Rh Variability in Multi-Ethnic Perspective

Consequences for *RH* Genotyping

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Aan mijn ouders
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Introduction

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1.0 Introduction to Blood Groups

Around 1900 Landsteiner noticed that plasma from some individuals agglutinated with the red cells of others.[1] This notion led the way to discovery of blood groups. The ABO blood group system was discovered by both Landsteiner[2] (A, B and C (later named O), groups) and Decastello and Sturli[3] (AB group) in 1901 and 1902 respectively, which made blood transfusion feasible. After this discovery more blood groups were identified. Nowadays 29 blood group systems are known with a total of 243 system antigens. Some systems contain only 1 antigen (e.g. P system) whereas others contain as much as 43 (MNS system) or 49 (Rh system) antigens.
The Rh blood group system was first described by Levine and Stetson in 1939. They investigated a hemolytic transfusion reaction in a woman transfused with her husband's red cells after giving birth to a stillborn baby. They found that an agglutinin in the woman's serum agglutinated her husband's red cells and those of 80% of ABO-compatible donors. Therefore, they suggested that the woman was immunized by a fetal antigen of paternal origin and that the hemolytic transfusion reaction was caused by the maternal agglutinin reacting with that antigen on the transfused red cells of her husband.[4] The following year, Landsteiner and Wiener found that sera from rabbits (later guinea pigs) immunized with red cells from Rhesus monkeys agglutinated 85% of human red cells.[5] The antibody responsible was named anti-Rh. In that same year Wiener and Peters found an apparently identical antibody in the sera of patients who experienced a transfusion reaction after receiving ABO-compatible red cells.[6] Initially it was thought that the human and animal antibody recognized a common factor on both Rhesus monkey and human red cells. However, in 1942 Fisk and Foord demonstrated that the human and animal anti-Rh were not identical and 20 years later it was demonstrated that human and animal anti-Rh do not recognize the same antigen.[7] The antigen defined by the animal anti-Rh was renamed 'LW' (after its' founders Landsteiner and Wiener). Not long after the discovery of RhD, the other major antigens of the Rh system (C, c, E and e) were identified.[8,9]

1.1 Blood Group Systems

The discovery of the ABO system made blood transfusions feasible and the discovery of RhD unravelled the mechanism of Hemolytic Disease of the Fetus and Newborn (HDFN) and facilitated the immunoprophylactic prevention of HDFN. The ABO and Rh systems are clinically the most relevant systems. However, many other blood group antibodies are capable of causing transfusion reactions and HDFN. A blood group system may be defined as a genetically discrete group of antigens controlled by a single gene or by a cluster of two or more closely linked homologous genes with virtually no recombination occurring between them.[10] Each system is genetically discrete from every other blood group system. Any two systems may be

shown to be different either by demonstrating that the genes segregate at meiosis through the analysis of families, or by the gene loci being allocated to different chromosomes or to clearly ditinct parts of the same chromosome.[10] Allmost all genes holstering blood group systems (except the P antigen) have been cloned and sequenced, providing more insight in the molecular mechanisms causing serological complexities like point mutations, gene conversions and unequal crossing over. Although most antigens belong to one of the 29 blood group systems, both low frequency antigens, or the 700 series,[11] like Batty (incidence of less than 1% in most populations tested) and high frequency antigens, or the 901 series,[11] like Langereis (incidence of at least 99%) can not always be assigned to a particular system. Also blood group collections have been introduced to harbour sets of genetically, biochemically or serologically related antigens that could not achieve the system status for various reasons (like the unability to shown a genetical distinction from all existing systems).[10] At the present time, five blood group collections are defined (e.g. the Cost collection).

