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The Genetic Polymorphism of RhD among Blood Donors in Gaza Strip and its Reflection on Blood Transfusion Strategy

A thesis submitted to the Faculty of Science in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences - Medical Technology

Thesis Prepared By

Mohamad I. Dawoud

Supervisors

Prof. Fadel A. Sharif **Prof. Mohammad Shubair**

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contain no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree of the university or other institute.

Mohamad I. Dawoud

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ABSTRACT

Rh system is one of the most and highly complex blood group systems, as many as over 45 different Rh antigens have been serologically defined. The Rh antigens are expressed by proteins encoded by a pair of highly homologous genes located at chromosome 1. *RHCE* gene encodes CcEe antigens, while the *RHD* encodes the D antigen.

RhD is the most important, immunogenic and polymorphic Rh antigen from the clinical aspects (comprises at least 30 epitopes), which plays a key role in transfusion medicine. Anti-D antibodies remain the leading cause of the hemolytic disease of the newborn, and antigen D compatible transfusion is a standard practice in transfusion therapy. Partial D lacks one or more D epitopes, and the partial D individual could be immunized on exposure to normal D positive during blood transfusion or pregnancy. The D^{VI} and DNB variants are the most frequent partial Ds that lack many of D epitopes, D^{VI} is usually typed as D negative while DNB is typed as D positive.

We have examined 102 genomic DNA samples derived from blood donors expressing D positive and negative phenotypes, to detect D^{VI} and DNB variants, and to investigate the molecular genetic of Rh negative phenotype. In addition 3 samples with discrepant RhD (weak D) were also investigated. To detect D^{VI} variant; simplex PCR was used to detect the presence or absence of *RHD* exon 10/intron 4, while PCR-SSP was used to detect DNB variant. Of these, 3 D^{VI} and 3 DNB samples were detected between blood donors. The PCR observations indicated a complete deletion of *RHD* gene in D negative specimens, and that the 3 weak samples were similar to normal *RHD* alleles but probably with weak *RHD* expression. A full concordance between phenotype and genotype was observed in D positive samples.

Key words: Rh system, RhD, D^{VI}, DNB, Anti D, Hybrid allele, PCR-SSP

الأنماط الجينية للعامل الريزيسي بين المتبرعين بالدم في قطاع غزة وانعكاساتها على سياسات نقل الدم

ملخص الدراسة

يعتبر نظام Rh من أهم وأكثر الفصائل الدموية تعقيدا، حيث يحتوي هذا النظام على ما يزيد عن 45 أنتيجين منها 5 أنتيجينات رئيسية، هذه الأنتجينات عبارة عن مواد بروتينية تخلق بواسطة زوج من المورثات الجينية المتشابهة والموجودة على الكروموسوم رقم 1، إحدى المورثات *RHCE* تنتج أربعة أنتجينات بينما المورثة الأخرى *RHD* تنتج أنتجين D والذي يعتبر الأساس في هذا النظام وإليه تعزى فصيلة الدم السالبة أو الموجبة.

ويعد RhD الأكثر أهمية وتنوعا وله أهمية طبية بالغة في مجالات نقل الدم فهو ذو قدرة عالية على إحداث رد فعل مناعي لتكوين أجسام مضادة وبتركيز عال، والتي تعد السبب الرئيس في مرض تحلل الدم عند الأطفال حديثي الولادة والكثير من حالات عدم التطابق أثناء عملية نقل الدم. الفصيلة السالبة للدم عادة ما تنتج عن غياب أو إزالة كاملة للمورثة *RHD*، بينما Partial D أو الفصيلة الجزئية فتنتج بسبب فقد واحد أو أكثر من المحددات الأنتيجينية كنتيجة لخلل في المورثة، والتي تنجم إما عن طفرة أحادية في المورثة أو حدوث عملية تهجين بين كلا المورثتين *RHD* و*RHC* في نظام Rh. فصيلة Partial D لها القدرة على حث جهاز المناعة لتكوين أجسام مضادة Anti-D والتي يمكن أن تسبب تحللا للدم المنقول كما أنها تسبب مرض تحلل الدم عند الأطفال حديثي الولادة.

هناك نوعان واسعا الانتشار من Partial D ويطلق عليهما D^{VI} وDNB ولهما أهمية طبية بالغة في مجال نقل الدم ومرض تحلل الدم لدى الأطفال حديثي الولادة. أما فصيلة weak D فقد وجد أنها تعود لخلل جيني في المورثة يؤدي إلى ضعف في إنتاج RhD ما يؤدي إلى تقليل كمية هذا الأنتجين على سطح الخلايا الحمراء بالرغم من أنه يحمل الصفات الطبيعة للأنتجين الكامل ولا يؤدي هذا النوع في العادة لأي من المضاعفات الطبية السابقة.

في هذه الدراسة قمنا بتحليل الحمض النووي DNA والمستخلص من 102 من المتبرعين بالدم والذين يحمل بعضهم الفصيلة الموجبة وبعضهم الآخر الفصيلة السالبة، وأجرينا فحوصات مسحية على كافة العينات لتحديد D^{VI} وDNB بين هؤلاء المتبرعين باستعمال تقنيات البيلوجيا الجزيئية PCR المتطورة، وكانت النتائج وجود 3 متبرعين يحملون النوع الأول و3 متبرعين يحملون النوع الثاني. بالإضافة لذلك، قمنا بتحليل ثلاث عينات لمرضى كان هناك صعوبة في تحديد فصيلة RhD لهم بالفحص المصلي، وهما امرأتان في حالة ولادة ومريض نقل دم وكانت نتيجة فحص الحمض النووي أنهم جميعا يحملون المورثة الطبيعية وأشارت النتائج أنهم يحملون فصيلة weak D. كذلك اشتملت الدراسة على تحديد البنية الوراثية للعينات ذات الفصائل السالبة لمجموعة المتبرعين بالدم الذين شملوا بالدراسة، ووجد أنها في الأغلب ناجمة عن إزالة كاملة للمورثة الجينية *RHD*. كما أشارت نتيجة البحث إلى وجود تطابق كامل بين الفحوصات المصلية وفحص الحمض النووي في العينات ذات الفصائل الموجبة.

مفاتيح الكلمات: النظام الريزيسي، العامل الريزيسي D، D^{VI}، DNB، Anti-D والمورثة الهجينة، PCR-SSP

Dedication

To the blessed sole of my father

To my dear warm-hearted mother

To My wife and kids, Sara, Abdullah, Yasser and Ammar

To my beloved brothers and sisters

To the Palestinian people who are steadfast and patient on the beloved land of Palestine

Thank you all. From the deepest of my heart, I express to you all my sincere love and appreciation.

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ABBREVIATIONS

a.a	Amino Acid
ASPA	Allele-Specific Primer Amplification
BFU-E	Burst Forming Unit-Erythroid
bp	base pair
CD	Cluster of Differentiation
cDNA	complementary DNA
CFU-E	Colony Forming Unit-Erythrocyte
DIC	Disseminated Intravascular Coagulation
DMA	D detected in Mali
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
epD	epitope D
Ex	Exon
F	Forward
gDNA	genomic DNA
GPB	Glycophorin B
H. A	Hemagglutination
HDN	Hemolytic Disease of the Newborn
HGH	Human Growth Hormone
HTR	Hemolytic Transfusion Reaction
IAT	Indirect Anti-globulin Test
IgG	Immunoglobulin Gamma
IgM	Immunoglobulin Mue
In	Intron
ISBT	International Society OF Blood Transfusion
kb	Kilo Base
kDa	Kilo Dalton
LW	Landsteiner, Winner
MoAb	Monoclonal Antibodies
mRNA	messenger Ribonucleic Acid
N.C	Negative Control
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PCR-SSP	Polymerase Chain Reaction – Sequence Specific Primers
R	Reverse
RBCs	Red Blood Cells
RFLP	Restriction Fragment Length Polymorphism
Rh	Rhesus system
RhAG	Rh Associated Glycoprotein
RHD^{Ψ}	RHD pseudogene
S.M	Size Marker
SMP1	Small Membrane Protein 1

SNP	Single Nucleotide Polymorphism
UTR	Untranslated Region
WBCs	White Blood Cells

Chapter I

Introduction

1.1. Overview

Before 1900, it was thought that all blood was the same, a misunderstanding that led to frequently fatal transfusions of animal blood into humans and hazardous transfusions of blood between people. Human blood is not the same, people belong to different blood groups, depending upon the surface marker antigens found on their red blood cells (RBCs). These antigens are either sugars or proteins, attached to various components in the RBCs membrane, and determine an individual's blood group. Over 25 blood group systems have been defined, but the main two blood group systems are called ABO and Rh systems.[1]

The blood group system is one or more blood group antigens produced by alleles at a single locus or by alleles at loci, and are so closely linked that crossing over between them may occur. Usually, those blood group antigens can be defined and classified serologically by specific antibodies, preferentially in sera of human origin. Several hundred such antigens have been defined and classified in different ways. Recently, DNA typing has become possible for many blood group antigens that are mostly defined by single amino acid polymorphisms and expressed by proteins of the RBCs surface. Variation in DNA sequence however, is not called a blood group unless there is a defining antibody to the translated protein [2,3].

Several classifications have been used to describe antigens, proteins and genes of blood group system, but the more common classification used for blood group systems is done according to the "International Society of Blood Transfusion" (ISBT). Each blood group antigen is assigned a six digits number by the ISBT. The first three digits represent the blood group (e.g., ABO is 001, Rh is 004), and the last three identify the antigen in the order it was discovered [1,2].

Immunologicaly, the blood group antigens are self antigens that are ignored by the immune system. The membrane of each RBC contains millions of those antigens and transfusing a patient with the incorrect blood group may have fatal consequences, because donor RBCs may be destroyed by antibodies in the recipient's plasma. The rapid intravascular hemolysis which occurs in incompatible blood transfusions can precipitate severe disseminated intravascular coagulation (DIC), prolonged hypotension, acute uremia and may cause death [2].

In case of incompatible blood of pregnant woman and the fetus, the situation seems to be similar and occurs in the same manner, where this incompatibility can cause hemolytic disease of the new born (HDN). When a woman becomes pregnant, and during delivery a small amount of fetal blood enters her circulation. The exposure to the fetal antigens can readily trigger immune response, which induce antibody formation. The acquired antibodies may threaten the next fetus, and expose him to high risk by causing HDN. To manage this complicated situation and prevent immune stimulation, administration of antibodies against transfused antigens to pregnant or mother, will neutralize and mask fetal RBCs antigens, consequently remove them from the mother circulation before her immune system recognizes them.[1,4,5]

It is not possible to completely remove the danger of adverse reactions when blood from two people is mixed, but the danger can be minimized. Therefore, before a blood is administrated, it must be typed and cross matched with the patient's blood, to ensure immune compatibility, and to achieve the clinical benefits of blood transfusion.[1]

To date, twenty five blood group systems with different antigens were defined. One of the most important blood group system between them is the Rh blood group system, which was discovered in 1940. A woman had a severe transfusion reaction when she was transfused with blood from her husband following delivery of a stillborn child with erythroblastosis fetalis. Her serum agglutinated RBCs from her husband and from about 80% of Caucasian ABO compatible donors. Later, Landsteiner and Wiener found that sera from rabbits and guinea pigs immunized

with RBCs from *Macaca mulatta* (*Macacus rhesus* in the original paper) agglutinated 85% of human RBCs samples and the detected antibody was named anti-D. From that discovery, the Rh blood group has become second in importance only to the ABO blood group in the field of transfusion medicine. It has remained of primary importance in obstetrics, and is still the main cause of HDN [1,2,6].

The Rh system is one of the most and highly complex blood group systems known in humans, as many as over 49 different Rh antigens have been serologically defined. These antigens are expressed as part of a protein complex in the RBC membrane. Its expression is only found in erythroid cell line, and therefore Rh antigens are only expressed in RBCs. The composition of the complex is unknown, but it is supposed to be a tetramer, consisting of two molecules of Rh associated glycoprotein (RhAG) and two molecules of Rh proteins. The Rh proteins may be RhD (carrying the D antigen) or RhCE (carrying the C or c antigen and the E or e antigen). It is unknown whether both RhCE and RhD can be in a single complex, but in D negative individuals the complex would only contain *RhCE* [1,7,8].

The significance of Rh system is related to the facts that: **1-** The Rh antigens are highly immunogenic and of great importance for transfusion medicine **2-** The complexity of its antigens, which stems from the highly polymorphic genes that encode them. **3-** The great differences among races in the frequencies of the *RH* gene complex. According to those facts, the Rh system remains the most polymorphic and immunogenic blood group system known in humans [1,7].

However, D antigen is the most important Rh antigen; it is a mosaic comprising at least 30 epitopes. Partial RhD phenotypes occur when there is absence of one or more of these epitopes, with the remainder expressed. The D^{VI} phenotype is the most common of partial D phenotypes, lacking most D antigen epitopes. D^{VI} mothers may become immunized by transfusion with D positive blood (if typed as D positive using polyclonal typing reagents) or by fetuses which have all of the D antigens. This situation can give rise to severe HDN [9].

DNB is another partial D that presents a normal D in routine typing, indicating that DNB carriers are generally able to produce strong allo-anti-D. Anti-D titers up to 128 were observed in DNB individuals[10].

Despite the importance of the Rh antigens in blood transfusion and HDN, the function of its proteins is speculative, and may involve in 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 [1,6,11].

Hitherto, no previous studies have been touched the field of blood transfusion medicine and its adverse reactions in Gaza Strip, Palestine. The RhD typing discrepancies of blood donors, patients, mothers and babies which complicate the transfusion process are encountered. To detect and characterize the more frequent partial Ds namely, D^{VI} and DNB, and to identify the molecular basis of Rh negative phenotype, PCR-SSP and monoplex PCR were used to screen 102 positive and negative RhD blood donors, and to investigate two discrepant RhD pregnant women and one blood recipient specimens.

1.2. Aim of the study

The study aims to highlight the genetic polymorphism of the Rh blood group in Palestinians residing in Gaza Strip, and to test the visibility of implementing molecular techniques for improving and developing the blood transfusion strategies.

1.3. Objectives

- To detect the presence of the common partial Ds; D^{VI} and DNB among blood donors in Gaza strip.
- To determine the molecular basis of Rh negative among Palestinians for the first time.
- To determine the frequencies of D^{VI} and DNB variants among Palestinians residing in Gaza Strip.

- To reconfirm the phenotype of partial D, weak D and D negative, in discrepant RhD individuals, by using molecular analysis.
- To determine the concordance between serological RhD typing and DNA typing.

Chapter II

Literature Review

2.1. Historical background

At the very beginning of the 20th century, Landsteiner´s landmark discovery of the ABO blood group system, paved the way for rational transfusion medicine. The 20th century was the starting age of serology that became a dominant methodology for almost all crucial tasks in transfusion medicine. As the century comes to a close, a new technology evolved, which appears to be gradually infiltrating the field of transfusion medicine, i.e., genotyping using an array of methods from molecular biology. Serology is considered too simple and reliable, too precise and cost effective, to be replaced for any major task by genotyping methods, which are perceived as being complex and trouble-prone [12].

Several decades later, the accumulation of knowledge in the field of genotyping was breathtaking, and the options to construct meaningful, precise and cost efficient diagnostic tools were improved accordingly. The molecular genetics of most blood group antigens is expected to be completed soon, and the application of molecular techniques and the exploration of the molecular basis were instrumental for almost all recent significant contributions to the basic and clinical aspects of blood transfusion. The molecular basis of weak D has just recently been resolved, and a major part of the future of blood grouping will clearly involve genotyping [13].

