for transfusion medicine. Secondly, Rh antigens are complex, which stems from the highly polymorphic genes that encode them and there are great differences among races in the frequencies of the *RH* gene complex. Thus the Rh system remains the most polymorphic and immunogenic blood group system known in humans until now. Additionally, D antigen is the most important Rh antigen; it is a mosaic comprising at least 30 epitopes.

Despite the importance of the Rh antigens in blood transfusion and HDFN, the function of its proteins is speculative, and may involve transporting ammonium ions

across the RBC membrane and maintaining the integrity of the RBC membrane. Substitutions of amino acids that are located in Rh transmembraneous segments may affect the function of the Rh protein (Avent & Reid, 2000). The clinical significance of the Rh antigen is related to the ability of D negative or some D variant types to form anti-D if exposed to D antigens. Anti-D is a major cause of haemolytic disease of the HDFN and can cause transfusion reactions (Brecher et al., 2005).

The Rh phenotype (D--) is an example of a rare phenotype but individuals having this phenotype can develop haemolytic diseases. Individuals with this Rh phenotype produce antibodies to RBC against common Rh antigens through pregnancies or transfusions (Bretcher et al., 2005). Furthermore, no blood will be compatible except of the same blood group and phenotyped as (D--). These individuals can develop alloantibody known as anti Rh 17 that can cause HDFN or HTR if other blood group is given (Denise, 1999).

In Malaysia, National Blood Centre (NBC) is the referral center for donors and patients with Immunohaematology issues where patients are referred for further investigation. Recently, NBC had identified 3 Bidayuh pregnant mothers who had been referred with anti Rh 17 antibodies requiring transfusion and no compatible blood was found. In this situation, their siblings were typed and the process was time consuming (Musa et al., 2008). In cases of emergencies, this may be a setback since blood is not readily available.

In current practice, serological method is used to identify the red cell phenotype. This method cannot detect complicated cases, especially the rare Rh blood group since sensitivity is not optimum (Anstee, 2005).

In NBC, all cases are investigated by serological methods and not by molecular analysis for the identification of Rh blood groups which may be time consuming. It is therefore timely to conduct a molecular and protein quantification study and establish our local database on Rh blood group system in the Malaysian population. Malaysia has a multiracial population comprising Malays, Chinese, and Indians who are the major races in Peninsular Malaysia with other ethnic groups especially in East Malaysia in the north of Borneo Island. In an effort to gain more insight into the molecular background and frequency of RH genotypes, a comprehensive and systematic study was undertaken to determine the distribution of Rh genotypes from selected ethnic groups among blood donors in NBC, Kuala Lumpur.

This research was conducted at NBC because of its status as the national referral centre and the high number of blood donations received daily. In addition, it is hoped that a policy for D antigen testing in NBC can be formulated based on the research findings.

1.2 Objectives

1.2.1 Main Objective

The main objective of this study was to characterize the RH genes and their corresponding Rh antigen proteins quantification among blood donors in the National Blood Centre (NBC), Kuala Lumpur.

1.2.2 Specific Objectives

i) To determine the frequency of Rh antigen of the various Rh phenotypes among blood donors in NBC.

ii) To determine the frequency of RH gene of the various RH genotypes among blood donors in NBC.

iii) To determine the RH gene polymorphism among blood donors in NBC.

iv) To determine the mutations of the Rh blood group system among blood donors in NBC.

v) To quantify the corresponding Rh antigen protein among blood donors in NBC.

1.3 Research Hypothesis

H1 – There is a difference in the Rh antigen frequency from the various Rh phenotypes among the blood donors in NBC.

H2 – There is a difference in the RH gene frequency from the various RH genotypes among the blood donors in NBC.

H3 –There is a variation of RH gene polymorphism among the blood donors in NBC.

H4 – There is heterogeneity in the molecular basis of the Rh blood group system among the blood donors in NBC.

H5 –There is a variation in concentration of Rh protein expression among the blood donors in NBC.