1.1.1 Molecular Basis of Blood Group Systems

Every blood group system is encoded by a single gene or a cluster of two or more highly homologous genes. Once a gene is experimentally shown to encode a protein that carries a blood group, focused genotyping of blood from donors with serologically defined antigen profiles is used to determine the molecular basis of the encoded protein (antigen). Blood group antigens of a certain system are encoded by different alleles of the gene coding for that particular blood group. Therefore, the diversity of alleles of that gene determines the number of different antigens of a system. Different genetic biological mechanisms account for the molecular diversity of blood groups systems like nucleotide substitutions, crossing over, alternative RNA-splicing, deletion of nucleotides, exons or genes, insertion of nucleotides, exons or genes and exon duplication events.[12] However, most bloodgroups are encoded by Single Nucleotide Polymorphisms (or SNP's), which means that a single nucleotide difference in the whole gene determines which blood group antigen is expressed on the protein of the red cell.

1.1.2 Blood Group Antigen Structure and Function in Red Cells

Most blood groups are synthesized by the red cell. Biochemical and molecular genetic studies have revealed most polypeptide antigen structures. The chemical nature of carbohydrate antigens (like ABO, Hh, Lewis and P) has been known for some time but the glycosyltransferases that direct the synthesis of these molecules have only been characterized more recently. Generally, blood group gene products can be classified into five functional categories: a) membrane transporters and channels, b) receptors for exogenous ligands, viruses, bacteria and / or parasites, c) adhesion molecules, d) enzymes and e) structural proteins. However, some molecules may fit

into more than one specified category (e.g. Band 3 is an anion exchanger, plays a role in membrane structure and also plays a role in the cytoadherence of *P. Falciparum* infected RBC's to the vascular endothelium). Most blood group systems have "null phenotypes", phenotypes in which the antigens of the system are not expressed. Null phenotypes are generally caused by inactivating mutations in the encoding gene. Therefore the protein is not present in any tissue, although exceptions are known (like the FY-GATA mutation which only hampers Fy antigen expression on red cells). Only a few null phenotypes are known to be associated with a clinical syndrome (like the Rh_{null}, McLeod and Leach null phenotypes) which, if present, is often mild or of low severity.[13]

Ad a) Blood group system antigens that function as membrane transporters and/or channels usually are polytopic and have an even number of -helical membrane spanning domains of about 21 amino acids each and have both termini inside the cytosol. They facilitate the transport of biologically important molecules into or out of the cell. This category includes the Band 3 anion exchanger (Diego system),14 the water channel AQP1 (Colton system)[15] and the urea transporter Kidd.[16]

Ad b) Some red cell surface proteins appear to function as receptors for exogenous ligands, viruses, bacteria and/or parasites. The biological significance of the interaction between microorganisms and red cells is unknown, except for some parasitic infections with a intra-erythrocytic stage of development (like malaria). Many microorganisms recognize sialic acid, a charged monosaccharide present on cell glycoconjugates and abundantly present on glycophorin A.[17] An example of a microbial interaction with an other carbohydrate structure is the parvovirus B19 that uses the P blood group glycolipid to infect human erythrocyte progenitor and in most cases causes benign anemia.[18,19]

Ad c) Other red cell surface proteins function as adhesion molecules and most of these proteins carry blood group activity. Cell adhesion molecules are involved in the adhesion of cells to other cells and to the extracellular matrix. CD44 is a wide-spread glycoprotein which carries the Indian (In^a/In^b) polymorphism and possibly also the high frequency antigen AnWj.[10] CD44H, the standard form of the molecule, is present on red cells and leukocytes. AnWj most probably is an isoform of the CD44 glycoprotein.[20] On epithelial cells, AnWj appears to be a receptor for Heamophilus Influanzae, a major cause of bacterial meningitis in young children.[21] Also the Lutheran (Lu) and LW antigens display adhesion activity. The Lu glycoprotein binds laminins (a family of ECM glycoproteins, present in all basement membranes)[22] and the LW antigens (expressed on the red cell membrane glycoprotein CD242 or ICAM-4) form a ligand for intergrins.[23]