2.2. Rh blood group system

Rh blood group system is a highly complex red cell blood group system. Besides the 5 major antigens (D, C, E, c, e), at least 45 independent antigens, and numerous phenotypes are identified by the corresponding antibodies [14].

The core of this complex is thought to be composed of Rh antigens and RhAG subunits, with accessory chains (CD47, LW, and GPB) that are associated with the complex by noncovalent linkages [15,16,17].

The Rh antigens and RhAG are referred to as the Rh protein family. The molecular weight of the protein family is estimated by density ultracentrifugation to be 170 000 Daltons, and is stabilized by both N-terminal and C-terminal domains. The association of the Rh protein family and the Rh accessory proteins is called the Rh complex. The mode of association of the core of complex with Rh accessory proteins, some of which interact directly with the membrane skeleton, remains undefined [6].

In the fetus, Rh antigens are expressed on RBCs from the 6th week of gestation, and seem to be expressed only in the erythroid cell line, because the attempts to express RhD on the surface of non-erythroid cells, which do not produce endogenous RhD protein, have met little success [6,18].

Allo-antibodies that recognize Rh antigens are usually IgG, and are detected by the indirect anti-globulin test (IAT). The test was formulated to detect the cell bound IgG (in vitro sensitized RBCs), by exposing them to an anti-globulin reagent. Allo-antibodies in the Rh blood group system can cause destruction of transfused RBCs and of fetal RBCs in HDN [6].

2.3. Molecular basis of Rh system

The ability to clone complementary DNA (cDNA) and sequence genes encoding the Rh proteins has led to an understanding of the molecular basis associated with some of the Rh antigens. An intense investigation has yielded considerable knowledge of the molecular background of Rh system. The genes encoding 2 distinct Rh proteins that carry the D antigen, and C or c together with either E or e antigens, have been cloned. In addition, a related gene that expresses RhAG protein which is essential for assembly of the Rh protein complex in the erythrocyte membrane and for expression of Rh antigens, and other related genes has been defined [3,19,20].

2.3.1. *RHD* and *RHCE* genes

The two closely and tightly linked homologous *RHD* (*RH* 30) and *RHCE* genes are located at chromosomal position 1p34.1-1p36, respectively. They are 97% identical, have opposite orientation with the 3' ends of the two genes facing each other and the two genes are inherited together (Figure 2.1.). Both genes encompass 10 exons ranging in size from 72 to 247 bp, distributed over 75 kb DNA sequence, and their structures exhibit similar exon-intron organization. Many genetic variations between *RHD* and *RHCE* genes have been recognized, for example *RHD* gene has a larger exon 10 than the *RHCE* gene, and the *RHCE* gene has a larger intron 4 than the *RHD* gene [1,21,22,23,24,25].

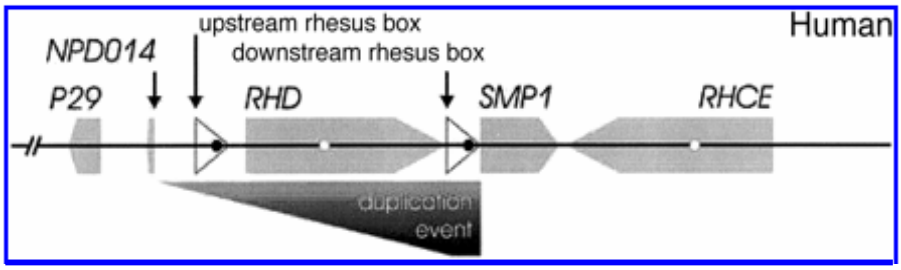

Figure 2.1. *RH* locus in human. The orientations and positions of genes are indicated by gray arrows, the rhesus boxes by white triangles. An open reading frame (ORF) in the rhesus box is depicted as a black circle. During the duplication event, *RHD* and its rhesus boxes are inserted between *NPD014* and *SMP1*[13].

The open reading frames of *RHCE* and *RHD* occur in opposite orientation, their 3' ends are separated by about a 30kb region that contains the *SMP1* gene (Small Membrane Protein 1). In addition, two 9 kb highly homologous sequences, named "*Rhesus Boxes*" flank the *RHD* gene and are located at the 5' and 3' ends of *RHD* gene. *RHD* gene encodes the D antigen, while the *RHCE* gene encodes CE antigens and exists in four allelic forms, each allele determines the expression of two antigens in Ce, ce, cE or CE combinations [23,26,27].

Previously, Rh Cc and Ee antigens have been predicted to be expressed on different polypeptide chains, by a mechanism of alternate splicing (exon

skipping), the Rh Cc transcripts are proposed to lack exon 5, which encodes residues critical for Rh E/e antigenicity. Recent evidence refutes this hypothesis; a full length Rh cE transcript was expressed with both Rhc and E antigens. In addition, Rh Cc proteins have been shown to be comprised of full length, not truncated polypeptide chains [25].

C/c polymorphism arises from four SNPs that cause four amino acid changes, and the E/e polymorphism arises from a single SNP (676 G→C) that causes a single amino acid change (Pro226Ala) in the Rhe polypeptide. The D/d polymorphism most commonly arises from a complete deletion of the entire *RHD* gene [28,29,30].

Also, there are many uncommon fusion genes, comprising parts of *RHCE* and parts of *RHD*, which may encode abnormal D and CcEe antigens and one or more low frequency Rh antigens [23,27].

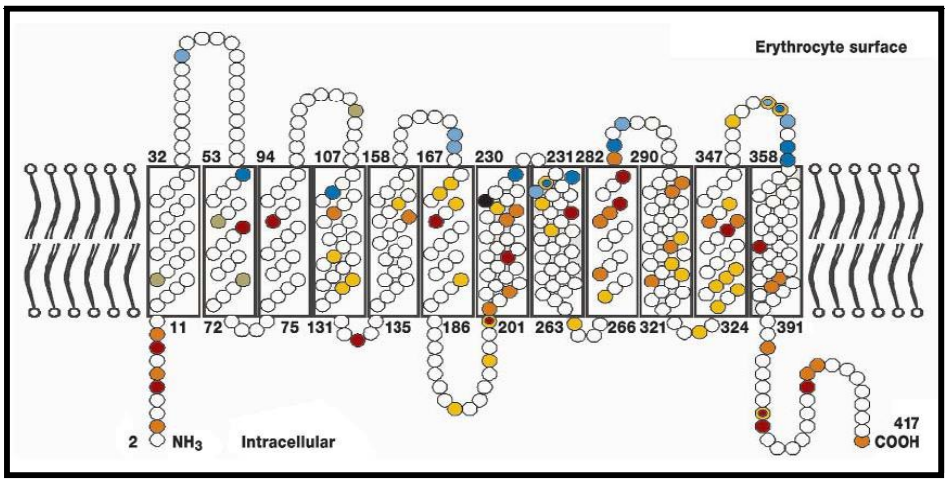

Figure. 2.2. Distribution of Rhesus protein in the erythrocyte membrane. Mature proteins in the membrane lack the first amino acid. The amino acid substitutions that distinguish the RhD from the RhCE protein are shown in yellow. Four amino acids code the C antigen in green, One a.a codes the E antigen in black. Amino acid substitutions that code partial D are in blue. Amino acid that codes for weak D is in red [4].

The transcription product of each *RHD* and *RHCE* is determined by approximately 1,251 bp, and each Rh product predicts a 30 kDa protein consisting of 417 amino acids (differ by 35 amino acids as a result of 44

nucleotide substitutions in the coding sequence), with 12 membrane spans, 6 extracellular loops, 7 intracellular protein segments and cytoplasmic N and C termini (Figure 2.2.) [24,26].

2.3.2. *RhAG* related gene

RhAG (Rh 50), the product of a single copy gene *RHAG,* is essential for the presentation of the Rh antigenic activity. *RHAG* gene is located at position 6p11-21.1, encompasses 32 kb, organized into 10 exons and shares 36% sequence identity with *RHD/RHCE* genes. *RhAG* protein product consists of 409 amino acids, the amino acid sequence homology (approximately 40%) of the Rh protein and RhAG protein indicates an ancestral relationship between the *RhAG* gene and *RHD/RHCE* genes [6,15,23].

While the *RHD/RHCE* locus harbors a large repertoire of allelic diversity at the level of population, alterations in the *RHAG* locus are relatively rare. *RhAG* is not known to possess a protein based blood group polymorphism, but several mutations in *RHAG* have been defined that cause the regulator type of Rh deficiency syndrome (Rh_{null}) [6,23].

RHAG gene mutation has been detected by sequencing cDNA clones from *RHAG* mRNA, which revealed a single base change (836 G→A) yielding a missense mutation (Gly279Glu) within a predicted hydrophobic domain for the membrane protein. The Rh_{null} encountered was a composite heterozygote for this mutation carrying two alleles with the A and G at nucleotide 836. Only the A836 mutant allele was present, suggesting that the second allele with G836 was apparently silent (no transcript detected). The *RH50* mutant allele (836A) was inherited maternally, whereas the silent *RH50* allele (836G) was inherited paternally. In Rh_{mod}, one other single nucleotide change in the *RHAG* gene has been detected. The nucleotide change resulted in a serine to asparagine change at position 79 along the RhAG glycoprotein. It is likely that a range of substitutions along the *RH50* gene may be responsible for the varying degree of

modulated Rh antigen activity. These observations promote the evidence that rare but diverse genetic alterations may occur along the *RHAG* gene [31].

2.3.3. *Rhesus box*

The *RHD* gene is flanked by 2 DNA segments, dubbed *Rhesus boxes*, with a length of approximately 9000 bp (Figure 2.1.). The two segments are 98.6% homologous, and have identical orientation. The upstream *Rhesus box* (5' of *RHD*) is approximately 9142 bp long and ends approximately 4900 bp 5' of the *RHD* start codon. The downstream *Rhesus box* (3' of *RHD*) is 9145 bp long and originates 104 bp after the *RHD* stop codon. While there is an overall 98.6% homology between both *Rhesus boxes*, a 1463-bp absolute identity region located between positions 5701 and 7163 bp is completely identical. The breakpoint region of the *RHD* deletion is located in the 903 bp identity region of the *Rhesus box* [21].

In haplotypes with an *RHD* deletion, the fusion of the two *Rhesus boxes* generates the single hybrid *Rhesus box* (Figure 2.3.).The detection of which has been applied for *RHD* zygosity determination. Aberrant *Rhesus boxes* can appear to be frequent among African individuals [32].

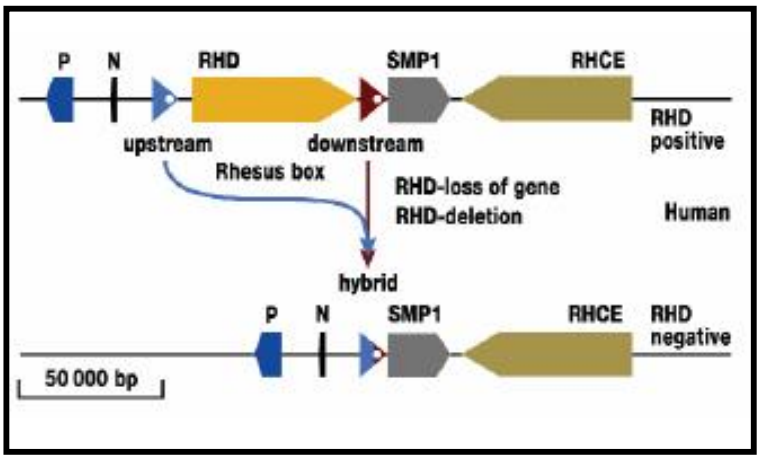

Figure 2.3. Deletion of the *RHD* gene.
Deletion of the *RHD* gene results from recombination between an upstream and a downstream Rhesus boxes. An individual homozygous for this haplotype is D negative [4].

2.3.4. *SMP1* gene

The *SMP1* gene has 7 exons with about 20 000 bp total gene size, located on chromosome 1 (Figure 2.1.). *SMP1* gene expression yields an 18 kDa small membrane protein, and its function is as yet unknown. Its position between both *RH* genes implies that, any polymorphism of the *SMP1* gene would be tightly linked to a specific *RH* haplotype. It might be speculated that mutations of the *SMP1* gene may cause selection pressure for or against specific *RH* haplotypes. Such factors might explain some unresolved issues of *RH* haplotype distribution, such as the high frequency of specific *RH* in the European population [21].

2.3.5. *RHD* pseudogene *(RHD^{Ψ})*

About two-thirds of D negative Africans have an inactive *RHD* gene called *RHD* pseudogene (*RHD^{Ψ}*). This *RHD^{Ψ}* gene has a 37 bp insert in exon 4 at position 210, which may introduce a reading frame shift, stop codon in exon 6 and premature termination of translation. In D negative African Americans and South African people of mixed race, 24% of African Americans and 17% of South African donors of mixed race have *RHD^{Ψ}* [33].

2.4. RhD antigen

The most important, immunogenic and polymorphic Rh from the clinical aspect is the RhD, which plays a key role in immunohematology and transfusion medicine [6,8].

2.4.1. RhD epitopes mapping

The D antigen comprises several different antigenic epitopes (at least 30 epitopes), which are conformation dependent. Specific amino acids are dispersed along the entire RhD protein. Eight amino acids are located on the exofacial surface, and seem to be crucial for epitope determination, for example, the following amino acids are crucial for epD3a (Asp350), epD3b (Asp350 +

Gly353), epD4a (Gly353 + Ala354), epD4b (Ala354), epD9a (Asp350 + Gly353 + Ala354) and epD9b (Asp350 + Ala354) expression. All of these amino acids reside on the predicted external domain of the RhD protein. Twenty four amino acids are predicted to reside in the transmembrane and cytoplasmic domains (Figure 2.2.). Any alteration of the specific amino acids as a result of genetic mutation will affect the D protein conformation and subsequently epitopes topology [6,7,34].

The number of copies of the D antigen may reach 16,000 per RBC, and it appears to be expressed early during erythropoiesis. Anti-D binds to approximately 3% of Burst forming unit-erythroid (BFU-E), 68% of Colony forming unit-erythrocyte (CFU-E), and to all of the more mature erythroid cells. The small d which was thought to be antithetical to D does not exist, but usually is used to indicate the D negative phenotype [6].

The genetic analysis of the Rh system, led to the design of therapeutically useful reagents that modulate antibody binding, and provide relevant information regarding the structural organization of RhD epitopes. There are at least 37 accepted RhD epitopes, defined by differential agglutination of RhD variant RBCs. The original model consisted of 9 D epitopes, but has been expanded to consist of 37 epitopes. Classically, these epitopes are believed to cover different regions of the RhD molecule. Most RhD epitopes differ, not spatially, but only in the number and arrangement of their potential contact residues. The absence of D epitopes may not always be a direct result of the change in molecular structure. The presence of Rh proteins encoded by *cis* e.g., *RHCE* and *trans* e.g., *RhAG* genes can effect the binding of certain monoclonal anti-D [6.,35,36].

2.4.2. Clinical importance of RhD

Individuals who do not produce the D antigen, will produce anti-D if they encounter the D antigen on transfused RBCs causing significant hemolytic transfusion reaction (HTR), or on fetal RBCs causing HDN resulting in hydrops fetalis, brain damage due to increased levels of bilirubin (kernicterus), and fetal death [27,37].