CHAPTER 2

LITERATURE REVIEW

2.1 Rh Factor

An individual either has, or does not have, the "*Rhesus factor*" on the surface of his/her red blood cells. This term strictly refers to the most immunogenic D antigen of the Rh blood group system. The status is usually indicated by *Rh positive* (Rh+ does have the D antigen) or *Rh negative* (Rh- does not have the D antigen) suffix to the ABO blood type (Avent et al., 2000). However, other antigens of this blood group system are also clinically relevant. In contrast to the ABO blood group, immunization against Rh occurs only through blood transfusion or placental exposure during pregnancy in women (Reid & Francis, 2004).

2.2 Rh Nomenclature

The Rh blood group system has two sets of nomenclatures: one developed by Ronald Fisher and R.R. Race, the other by Wiener (Westhoff, 2004). Both systems reflected alternative theories of inheritance. The Fisher-Race system, which is more commonly in use today, uses the CDE nomenclature. This system was based on the theory that a separate gene controls the product of each corresponding antigen. However, the d gene was hypothetical, not actual (Daniels, 2007).

The Wiener system uses the Rh-Hr nomenclature. This system was based on the theory that there is one gene at a single locus on each chromosome, each contributing to production of multiple antigens. In this theory, a gene R_1 is supposed to give rise to the "blood factors" Rh_0, rh', and hr" (corresponding to modern nomenclature of the D, C and e antigens) and the gene r to produce rh' and hr" (corresponding to modern nomenclature of the c and e antigens) (Denise, 1999). Notations of the two theories are used interchangeably in blood banking (e.g., Rho [D] meaning RhD positive). Wiener's notation is more complex and cumbersome for routine use. Easier to explain, the Fisher-Race theory has become more widely used (Denise, 1999).

On the other hand, DNA testing has shown that both theories are partially correct. There are in fact two linked genes: the *RHD* gene, which produces a single immune specificity (anti-D), and the *RHCE* gene with double immune specificities (anti-C or anti-c with anti-E or anti-e). The CDE notation used in the Fisher-Race nomenclature is sometimes rearranged to DCE to more accurately represent the co-location of the C and E encoding on the *RHCE* gene, and to make interpretations easier (Daniels et al., 2004).

2.3 Rh System Antigens

The proteins that carry the Rh antigens are transmembrane proteins, whose structure suggests that they are ion channels (Patnaik et al., 2012). The main antigens are D, C, E, c and e, which are encoded by two adjacent gene loci, the *RHD* gene which encodes the RhD protein with the D antigen (and variants), and the *RHCE* gene which encodes the RhCE protein with the C, E, c and e antigens (and variants)

(Patnaik et al., 2012). There is no d antigen. Lowercase "d" indicates the absence of the D antigen (the gene is usually deleted or otherwise nonfunctional).

Rh phenotypes are readily identified by identifying the presence or absence of the Rh surface antigens. Most of the Rh phenotypes can be produced by several different Rh genotypes (Daniels & Bromilow, 2007). The exact genotype of any individual can only be identified by DNA analysis. For transfusion, negative antigen blood is given only for the phenotype which is usually clinically significant to ensure a patient is not exposed to an antigen they are likely to develop antibodies against. A probable genotype may be speculated on, based on the statistical distributions of genotypes in the patient's place of origin (Westhoff, 2007).