Ad d) Blood group antigens may also be dependent on enzymatic activity that synthesizes the antigen. Typical examples of this are the glycosyltransferases responsible for the biosynthesis of carbohydrate antigens (ABO, Hh, Lewis, etc.). The Yt (Cartwright) glycoprotein represents acetylcholinesterase (AChE, a serine

hydrolase). Besides its GPI linked presence on red cells, AChE is also present in patients with PNH (Paroxysmal Nocturnal Haemoglobinuria) who are unable to couple GPI anchors.[24] The Kell glycoprotein protein is an endopeptidase capable of cleaving the biologically inactive big endothelin (40 amino acids) into the smaller active vasoconstrictor endothelin (21 amino acids).[25]

Ad e) Some blood group molecules are involved in the structure of red blood cells. The red cell architecture is maintained by the membrane skeleton, a network of glycoproteins beneath the plasma membrane. Red cell membrane glycoproteins that link the membrane with its' skeleton are Band 3 (also a red cell transporter), the Diego antigen and glycophorin C and its' isoform glycophorin D (carrying the Gerbich blood group antigens). Mutations in the genes of the above mentioned blood group antigens result in abnormally shaped erythrocytes. The Rh system also relates to the red cell shape by being firmly linked to the red cell membrane skeleton.[26,27] Although GPA and GPB are major erythrocyte membrane components, they are not critical to red cell membrane integrity.

1.1.3 Blood Group Immunogenicity

Transfused red cells are regarded as incompatible if their survival in the recipient's circulation is limited by antibodies. Incompatible transfused red cells may cause a hemolytic transfusion reaction. A distinction is made between an immediate hemolytic transfusion reaction (IHTR), in which destruction begins during transfusion, and a delayed hemolytic transfusion reaction (DHTR), in which destruction begins only after there has been an immune response, provoked by the transfusion. Almost all DHTR's are caused by secondary immune responses.[28]

Red cell destruction is classified as either intravascular, characterized by the rupture of red cells within the blood stream and liberation of Hb into the plasma, or as extravascular, characterized by phagocytosis of red cells by macrophages with subsequent liberation of bilirubin into the plasma.[29] Antibody-mediated intravascular red cell destruction is caused by the sequential binding of the complement components C1 through C9 following the formation of antibody-antigen complexes. This type of cell destruction is usually seen following a transfusion of major-side ABO incompatible blood. With extravascular red cell destruction, the abnormal (IgG or C3) coating of the red cell acts as a signal to macrophages that attempt to remove the abnormal protein from the red cell membrane. The macrophage activity may result in phagocytic destruction of the red cell within the liver and the spleen. However, if the macrophage succeeds in removing the IgG or C3 coating from the red cell, it also removes a part of the cell membrane creating a spherocyte.[30]

Hemolytic disease of the fetus and newborn (HDFN) is a condition in which the life span of the infant's red cells is shortened by the action of specific antibodies derived from the mother by placental transfer.[28] The immunoglobulin involved in HDFN is IgG which binds to the Fc receptor on the plasma membrane of the placenta.[31]

Although the commonest IgG red cell antibodies in human serum are anti-A and anti-B, anti-D is involved in most cases of moderate to severe HDFN.[28] The mechanism of HDFN due to anti-D is described in 1.2.4.

1.2 The Rh Blood Group System

1.2.1 Molecular Basis of the Rh System

The *RHD* and *RHCE* genes, encode the RhD and RhCcEe proteins respectively. Each gene consists of 10 exons with a total of 417 amino acids. The *RHD* and *RHCE* genes face each other with their 3'-end at the *RH* locus on chromosome 1p36.13-p34.3. The *RHD* gene is flanked by the upstream *Rh box* (5'-end) and the downstream *Rh box* (3'-end). Situated in between the downstream *Rh box* and *RHCE* is the *SMP1* (Small Membrane Protein 1) gene of which the exact function is not known (figure 1A).