Although, infant mortality due to HDN has been dramatically reduced by passive administration of anti-D immunoglobulin to women at risk, allo-immunization still occurs in 1% to 1.5% of women at risk. Moreover, aberrant *RHD* alleles encoding variant antigen D, can cause typing problems and permit immunization by a normal antigen D. For those reasons, the RhD status is routinely determined in blood donors, transfusion recipients, and in pregnant women [1,38,39,40].

Allo-antibodies that recognize RhD antigens are usually IgG, they rarely, if ever, bind complement, and therefore RBCs destruction is mediated almost exclusively via macrophages in the spleen (extravascular hemolysis) [1,6].

2.4.3. Genetic polymorphism of RhD

2.4.3.1. RhD negative trait

The frequency of RhD negative phenotype varies widely in different parts of the world. The highest percentage of RhD negative was found in white donors (17.3%), and depending on the population, it ranges between 3%-25%. RhD negative phenotype is found in less than 0.5% of Asian people, 3%-7% in Africans, in only 0.1%-0.4% of the population of China, in 0.5% of Japanese and in 17% of English people [1,21,22,27,41].

The RhD negative trait could be generated by multiple genetic mechanisms and is ethnic group-dependent. There are actually three major genetic mechanisms associated with the D negative phenotype: 1- Complete or partial deletion of *RHD* (Figure 2.3.). 2- An *RHD* pseudogene **(***RHD*$^{\Psi}$) containing a 37 bp insert. 3- A hybrid *RHD-CE-D* gene that probably produces an abnormal C antigen but does not produce a D antigen (Figure 2.4.). Generally, the *RHD* gene deletion accounts for almost all D negative phenotypes [7,24,41,42,43].

In addition, a point mutation in the intact *RHD* gene can generate a silent allele and subsequently Rh negative phenotype. Studying the genetic diversity of Rh, by analyzing intron 4 of the *RHCE* and *RHD* genes, and exon 10 of the *RHD* gene derived from genomic DNA samples (n= 357) expressing D negative phenotypes by using multiplex PCR assay showed that, five RhD negative

individuals had an apparently intact but dysfunctional *RHD* gene. Sequence analysis of transcripts obtained from one of these individuals illustrated the presence of full length *RHD* transcripts, which have a point mutation at nucleotide 121C→T, generating a premature stop codon (Gln41Stop). A nonsense mutation and in some cases four nucleotides deletion in exon 4 of the *RHD* gene can generate Rh negative phenotype [25].

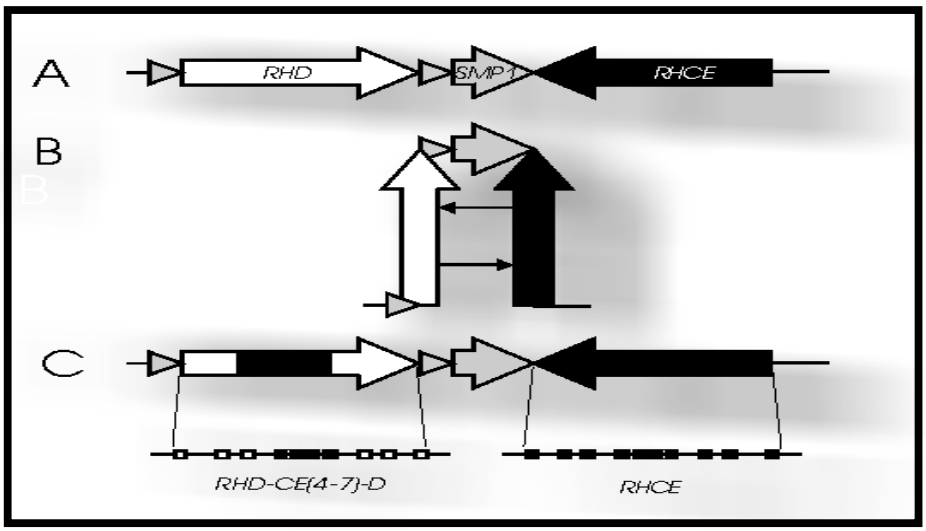

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Figure 2.4. Proposed mechanism of gene conversion in *cis*.

The *RHD* and *RHCE* genes are inversely orientated as typical for clustered genes. A putative hairpin formation of the chromosome allows the close proximity of homologous segments to be in identical orientation. This structural feature is instrumental for gene conversion events in *cis*. Resolving the hairpin yields an *RHD-CE-D* hybrid gene structure [13].

Among Europeans, the presence or absence of the antigen D on the RBCs correlates closely with the presence of the "standard" *RHD* allele or a deletion of the whole *RHD* gene respectively. Later, a new *RhD* negative allele has been detected between D negative Europeans, that may be generated by gene conversion, for which a mechanism triggered by hairpin formation of chromosomal DNA (illustrated in Figure 2.4.). Also, an intact but dysfunctional

RHD gene was reported in a small number of phenotypically D negative Caucasians [24,39,44,45].

In non-Whites, it has been found that D negativity can appear in individuals carrying the complete *RHD* gene, those individuals of Black or Asian origin, exhibit either an internal duplication or a deletion within the *RHD* gene, resulting in a premature stop codon in *RHD* transcripts [7,33].

In Asians, the RhD negative phenotype may be associated with a Gly314Val missense mutation, and several other *RHD-CE-D* hybrid alleles. In the African population, a significantly higher proportion (up to 60%) of serologically D negative individuals carry *RHD* genes compared with Europeans, the D negative Africans often carry *RHD* alleles like RHD^{ψ} which harbor large remnants of the *RHD* gene [24,39,45].

2.4.3.2. Rh_{null} phenotype

RhAG protein must be present to direct the Rh antigens to the RBCs membrane, if it is missing, none of the Rh antigens will be expressed. The RhD antigen polymorphism generally arises from complete absence of the *D* gene, rare individuals (approximately 1 in 6×10^6 individuals) lack all of the Rh blood group antigens and are called Rh_{null} or Rh_{mod} [1,6,31,46].

Rh_{null} phenotype is also known as Rh deficiency syndrome. This autosomal recessive disorder manifests chronic hemolytic anemia in which the RBCs have a stomatocytosis morphology, an increased osmotic fragility, an altered ion transport system and abnormal membrane phospholipids organization [23,31].

Two classes of Rh_{null} types exist and arise from independent genetic events and may be classified as regulator or amorph types. The amorph type is caused by homozygosity for a silent allele at the *RH* locus, whereas the more common regulator type is apparently caused by homozygosity for an autosomal *RhAG* gene. The Rh_{mod} phenotype reflects either incomplete penetrance of *RHAG* mutations or other unknown mutations [6,23,31].

Three types of mutations have been shown to cause the Rh_{null} phenotype, missense mutation, small exonic deletions or splice site mutations. Missense

mutations result in single amino acid replacements, whereas the other two types eventually lead to frame shift and premature chain terminations. In the amorph type of Rh_{null}, a homozygous mutation in the *RHCE* gene generally occurs on the genetic background of *RHD* gene deletion. In the regulator type Rh_{null}, the location of mutations appears to be clustered in the *RHAG* gene. In one subject, the Rh_{mod} phenotype has been shown to result from a defective translation initiation [23].

2.4.3.3. Weak D

The weak D phenomenon was first observed in 1946. This antigen was formerly called D^u, later weak D. The difference between the normal D and weak D phenotype was gradually realized to be quantitative, not qualitative. The weak D is found in less than 1% of Caucasians and is only slightly more common in African Americans. More than 20 weak D types can be distinguished, and the total number of weak D types may exceed 54 types [26,43,47].

In 1999, the molecular basis of weak D was found, and a highly prevalent genetic heterogeneity in the weak D phenotype was detected. Weak D alleles evolved independently in the different haplotypes, each distinct event being associated with a change in the RhD protein sequence. Typically, the weak D phenotype is caused by a single amino acid switch in the transmembrane region of the RhD protein, which leads to reduction of the expression level of RhD antigen. All weak D types displayed antigen densities between 70 and 4000 RhD antigens per RBC, and there was no simple relation of the type of substitution to the antigen density. The amino acid substitutions of weak D types are located in intracellular and transmembraneous protein segments (Figure 2.5.), and clustered in four regions of the RhD protein. Substitutions of amino acid are located at positions: 2 to 13, around 149, 179 to 225, and 267 to 397 of protein sequence [3,25,26,40,43,48].

Some weak D may carry structurally abnormal *RHD* alleles. *RHD* intron 4 was deleted, and its PCR products were not detected in 4 of 44 English weak D phenotype, and in one of 90 Northern German weak D, no *RHD* specific exon

5 PCR products had been detected. It is probably that, the weak D phenotype individuals who type as *RHD* intron 4 negative, are D^{VI} phenotypes [25,49].

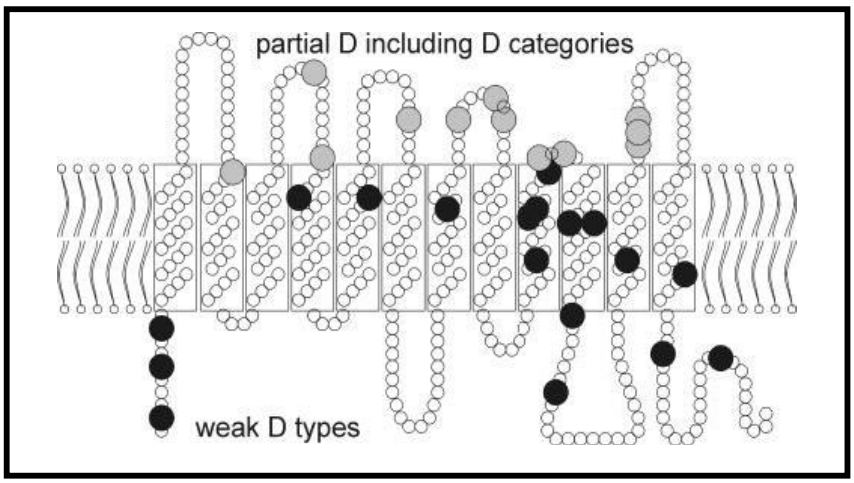

Figure 2.5. Specific amino acids location in weak D and partial D
Amino acid positions affected in weak D types are shown in black; those affected in partial D, are shown in gray. Amino acid substitutions in weak D are located in the intracellular or transmembraneous protein segments, those found in partial D phenotypes in the extracellular protein segments[43].

In more extensive molecular screening by polymerase chain reaction using sequence specific primers (PCR-SSP), about 2.5% structural abnormalities in 600 weak D samples were found. Another fraction of weak D is found to be caused by the suppressive effects of Cde haplotypes in *trans* position. Investigation of 161 weak D European DNA samples, based on sequencing all the 10 *RHD* exons, polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and polymerase chain reaction using PCR-SSP revealed that, none of the samples had a normal *RHD* exon. The exons sequencing has detected 16 different molecular weak D types between the examined samples [49].

Despite the increasing sensitivity of monoclonal anti-D reagents which often typed weak D as normal RhD, some weak D antigens like D^{el} antigen may not be agglutinated by all monoclonal anti-D. The D^{el} phenotype is the extreme form of weak D, in which the antigen D is expressed so weakly that it could only be demonstrated by adsorption and elution techniques [6,26,49].

In most weak D cases, adequate levels of D antigen are present and there has been no change in D epitopes, weak D RBCs have all D epitopes, which are however, weakly expressed. Based on this fact, the formation of anti-D is suppressed, therefore, individuals with the weak D phenotype can receive RhD positive blood, and no more needs for post partum anti-D as prophylaxis for weak D mothers [1,26,43].

2.4.3.4. Partial D

In the field of blood transfusion, it is surprising to find RhD positive individuals with anti-D. It's known that, the D antigen comprises several different antigenic epitopes. Rare individuals carry a partial D antigen and may produce allo-antibodies directed against D epitopes that are lacking in their RhD protein. Those D variants or partial D individuals lack one or more D epitopes, with the remainder expressed which can immunize them on exposure to normal D positive erythrocytes during blood transfusion or pregnancy. Initially, partial D individuals have been classified into seven distinct categories (D^{II} to D^{VII}, D^{I} being obsolete) based on the mutual reactivity with polyclonal anti-D sera from immunized partial D carriers. Today, characterization of partial D is performed by differential reactivity with monoclonal anti-D antibodies [1,8,9].

The study of partial D at the serological and molecular levels have resulted in the prediction of regions of the D protein involved in epitope presentation, however, these predictions are hindered by the fact that most partial Ds arise through multiple changes in the RhD protein sequence, some of which may have no effect on D antigenicity [37].

Some partial D alleles appeared to be quite frequent, for instance anti-D immunizations in D positive individuals were reported to be frequent in Europeans. Among Europeans, 17 variant *RHD* (partial D) alleles were found by a PCR-SSP screen, and the population frequency of all known partial D phenotypes combined is less than 1% [39,50].

In White population frequencies of D^{VII}, D^{VI}, D^{IV}, D^{V}, D^{II}, and DFR are 1:900, 1:6,800, 1:10,000, 1:30,000, 1:30,000, and 1:60,000, respectively, as established with serological methods [7].

The situation is more intricate in Africans, where the occurrence of aberrant *RHD* alleles and anti-D immunizations in D positive individuals is much more frequent than in Europeans. Anti-D immunization in transfusion recipients and pregnant women harboring African partial Ds is a continuing problem, for example, 11% of anti-D immunized pregnancies in the Cape Town area (South Africa), occurred in D positive women. The serologic testing of RhD is confounded by frequent African alleles that almost defy serologic recognition. Molecular analysis revealed that, in Africans, there are often multiple missense mutations, rather than single ones [48].

The molecular basis of the partial D phenotypes is often *RHD/CE* hybrid alleles, in which parts of the *RHD* gene were substituted by the respective segments of the *RHCE* gene, It can arise from replacement of *RHD* exons by their *RHCE* counterparts, as has been shown in D^{IIIb}, D^{IIIc}, D^{IVb}, D^{Va}, D^{VI}, DFR, and DBT. Partial D phenotype can also arise due to single missense mutations associated with amino acid substitution in exofacial positions (Figure 2.5.), or dispersed missense mutations in the *RHD* gene as exemplified in D^{II}, D^{IVa}, D^{VII}, DNB, DHMi, DNU, and DHR [7].

The D^{Va} (Hus) which was investigated through cDNA analysis, revealed an *RHD-CE(5)-D* hybrid allele. In D^{IVa} phenotype that lacks epD 1, 2, 3, and 9, there is a rearrangement of exon 3 and part of exon 7 of the *RHD* gene. All *RHD* genes in individuals expressing the D^{VI} phenotype appear to lack intron 4 of the *RHD* gene [20,25,51].

The structure of the *RH* gene locus strongly favors the generation of hybrid alleles of a *RHD-CE-D* type. More than 20 partial D alleles of this type have been described, and the substituted gene segments range from a few to more than 10,000 bp. The *RHCE* intron 4 contains an *Alu* repeat element, which is widely dispersed in mammalian genomes. The absence of such a repeat from the *RHD* gene could potentially explained why this gene serves as the acceptor of gene

conversion events. Moreover, inhibition of gene conversion events by *Alu* repeat elements may explain why the *RHCE* gene serves as the donor in the majority of *RH* gene conversion events generating partial D [25,43].

More than 10 partial D variants are caused by single missense mutations located in the extracellular loops of D protein, the phenotypes of these partial D are much more diverse than the phenotypes caused by gene conversion and determined by the localization and type of substitution. Generally, less epitopes are affected in missense mutations than in partial Ds that were caused by gene conversions. The presence of certain *RHD* regions in hybrid genes encoding partial D antigens may predict a D negative phenotype, and the presence of some *RHD* regions in genes encoding no D antigen may predict a D positive phenotype. In order to avoid these complications, methods which detect more than one region of *RHD* alleles have been introduced [33,43].