2.4 RH Gene Polymorphism

Rh antigens constitute a clinically important blood group system mainly because of their involvement in haemolytic reactions as extremely potent immunogens (Harvey & Anstee, 1993). Besides the major D, C/c, and E/e antigens, a large array of qualitative and/or quantitative Rh polymorphisms are encountered in the human population (Race et al., 1975). These surface-active markers are carried on Rh polypeptides, a family of transmembrane proteins that possess two unusual defining features, nonglycosylation and palmitoylation (Cartron, 1999). The Rh polypeptides appear to play important roles in RBC membrane structure and physiologic processes, as highlighted by the occurrence of chronic but moderate hemolytic anemia and stomatocytosis in patients with Rh deficiency syndrome (Agre et al., 1991). Since the cloning of two Rh complementary DNA (cDNAs), much

information has been obtained about their primary structure, erythroid expression, and complex interaction with other membrane components (Cartron et al, 1994). Whereas biochemical analyses showed that the D, C/c, and Ee antigens reside in at least three distinct polypeptides, molecular studies indicated that only two structural genes, D and non-D (including *ce, Ce, cE,* and *CE* alleles), occur in the human genome. Nevertheless, it has remained unclear whether *C/c* is coexpressed with or derived from the *E/e* allelic series by differential Ribonucleic Acid (RNA) splicing (Mouro et al., 1993). It is also yet to be established whether the Rh protein(s) alone would be sufficient or whether additional factor(s) would be required for the antigenic presentation on the RBC membrane.

Despite these uncertainties, studies on a number of Rh phenotypes at the molecular level have provided insight into the underlying genetic mechanisms. Analysis of the D category variants indicated that they have taken place via homologous recombinations between the D and non-D genes (Rouillac et al., 1995). However, the molecular basis for those variants lacking some of the major Rh antigens appears to be more heterogeneous. For instance, the absence of D antigen from RBCs, a status referred to as Rh-negative, may or may not be associated with D locus deletion (Huang et al., 1996). In addition, the D- variants occurred on the background of partial deletion or nondeletion of the non-D locus (Huang et al.,1995). The DC- and DCW- phenotypes were proposed to result from segmental gene conversion events (Cherif–Zahar et al., 1994). The structure and expression of Rh polypeptide genes in two unrelated individuals exhibiting the dCCee and DCW- phenotypes.

2.5 RH Gene Frequency

Wide racial differences are recognized not only in frequency of Rh phenotypes but also at the molecular level (Garratty, 2005). Several *RHD*, *RHC/c*, and *RHE/e* genotyping assays have been developed. A number of studies have been carried out to assess the frequency of these three molecular backgrounds in Africans and Caucasians (Mhammed et al., 2009). There are marked differences in the incidence of phenotypes associated with weak and partial D, even in different parts of one country (Garratty, 2005).

There are many differences in the incidence of phenotypes associated with weak and partial D seen in the African and Oriental population backgrounds but only 3 to 5 percent or less than one percent of individuals of African and Oriental background, respectively, type as RhD negative. In the African and Asian populations the RhD negative phenotypes, are often caused by inactive or silent *RHD* rather than the truly gene deletions as found in Caucasians. Approximately one third of RhD negative Oriental persons are Rh Del with deletion of the whole RHD gene (Garratty, 2005).

Previously, Hyland et al. (1994) applied restriction fragment length polymorphism (RFLP) patterns on Southern blots for Rh genotypes. However, they found a 100% correlation for 102 randomly selected blood donors for the RhC, Rhe and RhD phenotypes, but only 94.8% for the Rhc and 94.3% for the RhE phenotypes.

The sequence of RH genes may vary with different ethnic groups. It is important to be aware of the differences in genetic sequences in order to develop genotyping

methods that are reliable in a multiracial population. The Rh blood group system has shown significantly different distribution among ethnic groups. In Malaysia, it also found that the Rh blood group system had shown significantly different between the ethnic group of blood donors as in Table 2.1 (Musa et al., 2012). Table 2.2 shows the prevalence of phenotypes and genotypes in England, United Kingdom (UK).

Finally, it is important to remember that much of the recent data on weak D and partial D have come from Europe, and some of these reports emphasize major differences found in selected areas of a single European country. The incidence of various D variants may be different such in countries with larger Asian or African populations; they need to relate to statistics gathered in their own countries (Garratty, 2005). To date in the Asian population, there is limited data on the molecular basis of the Rh blood group system and other blood group systems especially in Malaysia.