1.2.1.1 RhD-negativity

RhD-negative RBC's usually lack the entire RhD protein on the erythrocyte surface due to an RHD gene deletion. This deletion is proposed to have occurred through a mechanism of unequal crossing over at the *RH* locus, triggered by the homology of the *Rh boxes* (figure 1B).[32]

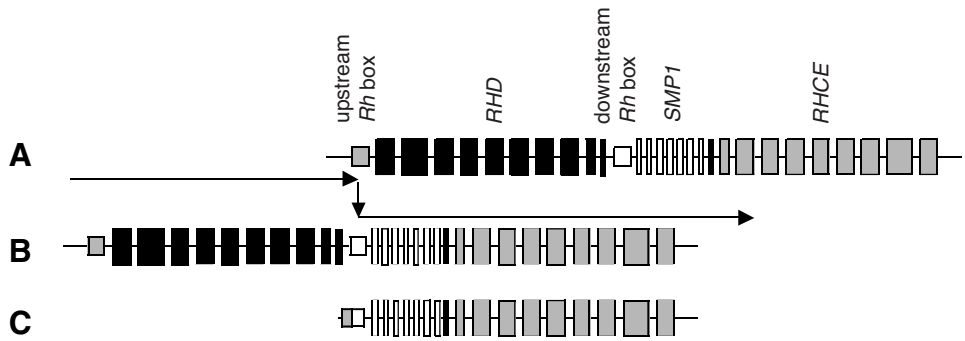

Figure 1. The structure of the *RH* locus (A) and the proposed mechanism of unequal crossing over (B) causing a *RHD* negative *RH* locus (C).

As a result of this unequal crossing over, the *RHD* negative *RH* locus carries a so called hybrid *Rh box*, with its' 5'-end identical to the upstream *Rh box* and its' 3'-end identical to the downstream *Rh box*. About 15% of the European population is RhD-negative whereas in blacks only 5% carry the RhD-negative phenotype and in Asians RhD-negativity is even more rare (<1%).[33] In whites, the *RHD* gene deletion is the major form of RhD-negativity. In blacks, RhD-negativity is frequently caused by either the *RHD*ψ allele or the *r's* (or (C)cde^s) allele. The *RHD*ψ allele is characterized by a 37 bp duplication of the intron 3/exon 4 boundary causing a frame shift

and a premature translation termination codon in exon 4, 609G>A, 654G>C and 667T>G missense mutations in exon 5 and 674C>T missense and 807T>G nonsense mutations in exon 6 and has a frequency of 0.0714 in blacks.[34] The *r's* is a hybrid *RHD* allele characterized by the presence of *RHCE* exons starting from nt. 455 (exon 3) to exon 7. The r's allele gives rise to a weak expression of RhC and has a frequency of 0.036 in blacks.[35,36] Figure 2 shows some of the most frequent forms of *RHD* negativity.[37]