It is important to identify partial D phenotype, and usually individuals with this phenotype are difficult to identify. In most cases false negative typing has been encountered. People who have been identified as having the partial D phenotype, should not receive RhD positive blood, because allo-antibodies may be produced against missing epitopes when the partial D individuals exposed to the complete antigen by blood transfusion or during pregnancy [1,7,26].

Therefore, for blood transfusion recipients and pregnant women, it is a common practice to use a procedure that will classify RBCs with partial D antigens as D negative. Thus, blood donated from such a person should be labeled as D positive, but the same person should be listed as D negative when they are recipients in need of transfusion [6].

2.5. Types of Partial D

Anti-D immunizations are known to occur infrequently in D positives, often in individuals with such a low antigen D density. Usually these cases could be traced to a few partial D phenotypes, especially D^{VI}. Most, but not all partial D can be identified by the lack of reactivity with certain monoclonal anti-D antibodies, which is interpreted as lack of certain D epitopes. The increasingly elaborated D

epitope schemes allowed the identification and classification of many new partial Ds [40].

The most common Partial Ds (Table 2.1.) that detected between different populations and ethnic groups are:

2.5.1. D^{VI}

D^{VI} phenotype is the most common and the most clinically significant partial D phenotype in transfusion medicine. D^{VI} lacks most epitopes compared to any other D variant. It is missing epD1, 2, 5-8 (using the 9 epitope model) or epD1-4,7-22, 26-29 (using the 30 epitope model), with very low RhD antigen density, (500 antigen/cell in D^{VI} type I) and (2,400 antigen/cell in D^{VI} type II) [8,9].

This partial D is still considered the major cause of allo-immunization with anti-D in RhD positive individuals. The D^{VI} phenotype has a frequency ranging between 0.02% to 0.05% in Caucasians and 1:6,200 (about 0.02%) in Germany, and it has also been observed in the English people [8,52,53].

The molecular basis of the D^{VI} phenotype has been proposed to occur by different genetic mechanisms. Individuals with D^{VI}Ec haplotype were initially assumed to carry a deletion of exons 4 and 5, but in fact, a gene conversion event generates a hybrid gene composed of exons 1-3 of the *RHD* gene, 4-5 of *RHCE* gene, and 6-10 of the *RHD* gene (classified as D^{VI} Type I). [8,9,54]

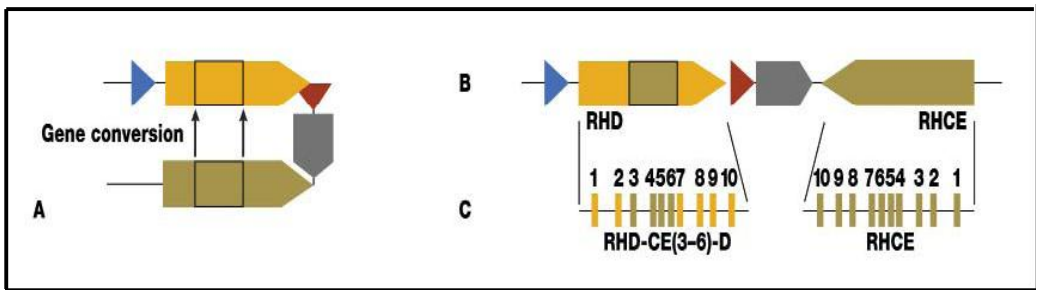

Figure 2.6. Category D^{VI} as a result of gene conversion.
The two *RH* genes lie on their chromosome locus in opposite directions. When the chromosome folds, the two *RH* genes are adjacent, the two loci in the same direction. This configuration allows gene conversion in *cis*, whereby a DNA segment is transferred from one gene to another. The middle section of the *RHD* gene (yellow) is substituted by the corresponding homologous section (green) of the *RHCE* gene [4].

D^{VI}Ccee individuals phenotype has a gene conversion event that generates a hybrid gene composed of exons 1-3 of the *RHD* gene, 4-6 of *RHCE*, and 7-10 of the *RHD* gene (classified as Type II), (Figure 2.6.). The D^{VI}Ccee phenotype is never generated by a partial *RHD* gene deletion [8,9,54].

In D^{VI} type II, the two breakpoints were identified precisely, they are located in intron 3 of *RHCE* gene within a 250-bp fragment comprising an *Alu* sequence, and within a 39 bp fragment in intron 6 of *RHCE* gene. This *Alu* sequence (and the 100 bp region immediately downstream) most likely defines a recombination hot spot [55].

The hybrid *RHD-RHCE-RHD* transcripts have been found in both D^{VI}Ce and D^{VI}cE haplotypes. The D^{VI}Ce transcripts are derived from an *RHD* gene where exons 4-6 have been replaced with *RHCE* equivalents (encoding Ala_{226}). The D^{VI}cE transcripts are derived from an *RHD* gene where exons 4 and 5 are replaced by *RHCE* equivalents (encoding Pro_{226}). This indicates that, residues encoded by *RHD* exons 4 and 5 are involved in epD1-2, 5-8 expression. Exon 6 of the *RHD* gene encodes only two amino acid differences to the *RHCE* gene (Val_{306} and Tyr_{311}), and these are likely to be located toward the internal face of the erythrocyte membrane, the amino acid residues encoded in exon 6 are not directly involved in expression of epitopes absent from D^{VI} phenotype erythrocytes [9,54].

A new D^{VI} {*RHD-Ce(3-6)-D*} hybrid allele dubbed D^{VI} type III has been detected in the Germans and is associated with an almost normal number of RhD proteins accessible on the RBCs surface (12,000 antigens/cell). PCR-SSP was used to determine the molecular basis of this new allele, complete exons 3, 4, 5, and 6 of *RHD* gene were replaced by the corresponding exons of the *RHCE* gene [8].

Another novel D^{VI} allele, named D^{VI} type IV (8,000 antigens/cell), was found in the Spanish population, and characterized as *RHD-Ce(3-5)-D* hybrid where exons 3, 4 and 5 of *RHD* gene were replaced by the counterparts of the *RHCE* gene. D^{VI} type IV is the most frequent D^{VI} variant in Spanish and has also been detected in the Portuguese population [53].

Based on the molecular structure, the clinical importance of D^{VI} becomes clearly apparent in severe cases of HDN that have occurred in D Positive babies born to D^{VI} mothers with anti-D. Transfusion strategies were devised to ensure D negative transfusion in carriers of D^{VI}. In the United Kingdom, D typing reagents (monoclonal anti-D) are adopted to type D^{VI} patients as D negative. Thus, after pregnancy D^{VI} phenotype mothers are given prophylactic anti-D [6,9,48,53].

2.5.2. DNB

DNB {The name derived from DNU-like and Bayern (Bavaria, Germany)} is a partial D that is caused by an *RHD* (Gly355Ser) allele associated with *CDe* haplotypes. Studying the molecular genetics of DNB by sequencing the 10 *RHD* exons and PCR-SSP to detect the abnormality in *RHD* exon 7, a single point mutation (1063G→A) that causes Gly to Ser substitution at codon 355 have been detected. The mutation causes loss of epitopes epD6 and epD31 as well as part of epD18 and epD23. The affected amino acid was located in the exofacial loop 6, adjacent to the mutations observed in D^{II} (Ala 354 Asp) and DNU (Gly 353 Arg). DNB phenotype presents a normal D in routine typing and the antigen density is about 6000 D antigens per RBC. Anti-D immunization events with allo-anti-D titers up to 128 were observed in DNB individuals from Central Europe, indicating that DNB carriers were generally able to produce strong anti-D antibodies. In routine D typing, DNB samples typed as normal RhD without noticeable weakening of the reaction. DNB, however, was the underlying partial D phenotype in a relevant fraction of anti-D immunizations occurring in Whites. Therefore, a serological strategy for detecting DNB would have to rely on a separate anti-D to discriminate DNB from normal D [10].

Screening of random samples of blood donors in 4 European populations (1118 donors from Germany, 500 D positive donors from Italy, 693 donors German speaking Switzerland and 768 donors from Denmark) revealed that, DNB was the most frequent partial D recognized so far in Whites, occurring with frequencies of up to 1:292 in Switzerland and 1: 351 in Germany [10.26].

2.5.3. DAR

This D variant consists of a *D* allele with 3 point mutations on polymorphic sites, in which *RHD* specific nucleotides are replaced by *RHCE* specific ones. The aberrant allele is linked with a variant *ce* allele, called *ceDAR*. Serologically, this D variant shows weaker reactions with a monoclonal anti-D and with polyclonal anti-serum used for routine screening, indicating weak D expression. DAR is highly prevalent in Blacks, 4.9% of South African Blacks are carriers of a *DAR* allele, and 1.5% had the homozygous gene or in combination with a D negative allele, but no Whites carrying this variant have been found. The DAR phenotype was characterized by complete loss of at least 9 of 37 RhD epitopes. DNA Sequence analysis showed a partial D allele with only 3 mutations 602C→G (exon 4), 667T→G (exon 5), and 1025T→C (exon 7). Screening 326 blood donors from South African Blacks by PCR showed that, five of these donors (1.5%) had the DAR phenotype, and they carried homozygous *DAR* allele. Immunogenicity of the D antigen for individuals with the DAR phenotype was proven, where individuals with DAR phenotype produced allo-antibodies against RhD after multiple transfusions with D positive blood. In a multiethnic society, it was recommended that, people expressing DAR should be carefully distinguished from D negative donors by the use of selected reagents [7,39].

2.5.4. RhD^{el}

D^{el} is defined by expressing trace amounts of antigen, and characterized as RhD negative by using a conventional serological test. The genetic characteristics of *D*^{el} and its expression show no differences between the *D* and *D*^{el} gene except that, at the *Bsp*HI site of exon 9 where the *D*^{el} gene lacks the *Bsp*HI site. More investigation showed that, *D* and *D*^{el} genes were similar at exon 7 and 8, but there was an exon 9 deletion of *D*^{el} gene. The *D*^{el} gene had a 1,013-bp deletion between introns 8 and 9, including whole exon 9. The allelic

polymorphism studies described three alleles representing the D^{el} phenotype, which were characterized by one missense and two splice site mutations [45,56].

The prevalence of the D^{el} phenotype among European populations is small. D^{el} individuals will develop anti-D antibodies when transfused RhD positive blood several weeks after transfusion. From this point RhD^{el} patients should be recognized as a type of RhD negative [47,56].

2.5.5. DAU

Five *RHD* alleles that shared a (Thr379Met) substitution were detected in Africans with partial D phenotypes. *DAU* alleles may be a major cause of antigen D variability and anti-D immunization in patients of African descent. Four of these alleles expressed a partial D phenotype characterized by the lack of distinct D epitopes or by an anti-D immunization event. These alleles constituted a cluster, because they shared a 1136C→T single nucleotide polymorphism (SNP) causing a (Thr379Met) substitution. Thr379Met was only found in *DAU-0* which represented the primordial allele of the *DAU* allele cluster, and the other 4 *DAU* alleles harbored one or 2 additional substitutions dispersed in the various segments of the protein (Figure 2.7.) [48].

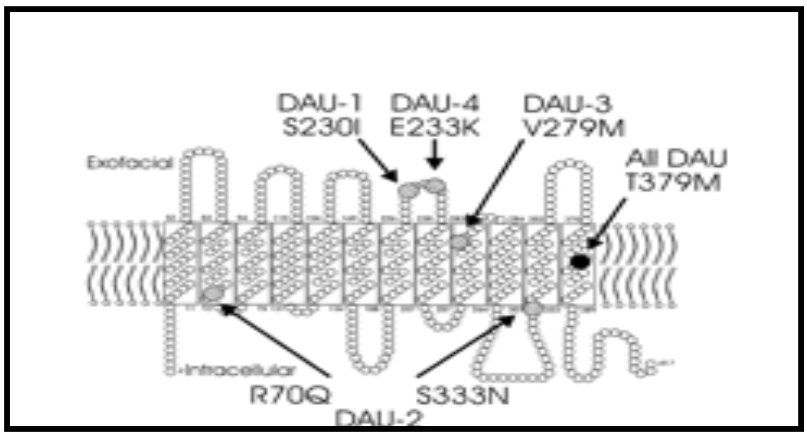

Figure 2.7. Schematic representation of the RhD proteins observed in 5 DAU phenotypes. All DAU types share a Thr379Met substitution (black disk) that is located in the transmembrane protein segment. DAU-1 to DAU-4 have additional substitutions located in exofacial loop 4, substitutions of DAU-2 position near the

border of intramembrane and intracellular protein segments, substitution of DAU-3 is located at an intramembraneous protein segment proximal to exofacial loop 5 [48].

The *DAU-5* allele was found among D positive Europeans, which represents a recombination between two known alleles of the *DAU* cluster. The antigen density of the DAU-0 phenotype was about normal, rendering it indistinguishable from the normal antigen D positive phenotype. DAU-0 had a normal D positive epitopes pattern. The other 4 DAU phenotypes however, are classified as partial D. The D epitopes patterns of the DAU phenotypes were distinct. Despite DAU-1 cells having a much higher antigen density than DAU-2 cells, there are more anti-D agglutinated DAU-2 RBCs than DAU-1. All *DAU* alleles are infrequent in Whites. The frequency of the DAU-0 phenotype in the German population was 1:3843 [26,48].

2.5.6. DNU and D^{II}

Two partial Ds, which both lack epD4 and epD9 were named the DNU and D^{II} phenotypes. Both have been originally classified as D^{II} phenotype, but subtle differences have been revealed in the serological profile of these two partial Ds. Differential reactivity and determination of the molecular basis of these phenotypes allowed the prediction of critical amino acids for epD3, epD4 and epD9 expression. The DNU phenotype arises from a single point mutation in exon 7 of the *RHD* gene resulting in a single amino acid change (Gly353Arg). Sequence analysis of exon 7 of the *RHD* gene derived from the D^{II} individual indicates that there is a single point mutation in this exon resulting in a single amino acid change (Ala354Asp)[34].

Table 2.1. A molecular based nomenclature for aberrant *RHD* allele and *RHCE* allele (Adopted from Flegel *et. al*., 1998)[3]

Allele	Location	Nucleotide change	Phenotype: Trivial name† Partial D	Phenotype: Haplotype(s)	Phenotype: Additional antigen(s)	Anti-D immunization‡	References
Missense mutations							
RHD(L110P)	exon 2	T→C at 329	D^{VII}	CDe	RH40 (Tar)	weak, infrequent	(Rouillac *et al*., 1995b; Flegel *et al*., 1996)
RHD(R229L)	exon 5	G→A at 686	DHR	cDE	–	not reported	(Jones *et al*., 1997)
RHD(T283I)	exon 6	C→T at 848	DHMi	c(D)E	–	strong, one sample	(Jones, 1995; Liu *et al*., 1996; Wagner *et al*., 1998b)
RHD(G353R)	exon 7	G→A at 1059	DNU	CDe	–	not reported	(Avent *et al*., 1997a)
RHD/RHCE alleles with single or multiple short conversions							
RHD(S103P)	exon 2	T→C at 307	D (G negative)	cDE	RH12 (G) neg.	anti-G, one sample	(Faas *et al*., 1996)
RHD(A354D)	exon 7	C→A at 1063	D^{II}	CDe	–	strong, rare	(Avent *et al*., 1997a)
RHD(N152T,T201R,*F*223V)	exon 3, 4 and 5	conversions	D^{IIIa}	cDe	RH20 (VS)	strong, few samples	(Huang *et al*., 1997; Huang, 1997b)
RHD(L62F,N152T,D350H)	exon 3 and 7	conversions	D^{IVa} (D^{IV} type I)	cDe, CDe, cDE	RH30 (Go^a)	strong, few samples	(Rouillac *et al*., 1995a; Huang, 1997b)
RHD/RHCE hybrid alleles (single larger conversions)							
RHD-CE(2)-D	exon 2	hybrid	D^{IIIb}	cDe	RH12 (G) neg.; RH20 (VS)	strong	(Rouillac *et al*., 1995c)
RHD-CE(3)-D	exon 3	hybrid	D^{IIIc}	CDe	–	strong, several samples	(Beckers *et al*., 1996a)
RHD-CE(3–5)-D§	exon 3–5	hybrid	DHMii	cDE	–	not reported	(Jones, 1995; Liu *et al*., 1996)
RHD-Ce(3–6)-D	exon 3–6	hybrid	D^{VI} type III	C(D)e	RH52 (BARC)	strong, one sample¶	(Wagner *et al*., 1998a)
RHD-CE(4)-D	exon 4 partial	hybrid	DFR	CDe > cDE	RH50 (FPTT)	weak, one sample	(Lomas *et al*., 1994; Rouillac *et al*., 1995a)
RHD-cE(4–5)-D	exon 4–5	hybrid	D^{VI} type I	c(D)E	–	strong, frequent	(Maaskant-van Wijk *et al*., 1997a; Avent *et al*., 1997b; Huang, 1997a)
RHD-CE(4–6)-D	exon 4–6	hybrid	D^{VI} type II	C(D)e	RH52 (BARC)	strong, frequent	(Mouro *et al*., 1994)
RHD-CE(5)-D	exon 5	hybrid	D^{Va}	cDe, CDe, cDE	RH23 (D^w)	strong, few samples	(Rouillac *et al*., 1995a)
RHD-CE(5–7)-D	exon 5–7	hybrid	DBT	CDe	RH32	strong, few samples	(Beckers *et al*., 1996b; Wallace *et al*., 1997)
RHD-CE(6–9)-D	exon 6–9	hybrid	D^{IV} type III	C(D)e	–	not reported	(Wagner *et al*., 1998b)
RHD-CE(7–9)-D	exon 7 part to 9	hybrid	D^{IVb} (D^{IV} type II)	CDe	–	strong, rare	(Rouillac *et al*., 1995a)
RHCE/RHD hybrid alleles							
RHCE-D(5)-CE	exon 5	hybrid	Rh33, R_0^{Har}	c(D)(e)	RH33, RH50 (FPTT)	weak, few samples	(Beckers *et al*., 1996c; Beckers *et al*., 1996d)

2.6. Genetic diversity of RhD

The *RH* genes appear to be a source of massive diversity, combination of different genetic rearrangements appears among all racial groups. More than 50 known aberrant *RHD* alleles expressing variant D antigens have been defined over the world. More than 25 partial D alleles are predominantly observed in Europeans, and 5 partial D alleles were typical for Africans. This allelic diversity indicates clearly that racial differences exist in the genetic background of the Rh D antigen [22,41].

The frequency of *RHD* alleles between Brazilian D negative blood donors of heterogeneous ethnic origins (50 Caucasian descent and 156 African descent) has been determined by using Multiplex PCR and Sequence analysis. The *RHD* genotype was determined by comparing the size of amplified products associated with the *RHD* gene in both intron 4 and exon 10. Eleven percent of the D negative Brazilians had the RHD^{Ψ}, 2% had the *RHD-CE-D* hybrid gene, and 87% entirely lacked *RHD*. All individuals of Caucasian descent had a complete deletion of *RHD*. Among the individuals of African descent, 14% had inactive *RHD* (RHD^{Ψ}) and 3% had the *RHD-CE-D* hybrid gene [33].

In Han Chinese, the situation is likely to be different. DNA samples from 131 RhD negative blood donors were investigated. PCR-SSP was used to amplify exons 2, 3, 4, 5, 6, 7, 9 and 10 of *RHD* gene, exons 1, 2 and 5 of *RHCE* gene, and intron 4 of each. Three classes of RhD negative polymorphisms were observed: 63.4% had a complete gene deletion, 19.9% had an intact but dysfunctional gene, and 16.7% carried at least one *RHD* exon [41].

The situation in Africans is more complicated, the variety of *RHD* alleles in people of African descent was less well defined than the allele distribution in Europeans. D negative blood donors (n=58) of native West African population of Mali were genotyped and screened for different *RHD* alleles. The regular normal *RHD* allele was most frequent and found in 42 donors. Among the aberrant *RHD* alleles, the *DAU* cluster was predominant with *DAU-0* observed in 18 donors. Partial *RHD* deletion was observed in two *RHD* negative alleles and RHD^{Ψ} was found in 7 donors. Three novel *RHD* alleles were detected, the first is *DMA* allele

(D detected in Mali), which encodes a leucine to phenylalanine substitution, and located in the seventh transmembraneous segment adjacent to the intracellular part of the RhD protein. The two other alleles were *RHD* (384T→C) and *DAU-0.1* and they are variants of regular *RHD* and *DAU-0*, respectively, carrying one silent mutation each [39].

The picture in Far East is different, among Japanese people that are typed as D negative by standard serology; two different *RHD* genotypes were defined. The first group of individuals lacked the *RHD* gene (similar to D negative Caucasians) and the second group possessed the *RHD* gene. PCR was used to amplify exons 4, 5, 10, specific sequence in the 3′ non-coding region of *RHD,* and intron 4 of the *RHD* and *RHCE* genes, in 130 RhD negative unrelated Japanese blood donors. The result showed that, 36 (27.7%) donors demonstrated the presence of normal *RHD* gene, the *RHD* gene detected in the RhD negative donors seems to be intact, which suggests that, another mechanism preventing the expression of RhD antigen is responsible. Other donors showed gross or partial deletions of the *RHD* gene [22,24].

In the Australian population, one of three RhD negative samples investigated by molecular techniques was found to have a normal *RHD* gene [22].

2.7. Discrepancies of RhD Typing

Blood group genotyping is increasingly utilized for prenatal diagnosis and after recent transfusions, sometimes serologic blood group typing cannot be performed with its usual ease. In the case of patients who have been recently transfused, and who harbor a large quantity of donor blood cells, it is difficult to determine the Rh phenotypes with accuracy. *RHD* genotyping with a specificity and sensitivity comparable to serologic methods is of practical importance [3,22,45].

To make a rapid diagnosis and achieve a good prognosis, especially in HDN, it is necessary to investigate fetal RhD type before birth. With current molecular technology, it is possible to perform analyses on fetally derived DNA to predict

the blood type of a fetus. Antigen D prediction by PCR was applied to fetus at risk of HDN [6,45,57].

Discrepancies between standard serological RhD typing (phenotype) and PCR typing (Genotype) have been observed, with both false positives and false negatives. The differences of molecular genetic of *RHD* and *RHCE* are used in RhD genotyping assays, these assays are dependent on the complete absence of the region of the *RHD* gene being detected in D negative individuals. The fact that the *RHD* gene has a larger exon 10 than the *RHCE* gene, the *RHCE* gene has a larger intron 4 than the *RHD* gene (The *RHD* intron 4 is composed of 426 nucleotides, while the *RHCE* intron 4 of 1077 nucleotides), and also that sequence differences exist between exon 7 of the *RHD* and *RHCE* genes is used to genotype *RHD* alleles from genomic DNA [25].

The use of at least two *RHD* typing PCR assays directed at different regions of *RHD* is advised to prevent discrepancies between phenotyping and genotyping. Serologic typing can not be used for discrimination between RHD^{+}/RHD^{+} homozygous and RHD^{+}/RHD^{-} heterozygous individuals, but molecular methods can. There are two applicable technical approaches, either testing for the hybrid *Rhesus box*es, or quantifying the *RHD* gene dose. Heterozygous *RHD* carriers have a lower quantity of *RHD* and a smaller ratio of *RHD* to *RHCE* than those that are homozygous for the *RHD* gene. This discrimination is of particular clinical interest in D negative mothers with an anti-D, the risk of an affected child is 100% with an RHD^{+}/RHD^{+} homozygous father, but it is only 50% with an RHD^{+}/RHD^{-} heterozygous father [21,42,47].

Chapter III

Materials and Methods

3.1. Study design

This descriptive study was designed to detect and determine the D variants that are frequent in other ethnic groups, namely; D^{VI} and DNB, and their frequencies in Palestinian population. In addition, to determine the molecular causes of RhD negative phenotype among blood donors.

The detection of D^{VI} variant is based on utilization of the differences that exist between *RHD* and *RHCE* genes, where, *RHCE* has a larger intron 4 while, the *RHD* gene has a larger exon 10. PCR was used to amplify and detect the presence of *RHD* exon 10/ intron 4, and *RHCE* intron 4 as an internal control. D^{VI} phenotype is characterized by absence of *RHD* intron 4. The presence of *RHD* exon 10 with *RHCE* intron 4 along with absence of *RHD* intron 4 is a strong indicator for the existence of the RH *D/CE/D* hybrid allele and consequently the presence of D^{VI} phenotype.

Amplification of both *RHD* exon 10/ intron 4 is used for investigating the molecular basis that generates the RH negative phenotype.

To define the DNB variant, PCR-SSP was used to detect the point mutation in *RHD* exon 7 that causes Gly to Ser substitution at codon 355.

3.2. Ethical consideration

The study was approved by the Palestinian ethical committee (Helsinki ethics committee).

3.3. Blood samples

The D negative and D positive blood samples enrolled in this study were collected at random from 102 unrelated Palestinian blood donors: 79 RhD positive and 23 RhD negative. Ninety blood donor samples were obtained from

El Shifa hospital complex-Central Blood Bank, and 12 blood donor samples from the Central Blood Bank Society. The samples were collected from January to October 2007, according to the approved protocol of blood collection of those blood bank centers.

Three samples with discrepant Rh, which were noticed because of weak D expression {weak D reactive with monoclonal antibody (MoAb)} were also obtained, two samples belonged to women who have discrepant RhD typing during routine RhD screening after delivery, and the third sample was obtained from a patient (3 years old child) who receives blood transfusion and had problems with RhD typing. All blood samples (3 ml each) were collected into ethylenediaminetetraacetic acid (K_2EDTA) anticoagulant.

3.4. Equipments

All equipments used in this study belong to the genetics laboratory at the Islamic University of Gaza, and they are listed in Table 3.1.

Table 3.1. Equipments used in the study

Item	Manufacturer
Thermocycler	Eppendrof, Germany
Electrophoresis chambers and tanks	BioRad, USA
Electrophoresis power supply	BioRad, USA
Microcentrifuge	Sanyo, UK
Microwave Oven	L.G, Korea
Bench top centrifuge	LWScentific, USA
Safety cabinet	Heraeus, Germany
Automatic pipettes	Eppendrof, Germany
UV Transilluminator	Vision, Scie-plas Ltd, UK

3.5. Serological RhD typing

Phenotype of the RhD for all samples was performed in blood bank centers (by blood bank staff as a part of blood donation process), using commercial monoclonal anti-D (LORNE Laboratories LTD). We retyped the samples to confirm the RhD phenotype. Blood samples were serologically typed for RhD by the method of direct hemagglutination (H.A) test (slide method) using the manufacturer's instructions, and according to the standard protocols employed in blood bank centers in Gaza Strip.

3.6. Chemicals and reagents

All Chemicals and reagents used in the study are listed in Table 3.2.

Table 3.2. Reagents and Chemicals used in the study

Reagent	manufactured
Wizard® Genomic DNA purification Kit	Promega, Madison, WI, USA
PCR primers	Operon Biotechnology GmbH
PCR Master Mix	Promega, Madison, WI, USA
Ladder (size marker) 100bp	Promega, Madison, WI, USA
Ladder (size marker) 50 bp	Promega, Madison, WI, USA
Agarose gel	Promega, Madison, WI, USA
Ultra pure water (nuclease free)	Promega, Madison, WI, USA
Ethidium bromide	Promega, Madison, WI, USA
Anti-D reagents	LORNE Laboratories LTD, U.K
Tris acetate EDTA	
Isopropanol	
Ethanol 70%	

3.7. Molecular analysis

3.7.1. DNA Isolation

Genomic DNA was isolated and purified from white blood cells (WBCs) of whole blood by using Wizard® Genomic DNA purification Kit (Promega, Madison, WI. USA), according to the manufacturer instruction protocol.

The Promega isolation kit consists of the following components:

1	Nuclei lysis solution	A7941
2	Cell lysis solution	A793A
3	RNas solution	A797A
4	Protein precipitation	A795A
5	DNA rehydration solution	A976A

3.7.2. Extraction and purification of genomic DNA

3.7.2.1. Cell Lysis

1. To lyse all blood cells, 900 µL cell lysis solution were added to 300 µL whole blood in a microfuge tube, the contents of the tube were well mixed by inversion several times.
2. The lysate was then incubated for 10 minutes at room temperature and gently mixed for several times .
3. The lysate was centrifuged at 13,000 rpm for 20 seconds, and the supernatant was discarded carefully as much as possible without disrupting the pellet .
4. The pellet was vortexed vigorously for 15 seconds to resuspend it again.

3.7.2.2. Nuclei Lysis and Protein Precipitation

5. To lyse the nuclei membrane of WBCs, 300 µL Nuclei lysis solution were added to the pellet suspension, and mixed by inversion several times until the solution became viscous.

6. To precipitate protein molecules, 100 µL Protein precipitation solution were added to the mixture and vortexed vigorously for 20 seconds.
7. The mixture was centrifuged at 13,000 rpm for 3 min.

3.7.2.3. DNA Precipitation and Rehydration

8. The supernatant was transferred to a new microfuge tube containing 300 µL Isopropanol, and mixed gently until the visibly DNA precipitates as a thread.
9. The tube was centrifuged at 13,000 rpm for 1 min, and the supernatant was discarded before the addition of 300 µL of 70% Ethanol (same volume as the original sample) to the pellet, the mixutre was mixed again by gentle inversion .
10. The tube contents were centrifuged at 13,000 rpm for 1 min, ethanol was aspirated, the tube was inverted on a clean tissue and the pellet was air dried for 15 minutes.
11. The DNA was re-hydrated in 100 µL of DNA rehydration solution for 1 hour at 65 ^{o}C.
12. The quantity and quality of isolated DNA was measured on Ethidium Bromide-stained 1.5% agarose gel.
13. DNA was stored in refrigerator at 2-8 ^{o}C until performing PCR

3.7.3. Primers reconstitution

The primers were reconstituted at 2.0 µmol concentration. Primer containers were first centrifuged at 13,000 rpm for 3 minutes, and then reconstituted with ultra pure water, vortexed and diluted by transfer of 5 µL of each reconstitute to a new clean sterile labeled microfuge tube containing 45 µL of ultra pure water, to be at a final concentration of 2.0 µmol.

PCR primers employed in the study are listed in Table 3.3.

Table 3.3. Primers sequences used for D^{VI} and DNB detection

Specificity		Nucleotide sequence	Position	Amplicon size
DNB re77	(F)	TCTCCACAGCTCCATCATGGG	966 to 986	118 bp
DNBb	(R)	cagtgacccacATGCCATTACT	11 to 1063	
HGH ($hgh1^3$)	(F)	tgccttcccaaccattccctta	665 to 686	434 bp
($hgh2^3$)	(R)	ccactcacggatttctgttgtgtttc	1098 to 1073	
RH intron 4	(F)	CGATACCCAGTTTGTCTGCC	608 to 627	RHD 478 bp
	(R)	AGAACATCCACAAGAAGAGGG	658 to 638	RHCE 1126 bp
RHD exon 10	(F)	TTGGATTTTAAGCAAAAGCATCC	1224 to 1266	185 bp
	(R)	ATTCTCCTCAAAGAGTGGCAG	1429 to 1409	

The positions of the synthetic oligonucleotides are indicated relative to their distances from the first nucleotide position of the start codon ATG for all primers in the exons, or relative to their exon/intron boundaries.

The primer sequences were obtained from the following published articles:

***** The DNB (re77, DNBb) and HGH ($hgh1^3$, $hgh2^3$) primer sequences were obtained from Chen. Q. (2004)[26].

***** The RH intron 4 and RHD exon 10 primer sequences were obtained from Qun. X. et. al (2003)[41].

3.7.4. Monoplex PCR for D^{VI} Detection

All D positive and D negative genomic DNAs were examined for D^{VI}. The method is based on analyzing two *RHD* regions by using two separate monoplex PCR (Simplex PCR) assays. Briefly, the assay involves the amplification of *RHD* exon 10/ intron 4, and *RHCE* intron 4, the latter was used as an internal control.

The first region that was subjected for PCR investigation was the *RH* intron 4. Depending on the size of the PCR products, specific primers intron 4 F (CGATACCCAGTTTGTCTGCC) and 4 R (AGAACATCCACAAGAAGAGGG) were used to distinguish and identify intron 4 of both *RHD* and *RHCE*. The two *RHD* and *RHCE* PCR fragments were obtained from one primer, when the

primers paired with the *RHD* specific intron 4, it amplified a product of 478 bp, where, when paired with the same *RHCE* specific intron 4, a product of 1126 bp was produced. Each PCR run was performed with negative controls without template. The positions of the PCR primers used for *RH* gene fragments detection are shown in Figure 3.1.

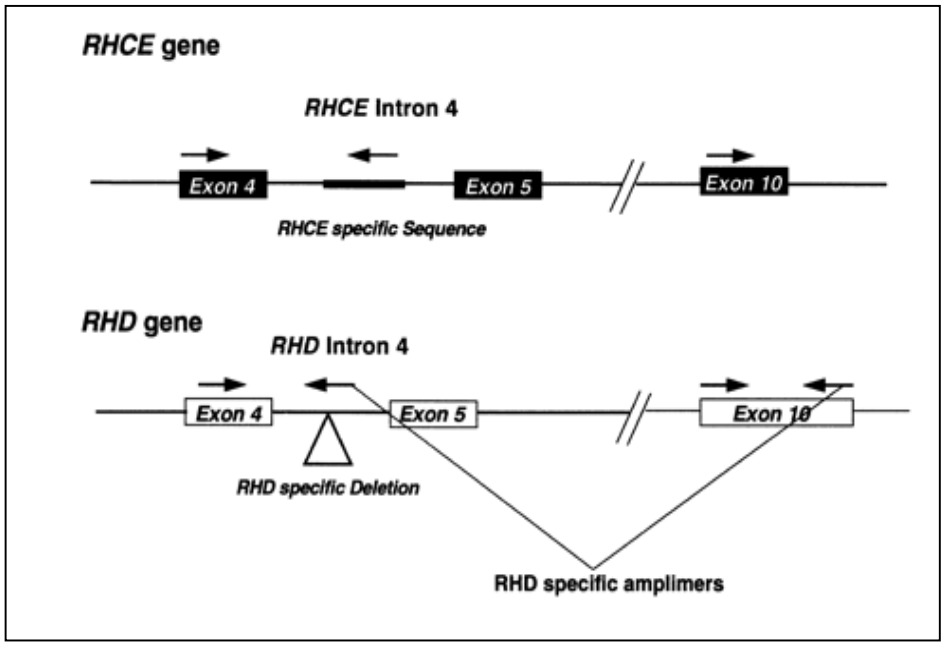

Figure 3.1. Schematic representation of the *RHD*^{VI} assay.

A diagrammatic representation of the PCR assay used in this study. The approximate positions of the primers are shown. The location of the *RHD* specific deletion or *RHCE*-specific insertion located within intron 4 of these respective genes are highlighted on the figure [9].

The second target region that was subjected for PCR was *RHD* exon 10. The specific primers; exon10 F (TTGGATTTTAAGCAAAAGCATCC) and exon 10 R (ATTCTCCTCAAAGAGTGGCAG) were applied to amplify and detect a sequence located between *RHD* specific 3' untranslated region (UTR) and *RHD* exon 10, which produced a product of 185 bp.

The two separate PCRs were performed in similar conditions and carried out in 0.2 ml PCR microfuge tube with a final volume of 20 µl, each containing, 2 µl of 2.0 µmol of each specific primer, 10 µl of PCR Mater Mix (Promega Madison, WI, USA), 2.0 µl of template genomic DNA (100-200 ng), 4.0 µl of nuclease free, sterile ultra pure water.

3.7.4.1 PCR condition

The following profile was used for both RHD exon 10 and *RHD*/*RHCE* intron 4 PCR assays. After initial denaturation for 10 minutes at 94°C, the samples were subjected to 30 cycles of PCR in a DNA programmable thermal cycler (Eppendrof, Germany). Each cycle was done at different conditions including 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1.5 minutes, followed by a final extension at 72°C for 5 minutes, and the product was hold at 4°C.

The *RH* intron 4 and *RHD* exon 10 PCR products of each sample were mixed carefully by gentle automatic pipeting, and then analyzed by electrophoresis where, 10 µl PCR products of each sample were stained with 2 µl bromophenol blue and analyzed by electrophoresis on 1.5 % agarose gels containing ethidium bromide in Tris-acetate EDTA buffer.

3.7.5. PCR-SSP for DNB Detection

A polymerase chain reaction with sequence specific priming {(PCR-SSP), also known as allele-specific primer amplification (ASPA)}, was used to detect or to confirm the 1063G→A substitution in the *RHD* exon 7 (DNB alleles) using the method described by *Wagner et.al*. (2002)[10] and *Chen et.al*. (2004)[26].

DNB specific sense primer re77 and D specific antisense primer DNBb at a concentration of 2.0 µmol were used to detect the specific mutation in *RHD* exon-7. The re77 sense primer (TCTCCACAGCTCCATCATGGG) corresponds to nucleotides sequence from 966 to 986 (exon/exon), while the antisense DNBb primer (CAGTGACCCACATGCCATTACT) corresponds to nucleotides 11 to 1063 (intron /exon). PCR should amplify a fragment with 118 bp product across *RHD* exon 7 **(Figure 3.2.)**

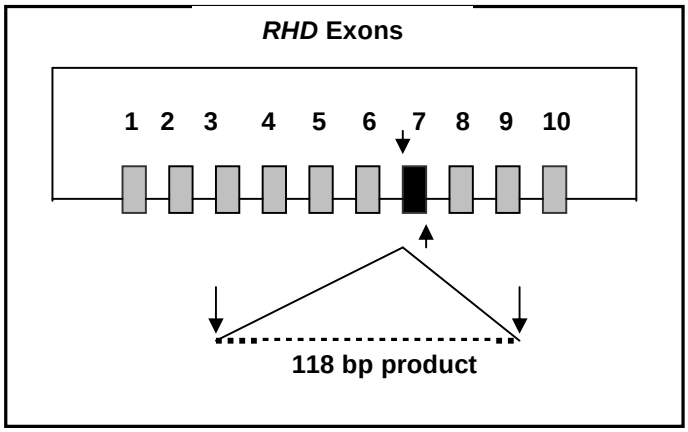

Figure 3.2. **A diagrammatic representation of the DNB PCR - SSP assay.** Arrows indicate the positions of the specific primers used to amplify a 118 bp cross *RHD* exon 7 that detect the missense mutation (Gly355Ser) in DNB allele.

Human growth hormone (HGH) positive control primers (hgh1[3] F) (tgccttcccaaccattccctta) (intron/intron) and (hgh2[3] R) (ccactcacggatttctgttgtgtttc) (intron/intron) were used at a concentration of 2.0 µmol as an internal control and were included in all PCR reactions, the positive control yields a 434 bp fragment.

Amplification was carried out in 0.2 ml PCR microfuge tube with a final volume of 20 µl, each containing 2 µl of 2.0 µmol DNB primers (DNBre77, DNBb), 0.5 µl of 2.0 µmol HGH primers (hgh1[3], hgh2[3]), 10 µl of PCR Mater Mix (Promega Madison, WI, USA), 1.5 µl of template genomic DNA (100-200 ng), 3.5 µl of nuclease free, ultra pure water. Each PCR run was performed with a negative control without template.

3.7.5.1. PCR condition

Thirty two PCR cycles were performed with the genomic DNA consisting of an initial denaturation of 2 min at 94°C, followed by ten cycles of 10 seconds denaturation at 94°C and 1 min annealing/extension at 65°C, and another 22 cycles of 30 sec denaturation at 94°C, 1 min annealing at 61°C, 30 sec extension at 72°C and final annealing 4 min at 72^{o}C. The PCR product was hold at 4°C.

After amplification, 10 µl of the final PCR products were stained with 2 µl bromophenol blue and analyzed by electrophoresis on 1.5 % agarose gels containing ethidium bromide in Tris-acetate EDTA buffer. The amplification was performed in programmable thermal cycler (Eppendrof, Germany).

3.7.6. Agarose gel electrophoresis

Dried agarose gel (0.75 mg) was dissolved in 50 ml 1x Tirs-Acetate-EDTA buffer (2M Tris base 1M Glacial Acetic Acid, 0.05 M EDTA) by heating. After the gel solution cooled to 50°C – 60°C, 1.5 µg/ml Ethidium Bromide was added and mixed, the gel was casted into a mold which was fitted with a well forming comb. The agarose gel was submerged in electrophoresis buffer within a horizontal electrophoresis apparatus.

After amplification, the PCR products and a DNA ladder size marker (Promega, Madison, WI, USA) were loaded into the sample wells to aid in fragment size determination. PCR fragments were detected by size in the agarose gel. Electrophoresis was performed by using Electrophoresis power supply (BioRad, USA) at 70 volts for 40 min at room temperature, and the DNA bands were visualized and documented using a UV trans-illuminator documentation system UV (Vision, Scie-plas Ltd, UK)

Chapter IV

Results

4.1. Immunohematology

All 102 Palestinian blood donors and the three patients involved in this study were typed for RhD by the routine methods of the relevant transfusion services. The phenotyping was performed by monoclonal anti D. The RhD negative and positive blood samples were retyped again in El Shifa hospital-central blood bank to confirm the RhD serotype, using the common commercial MoAb that is widely used in blood bank centers in Gaza Strip. Seventy nine samples were D positive, 23 were D negative and three samples had a discrepant RhD typing. The RhD serological typing results of the blood samples are illustrated in **table 4.1.**

Table 4.1. The RhD typing results of the samples

RhD phenotype	No. of Sample
Positive	79
Negative	23
Discrepant	3
Total	105

4.2. Molecular screening for RhD^{VI}

We performed a molecular screening on the 102 RhD negative and positive blood specimens to detect and determine the frequency of D^{VI} phenotype among these samples.

It is essential that performing RhD typing assays use the amplification of at least two different regions of the *RHD* gene. Intron 4 and exon 10 are sufficiently distant to be good candidate regions to detect the D^{VI} allele, which depends on the absence of *RHD* intron 4 with concomitant presence of *RHD* exon 10.

We screened all donor samples (n=102) for D^{VI} phenotype by using two independent PCRs: one PCR coamplifies introns 4 of the *RHD* and *RHCE* genes, and was performed by specific primers corresponding to specific common sequences located in both introns. This PCR produces two products, 478 bp corresponding to *RHD* intron 4 and 1126 bp from *RHCE* intron 4 (was also used as an internal control) from D positive genomes (two bands were detected). The D^{VI} which is characterized by absence of *RHD* intron 4 should yield one product (1126 bp), and the same applies for RhD negative specimens.

The second PCR primer pair we used is specific for exon 10 of the *RHD* gene only, and amplifies a product of 185 bp in D positive and D^{VI} but not in D negative samples (Figure 4.1.)

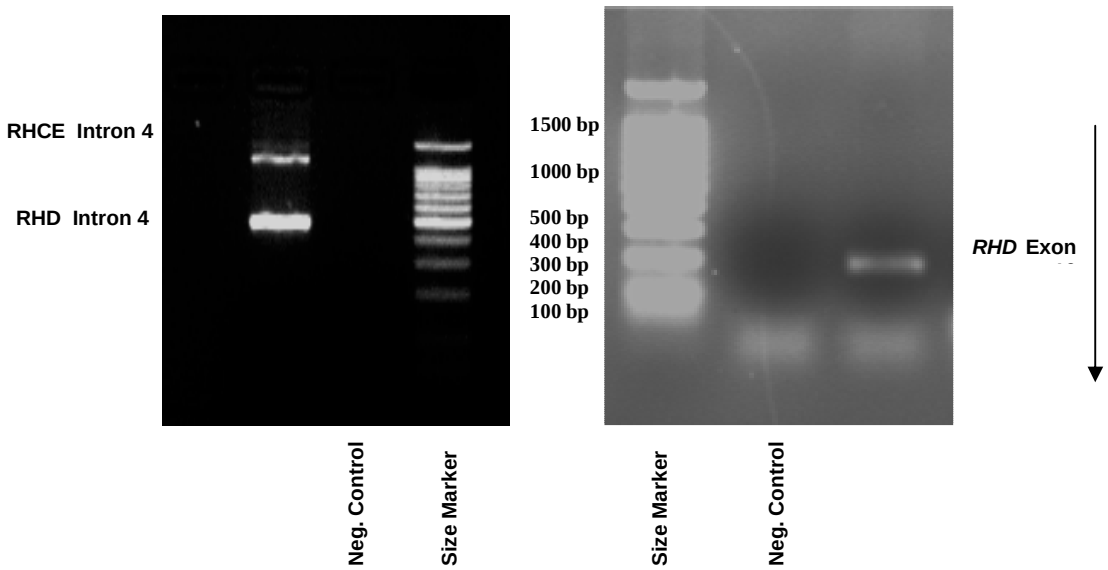

Figure 4.1. Simplex PCR amplification of *RH* intron 4 and *RHD* exon 10. Two independent PCRs amplified 478 bp and 1126 bp products corresponding to intron 4 of *RHD* and *RHCE* respectively (Left), and *RHD* exon 10 product 185 bp (Right) in D positive samples. The *RHCE* intron 4 was used as internal positive control.

Three patterns of PCR products were apparent when the two PCRs were performed: 1- Detection of both *RHD* intron 4/ exon10 regions. 2- Absence of both *RHD* regions, 3- Presence of *RHD* exon 10, but absence of *RHD* intron 4.

Of the 102 blood donors tested, 79 samples (77.5%) had both regions of *RHD*, 20 samples (19.6%) lacked both *RHD* regions and 3 samples (2.9%) had *RHD* exon 10 only (Table 4.2.). *RHCE* intron 4 was detected in all samples.

Table 4.2. Percentage of PCR patterns in D Pos/Neg blood donors (n=102)

PCR pattern	n	%
Detection of E10/In4 (*RHD* +)	79	77.5
Absence of E10/In4 (*RHD* -)	20	19.6
Detection of E10 only (D^{VI})	3	2.9

Ex : Exon
In : Intron

Three PCR products (1128 bp, 478 bp and 185 bp) were obtained in D positive samples, only one product (1128 bp) in D negative samples (Figure 4.2) and two products (1128 bp,185 bp) in D^{VI} samples.

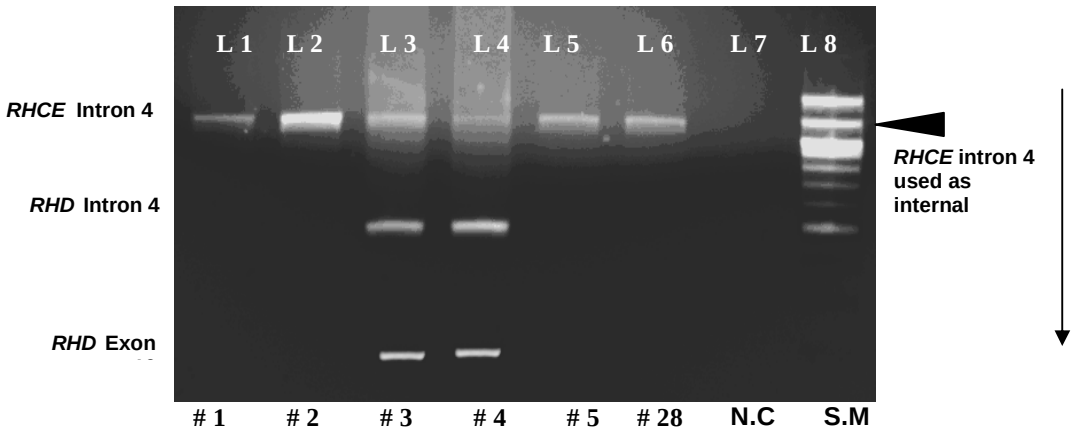

Figure 4.2. PCR pattern of D positive and D negative Three bands were detected in RhD positive (L 3, 4), and one band in RhD negative (L1,2,5,6), and no template negative control lane (L 7). *RHCE* intron 4 band indicated the successful PCR amplifications.

4.2.1. Screening D positive donors for *RHD* exon 10 and intron 4

The first pattern was observed in RhD positive blood donors. When we screened the 79 RhD positive donors, all samples (100%) exhibited a pattern of both introns 4 and exon 10 of *RHD gene*. Intron 4 of *RHCE* gene was always detected and indicated the success of PCR amplification (Figure 4.3).

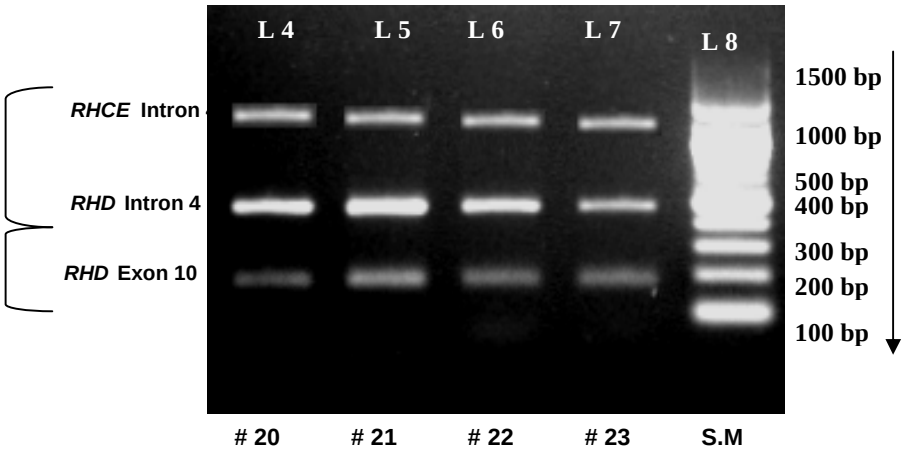

Figure 4.3. PCR products of representative RhD positive samples
Results of detection of *RHD* intron 4 and exon 10 in RhD positive samples, *RHCE* intron 4 was used as an internal control. Three PCR products were observed in each.

4.2.2. Screening D negative donors for *RHD* intron 4*/* exon 10

Genetic basis of the remaining 23 D negative blood samples were investigated and screened for D^{VI} in the same manner, where the genomic DNAs of the D negative donors were tested by PCR to determine the presence of exon 10 and intron 4 of *RHD* gene.

Two patterns of PCR products were observed in the D negative blood specimens, the first pattern was represented by the absence of both intron 4/exon 10 of *RHD*, and the second was represented by presence of *RHD* exon 10 with concomitant absence of *RHD* intron 4 (Figure 4.4).

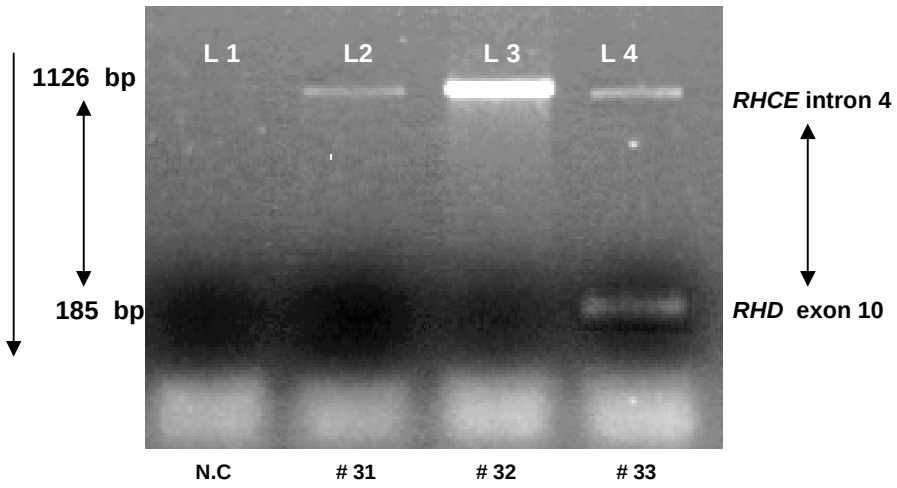

Figure 4.4. Detection of *RHD* intron 4/exon 10 in D negative samples. *RHD* intron 4/exon 10 were absent in most D negative (L 2,3), while exon 10 was only detected in some D negative samples (L 4).

4.2.2.1. RhD negative

Of the 23 D negative blood specimens examined, twenty D negative (87%) lacked both *RHD* regions but retained the *RHCE* intron 4 band (the internal control). The specific primers could not recognize the obviously deleted sequences of *RHD* gene, and no *RHD* exon 10 or intron 4 PCR products were detected (Figure 4.4.).

4.2.2.2. D^{VI} variant

The assay was discordant in some D negative phenotypes. Of the 23 D negative samples three (13%) had *RHD* exon 10 only, indicating the presence of D^{VI} variant. The PCR was intron 4 negative and exon 10 positive while retaining the control *RHCE* intron 4 (Figure 4.5). The *RHD* specific primers did not recognize the specific sequence of intron 4 and succeeded to amplify exon 10 of *RHD* gene.

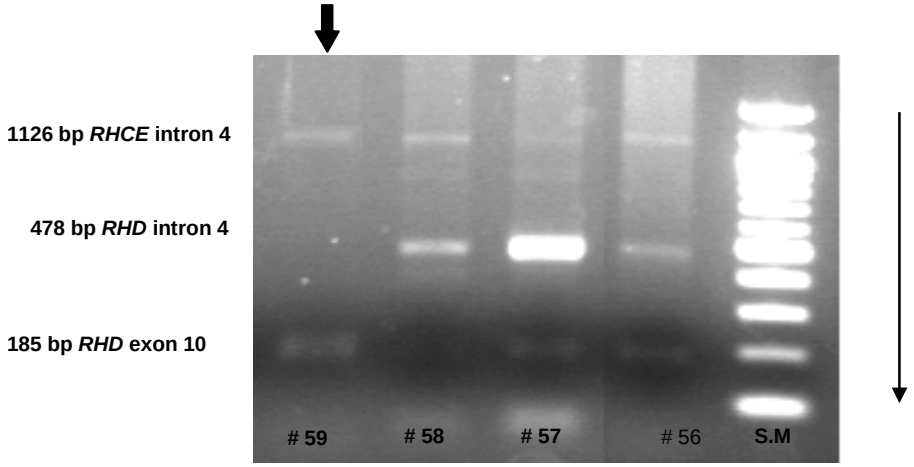

Figure 4.5. **PCR products of D^{VI} and D positive samples.** Two PCR patterns represent D positive samples (# 56, 57, 58) and D^{VI} phenotypes (# 59). Three *RH* products detected in RhD positive as a normal allele and two products detected in D^{VI} with absence of *RHD* intron 4.

4.3. Genotyping of weak D patients

RBCs of three patients showed weak reactions with anti-D MoAb as compared to normal RhD positive. Therefore, with restricted screening protocols, these donors might be considered as expressing partial D or weak D phenotype.

We investigated the three samples at the molecular level by PCR to distinguish whether they carry partial D variant or weak D phenotype. Discrepancies and significant discordance between standard serological typing and PCR typing have been observed when gDNA derived from those patients were investigated.

RHD intron 4/exon 10 and *RHCE* intron 4 were amplified, and the three bands were obtained and the amplification products were detected from the three samples, where *RHD* intron 4 and exon 10 were positive (Figure 4.6.).

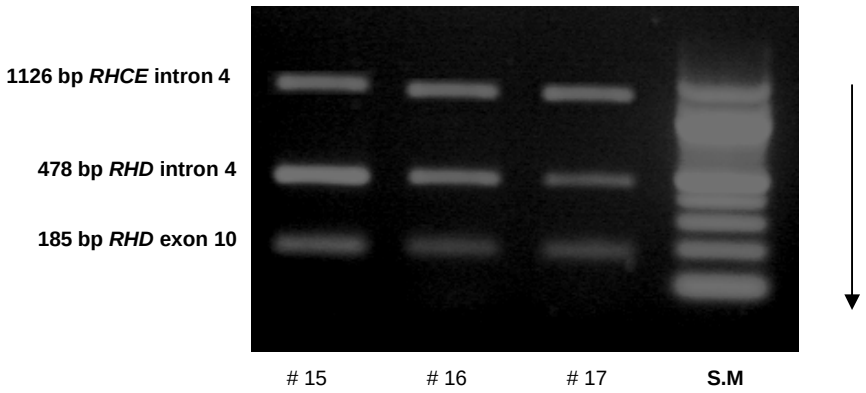

Figure 4.6. PCR products of weak D samples. PCR amplification results of weak D after separation on 1.5% agarose gels. Products of 1126 bp (*RHCE* intron 4) 478 bp (*RHD* intron 4) and 185 bp (*RHD* exon 10) were shown.

4.4. Molecular screening for DNB

All 79 D positive blood samples and the three weak D specimens were examined for DNB variant. The PCR-SSP amplified a product of 118 bp across *RHD* exon 7. The 434 bp product of human growth hormone gene *(HGH)* served as an internal control.

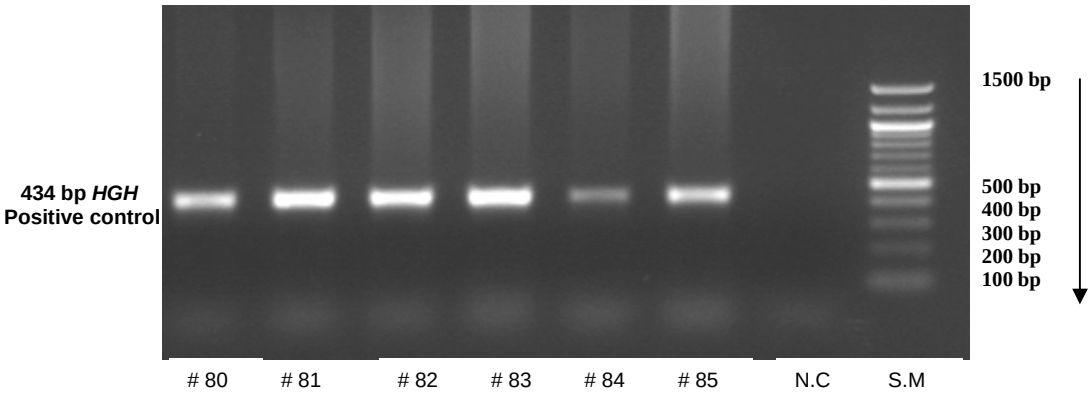

Figure 4.7. Negative DNB PCR assay. PCR-SSP produced no DNB product in wild *RHD* alleles, while retained 434 bp *HGH* positive control.

In normal *RHD* alleles, re77 and DNBb primers gave no product and PCR could not amplify any sequences. HGH positive control was amplified in each sample (Figure 4.7.).

Of the 82 gDNA derived from leukocytes, three samples (3.7%) showed 118 bp PCR products, which confirm the presence of DNB alleles. PCR-SSP patterns were compatible with the molecular basis of DNB previously described (Figure 4.8.).

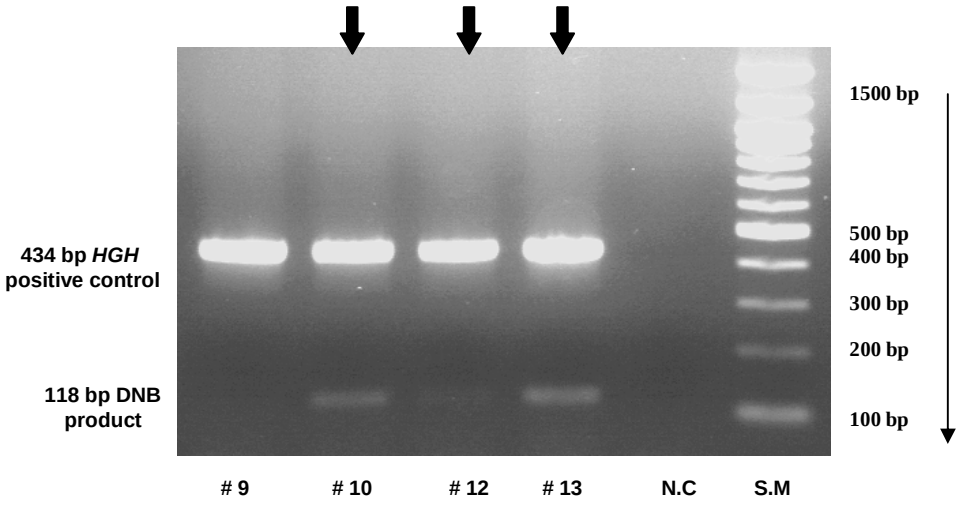

Figure 4.8. Positive DNB PCR assay. Positive PCR-SSP yielded 118 bp product representing DNB allele (#10,12,13) and negative PCR-SSP with no product (#9). The control band represents a 434bp product of the human growth hormone gene.

These 3 DNB alleles were observed in the RhD positive specimens.

Chapter V

Discussion

5.1. Genotyping versus phenotype

Genotyping strategies are increasingly utilized for blood grouping and may be devised to meet predefined specificity criteria. Significant progress has been made in the last decade in defining the molecular basis of human blood group antigen expression. The initial report of the molecular basis for the RhD phenotype was followed by the publication of a number of PCR based tests for predicting D phenotype from genomic DNA. Such techniques have proved valuable for predicting the D phenotype. Most *RH* DNA typing data are derived from Caucasian populations [3,4,17,10,26].

Our study had proven the correlation between immunohematologic features of Rh blood group and the molecular structures of the *RHD* gene and the relevant alleles. We determined the genetic polymorphism of RhD phenotype among blood donors in Gaza Strip according to the molecular characterization. The recombination event of *RHD* and *RHCE* genes leading to a hybrid *RHD*/*RHCE* allele was observed through the unique pattern of PCR amplification products [3,8].

We detected discordance between RhD genotyping and phenotyping in 9 samples. Allelic variability was observed in 6 donors and different genetic mechanisms seem to have contributed to allelic variability including DNA recombination events and missense mutations [3].

When we performed PCR based molecular analysis on blood donors' specimens, the two more frequent partial Ds namely D^{VI} and DNB were detected, and *RHD* gene regions were detected in 3 D negative individuals. In weak D samples, the molecular analysis showed the existence of normal allele with presumably weak expression [10,53].

Our findings apparently promote the suggestion that, high polymorphic and allelic diversity of *RHD* gene are present in the Palestinian population.

5.2. Intron 4/exon 10 PCR assay

We screened the 102 DNA samples derived from D positive and D negative individuals for *RH* intron 4 and *RHD* exon 10, to detect the hybrid D^{VI} allele and to correlate between RhD genotype and phenotype. We found that 97% of the samples (79 RhD positive and 20 RhD negative) had a compatible genotype with the phenotype.

5.2.1. ***D positive blood samples:*** In individuals, expressing normal D phenotype and serologically typed as D positive, a full concordance was observed In all D positive samples, where the two *RHD* regions and the *RHCE* intron 4 were detected in the 79 D positive samples. The two amplified bands of intron 4 originated from normal *RHD* and *RHCE* alleles. This observation revealed that, those donors seem to be identical to the normal RhD phenotype and have an intact *RHD* gene. The D^{VI} variant was not detected in any of the D positive samples, and the PCR assay was compatible with RhD serotype in all D positive blood donors [4].

The frequency of D positive/negative is not yet estimated in the Palestinian or other Arab populations and no data was published or reported before, but in other ethnic groups like Caucasians the D positive frequency was found to be about 82%-85%, in Africans 93%-97% and about 99% in the Far East [2,8,21,22,27].

5.2.2. ***D negative phenotype:*** The D negative phenotype is generally derived, from homozygosity of *RHD* gene deletion. The vast majority of D negative phenotypes lack the whole *RHD* gene [3,6,21].

Our findings showed that, of the 23 D negative donors examined, 20 D negative samples (87%) were completely concordant with both exon 10 and

intron 4 bands absence pattern, which should indicate a complete deletion of *RHD* gene (gross *RHD* gene deletion).

The examination of *RHD* intron 4 and exon10 of negative samples by using the specific primers excluded the confusion of either *RHD* pseudogene *(RHD^{ψ})* or any other unexpected molecular basis with grossly intact *RHD* genes [2].

The results of *RHD* intron 4/exon 10 PCR assay, support the hypothesis that, the majority of D negative genomes examined, appear to lack all portions of the *RHD* gene, and therefore probably have a complete *RHD* gene deletion event that generates the most RhD negative phenotype of blood donors in Gaza Strip.

Other workers have described D negative English and Scottish individuals with an apparently intact *RHD* gene, and intact *RHD* transcripts, the molecular basis was explained by a point mutation in the *RHD* gene at codon 41 generating a premature stop codon. This pattern was also shown in Japanese, where normal *RHD* gene was found in 27.7% of RhD negative Japanese donors [22,24,25].

Other investigators found a 37 bp insertion in *RHD* exon 4, which may introduce a stop codon at position 210. The insert is sequence duplication across the boundary of intron 3 and exon 4. Two-thirds of D negative Africans were found to carry this inactive *RHD* gene, and about 16.5% appear to be homozygous for an *RHD* gene deletion. In D negative African Americans and South African people of mixed race, 54% of African Americans and 81% of South African donors of mixed race were shown to have no *RHD* gene [27].

Further molecular investigations should be conducted on our D negative specimens to rule out *RHD* exon 4 insertion and point mutations in the *RHD* gene, though the latter is unlikely.

5.2.3. *D^{VI} variant:* The RhD positive/negative polymorphism is found to be generated by the complete absence of the RhD protein, which might be the result of entire *RHD* gene deletion [25].

RHD gene positive antigen D negative phenotype was previously reported. The nature and frequency of the mutant allele however, differ among populations, e.g., in USA, 6 of 26 *RHD* gene positive were shown to be antigen D

negative. Moreover in African and Japanese people, there is a large fraction of RhD negative alleles that harbor *RHD* specific sequences [3,45].

RHD gene rearrangements (gene conversions, point mutations) which alter the RhD protein structure significantly may be expressed at the erythrocyte surface. These alterations may generate partial D (D variant) phenotypes, many of which have been defined at the molecular level [3,7].

D^{VI} phenotype is the most common and the most clinically significant partial D phenotype in the transfusion medicine. It lacks most epitopes compared to any other D variants, with very low antigen density. This variant is still considered as the major cause of allo-immunization with anti D [8,9,52,53].

The notion of D^{VI} detection basically depends on detecting the molecular features of D^{VI} hybrid allele. The absence of *RHD* intron 4 with concomitant presence of *RHD* exon 10 is considered a good indicator [8].

Of the 23 blood donors involved in this study and who expressed D negative phenotype, the PCR assay of 3 samples (13%) showed apparent incompatibility between genotyping and phenotyping. Amplification products of *RHD* exon 10 and *RHCE* intron 4 were detected in the three samples, whereas *RHD* intron 4 was not amplified. This PCR pattern is different from either the complete deletion of *RHD* that generates negative phenotype, or from the normal *RHD* allele type that generates positive phenotype.

The absence of *RHD* intron 4 may indicate that, the amplified product of *RHCE* was derived from the two copies of *RHCE* intron 4 that exist in both *RHD* alleles. This result apparently indicates a recombination event (e.g., gene conversion) between *RHD* and *RHCE* alleles, and is most probably compatible with *RHD-CE-D* hybrid alleles of D^{VI} variant [8,24].

The PCR pattern was identical to that described for the *RHD-RHCE-RHD* hybrid genes, and congruent with partial D characterization. Consequently, the genotype of these three D negative donors appear to carry the D^{VI} phenotype [4,9].

Many investigators confirmed that all *RHD* genes in individuals expressing the D^{VI} phenotype lack intron 4 of the *RHD* gene. A hybrid *RHD-RHCE-RHD* gene, resulting from a probable gene conversion event in which the complete *RHD*

gene exons 4, 5, and 6 were replaced by the corresponding exons of the *RHCE* gene to generate the D^{VI} phenotype is the most accepted mechanism [8,9,54].

Other D variant generated by recombination events like D^{Va} (Hus) is excluded, because the hybrid allele of D^{Va} involves the replacement of whole exon 5 and intron 5 of *RHD* by their *RHCE* counterparts [51].

The negative phenotype of the D^{VI} cannot be explained by the lack of the RhD epitopes only, but may be due to a reduced number of RhD proteins accessible on the red blood cells' surface. The four D^{VI} types (I, II, III, IV) have the same clinical significance, because all types share *RHCE* exons 4 and 5 and their negative reaction patterns don't differ with polyclonal and most monoclonal antibodies and all types can readily cause allo-immunization [8,53].

The frequency of D^{VI} variant differs among populations, the phenotype has a frequency ranging between 0.02% to 0.05% in Caucasians, about 0.02% in Germany, and 0.04% in the English people [8,52,53].

The results of our study show that the frequency of the D^{VI} phenotype in Palestinian or Arab population may be greater than expected.

5.2.4. *Weak D phenotypes:* weak D phenotypes are not partial D phenotypes, but represent any D positive erythrocyte sample with a depression in the apparent numbers of D antigen sites, which has been found to be due to a depression of *RHD* mRNA and/or expression levels. Many reports described different alleles that can generate weak D phenotype. The weak D alleles generally possess mutations in *RHD* exon sequences. Missense mutations observed in most weak D alleles, are the probable cause for the reduced antigen D expression [25,49].

Serologically there is no well defined borderline between weak D and partial D that has an aberrant *RHD* coding sequence, lacks specific D epitopes, and may be associated with an allo-anti-D immunization [21,25].

The three weak D samples encountered in this study, showed weak reactions with anti-D MoAb than produced normal D positive reaction. Phenotypic differences among weak D samples have been also noted, one sample was +1

reactive and the other two were +2. The serologic test is known to indicate quantitative changes, antigen density variation, and qualitative changes [40].

The PCR product patterns of the three samples were consistent with normal *RHD* allele, and the presence of both *RHD* intron 4/exon 10 regions and *RHCE* intron 4, predicts that they possess intact *RHD* gene. We didn't investigate the mutation that has been proposed for these weak D and the molecular basis of these samples deserves further investigation (eg., *RHD* exons sequencing) to understand the mutation mechanism that generates the weak expression of the D antigen.

Our findings implied that, those individuals typed as *RHD* intron 4/exon 10 positive and don't carry either D positive antigen or negative phenotype, promote the assumption that they can be classified as weak D.

5.2.5. *DNB variant*: Several partial D antigens permit anti-D immunization in their carriers and DNB variant is one of these. It has the ability to produce strong anti-D, titered from 4 to 128. In routine D typing, DNB samples are usually typed as positive RhD and are agglutinated by most anti-Ds, including almost all commercial anti-D typing reagents [3,10]..

Although the DNB variant loses 4 epitopes (epD6 and epD31 as well as part of epD18 and epD23), the three samples observed in this study were serologically typed as D positive without noticeable weakening of the antigen D reaction.

Of the 79 D positive donors and the 3 weak D samples screened for DNB, 3 of the 82 samples (3.7%) were positive with PCR-SSP using the re77 and DNBb specific primers which amplify a product of 118 bp in each.

This finding confirms one of the mechanisms that generate the partial D phenotype, where a point mutation in the exofacial loop 6 characterizes the DNB features and classifies it as a partial D [10].

5.3. Impact of RHD genotyping on transfusion strategies

The application of molecular approaches in determining the genetic polymorphisms of *RHD*, their frequencies and distribution in the population have critical importance for the practical application of blood transfusion, for example, *RHD* PCR may allow the identification of D positive blood units missed by routine serology [10,26,51].

In instances where D^{VI} phenotype blood donor are identified as D negative by use of inappropriate D typing reagents, the recipient will be at risk of allo-immunization, so appropriate anti-D reagent must be used to type the D^{VI} blood donor as a D positive [4,53].

In the other hand, D^{VI} recipient should be treated and typed as D negative, triggering D negative transfusions, which is the clinically favored management [4,22,53].

This policy ensures D negative transfusions in D^{VI} patients that are at risk of anti-D immunization. D^{VI} recipients should be transfused with RhD negative blood to limit anti-D immunization. Because the frequency of partial D is low, the D negative transfusion would not compromise the D negative blood supply [8,10,40].

Alleles detection is also important for fetal genotyping assays using fetal DNA in maternal plasma, because false positive results will be obtained in mothers harboring *RHD* positive alleles D negative phenotype. When D^{VI} phenotype mothers are identified as D positive by use of inappropriate D typing reagents (eg, polyclonal anti-D), D^{VI} mothers may become alloimmunized against their normal D positive infants where prophylactic anti-D is not administered. In cases where alloimmunization has occurred prenatal detection of a fetus carrying a normal *RHD* gene can be achieved by analysis of fetal DNA with the PCR assay.

A normal *RHD* intron 4-derived band would indicate that the fetus carries a paternally inherited *RHD* gene. The absence of the *RHD* intron 4 derived band and the presence of the RHD exon 10 derived band would indicate that the fetus carries only the maternally derived RhD^{VI} gene, and would hence not be at risk of HDN [4,45,54].

A strategy based on two monoclonal anti Ds that do not react with D^{VI} to type blood recipients and mothers is advantageous. Today, D^{VI} may be detected specifically by suitable combinations of monoclonal anti-D antibodies. This strategy became mandatory in Germany from 1996, and in the United Kingdom as well, where, D typing reagents are adopted to type D^{VI} patients and mothers as D negative. Therefore, the transfusion recipient will receive D negative RBC products, and the pregnant woman will receive prophylactic Rh immunoglobulin, thereby preventing allo-immunization [8,9,41,52].

All commercial monoclonal anti-Ds reagents that don't bind D^{VI} can agglutinate DNB, therefore, a serologic strategy for detecting DNB would have to rely on a separate anti D that discriminates DNB from normal D. D negative transfusion strategy for DNB may be advantageous [10].

The transfusion strategy in weak D patients is not controlled and depends on the accidental sensitivity of the preferred typing reagents used. Strategies of transfusing D negative blood to patients carrying weak D is today considered wasteful, as it became apparent that most weak D patients may be safely transfused RhD positive. The risk of allo-anti-D immunization in the frequent weak D types is considered low [8,40].

Carriers of most weak D alleles don't stimulate immune system, and should be transfused with D positive blood to avoid the common practice of wasting D negative red cell units. Transfusion recipients are currently typed and transfused D positive, if their red cells are agglutinated by 2 IgM monoclonal anti D that do not react with D^{VI} [40,50].

Chapter VI

Conclusions and Recommendations

6.1. Conclusions

In our study, we can conclude the following:

- The RhD positive/negative polymorphism is generated by the complete deletion of the RhD gene.
- Many RhD antigens result from recombination events or point mutations generating different allelic forms of the *RHD* genes.
- The results are consistent with our expectations, the most two frequent D variant alleles, D^{VI} and DNB, are detectable among Palestinian blood donors.
- The weak D allele is identical to normal *RHD* alleles, with respect to *RHD* intron 4/exon10 PCR assay.
- The discordance between genotyping and phenotyping is confined only to D^{VI} DNB and weak D phenotypes.
- With D negative phenotypes, the use of two different regions in PCR assay minimized the risk of false negative results.
- Such systematic knowledge could have considerable impact for typing and transfusion strategy in Palestine.
- The selection of appropriate blood samples for studying the RhD alleles/variants at the molecular level must be taken into consideration.

6.2. Recommendations

According to our findings we strongly recommend the followings:

1. It is necessary to apply the RhD genotyping in clinical medicine. DNA typing will contribute to reducing the risk of alloimmunization, avoiding the common practice of wasting RhD negative blood units and minimizing the wastage of anti D prophylaxis.
2. Applications of RhD genotyping would be a useful tool when serotyping is not available, for example, in cases of massive transfusion and prenatal RhD typing of fetus at risk of HDN.
3. The rules that govern blood transfusion and blood donations strategies should be re-modulated to achieve the beneficial and maximum safety in the field of blood transfusion. For example, D negative transfusions is recommended if a recipient is known to carry D^{VI}, DNB or any other partial D phenotype, and all potentially immunogenic donors should be recognized as D positive.
4. Parameters for anti Ds selection must be reviewed to meet the developed and current knowledge's.
5. Further investigations of the genetic polymorphism and allelic diversity of RhD among Palestinian population, is strongly recommended. Other partial D alleles are difficult to discern by serologic means and need more investigations by genotyping approaches.
6. Finally blood transfusion is a critical practice requires that comprehensive policies and procedures for blood administration be designed, to prevent or reduce adverse reactions. The development of these policies should be a collaborative effort among all personnel involved in blood administration. Policies and procedures must be accessible, periodically reviewed for appropriateness and monitored for compliance.

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