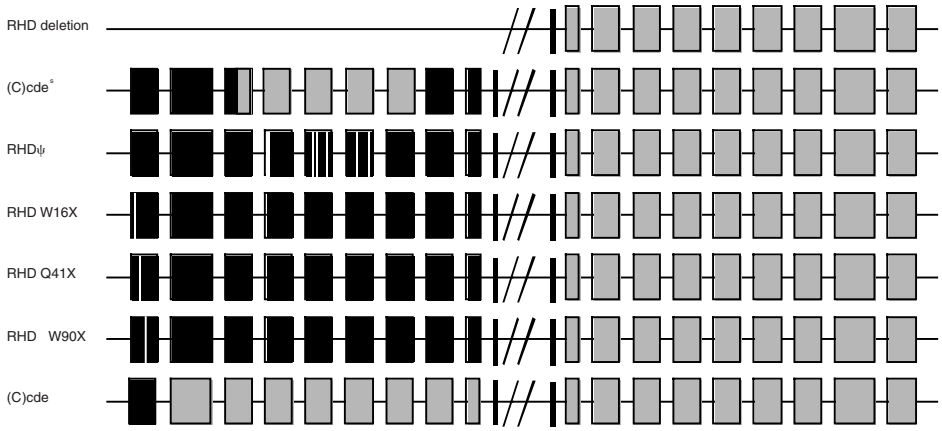

Figure 2. Different forms of *RHD*-negativity

1.2.1.2 Weak D Expression

Normal RhD-positive cells carry an antigen density ranging from 15,000 to 33,000 sites per cell (from R1r to R2R2). However, phenotypes have been identified with (type-specific) antigen densities between 70 and 5200 RhD antigens per cell. These phenotypes are called weak D and are caused by amino acid substitutions on the intracellular and transmembraneous segments of the RhD protein. RBC's with the weak D phenotype carry a presumably intact RhD antigen and occur in 0.2% to 1% of whites. Until now, 40 different weak D alleles have been identified at the molecular level[38] of which weak D types 1 and 2 are most frequent (70% and 18% of all weak D's, respectively) and types 1 to 3 represent the vast majority of all weak D.[39] In Asians, a very weak form of RhD is frequent among the RhD-negative typed population, called Del. The RhD-Del expression can only be detected by the adsorption/elution technique which is not a standard diagnostic procedure in most laboratories.

Since the weak D phenotype carries a presumably intact RhD antigen alloanti-D formation is not likely. However, one case of weak D type 15 has been reported to have alloanti-D and the weak D type 4.2 is functionally identical to the DAR partial RhD and known for its alloanti-D formation.[40] Additionally, an anti-D was observed after a RhD-positive transfusion in an individual with the weak D type 1 pheno- and geno-

type.[41] Therefore, the distinction between partial and weak D cannot be made on alloanti-D production. For the prediction of anti-D immunization risk in weak D Wagner et al. have developed the Rhesus index. This index is based on the antigen density of several different MoAb's and may theoretically range from 1.0 (low immunization risk, e.g. normal RhD) to 0.0 (high immunization risk, e.g. partial RhD lacking many epitopes). Weak D types 4.2 and 15 indeed show Rhesus indexes of 0.21, which is lower than all other weak D's with the single exception of weak D type 7 (Rhesus index of 0.03).[40] However, it has been shown that the Rhesus index is not only dependent on the quantity of the antigen but also on the affinity of the antibody.[42]

1.2.1.3 Partial RhD Expression.

The Rh system is highly diverse. This diversity is probably caused by the tandem arrangement of the *RHD* and *RHCE* genes (figure 3). This genetic configuration has led to the frequent creation of aberrant *RH* alleles caused by unidirectional replacement of *RHD* or *RHCE* gene fragments with their *RHCE* or *RHD* counterparts, leading to the formation of *RHD-CE-D* or *RHCE-D-CE* alleles, respectively.

Templated mutations, probably also caused by very short gene conversions, in which an isolated *RHD* or *RHCE* nucleotide is replaced by its *RHCE* or *RHD* specific counterpart have been found frequently. Finally, single point mutations in the *RHD/CE* genes that do not match the *RHD/CE* homologs have been described. These three mechanism have led to a large number of variant alleles. These alleles have mostly been described in non-White populations and give rise to an altered Rh protein.[10,43] Mutated *RHD* alleles cause altered RhD antigens, usually missing one or more epitopes (therefore this category is also referred to partial RhD). Sometimes new Rh antigens are expressed due to the different protein loop configuration.

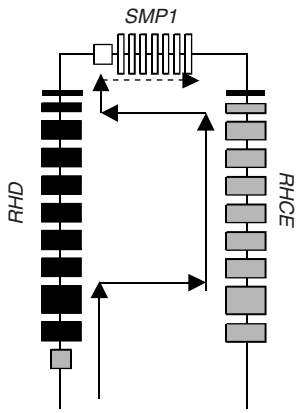

Figure 3. Tandem arrangement of the *RHD* and *RHCE* genes, leading to *RHD*(1-2)*RHCE*(3-8)*D*(9-10).

Carriers of a partial RhD protein most often produce anti-D antibodies against the RhD epitopes missing on their own RhD protein, when they are exposed to the complete RhD protein. The extant knowledge of the molecular basis of partial and weak D phenotypes enabled the development of the phylogeny of *RHD* in humans in which most "African" alleles are represented with the representative "Eurasian" (European-Asian) alleles (figure 4).[44,45,46]

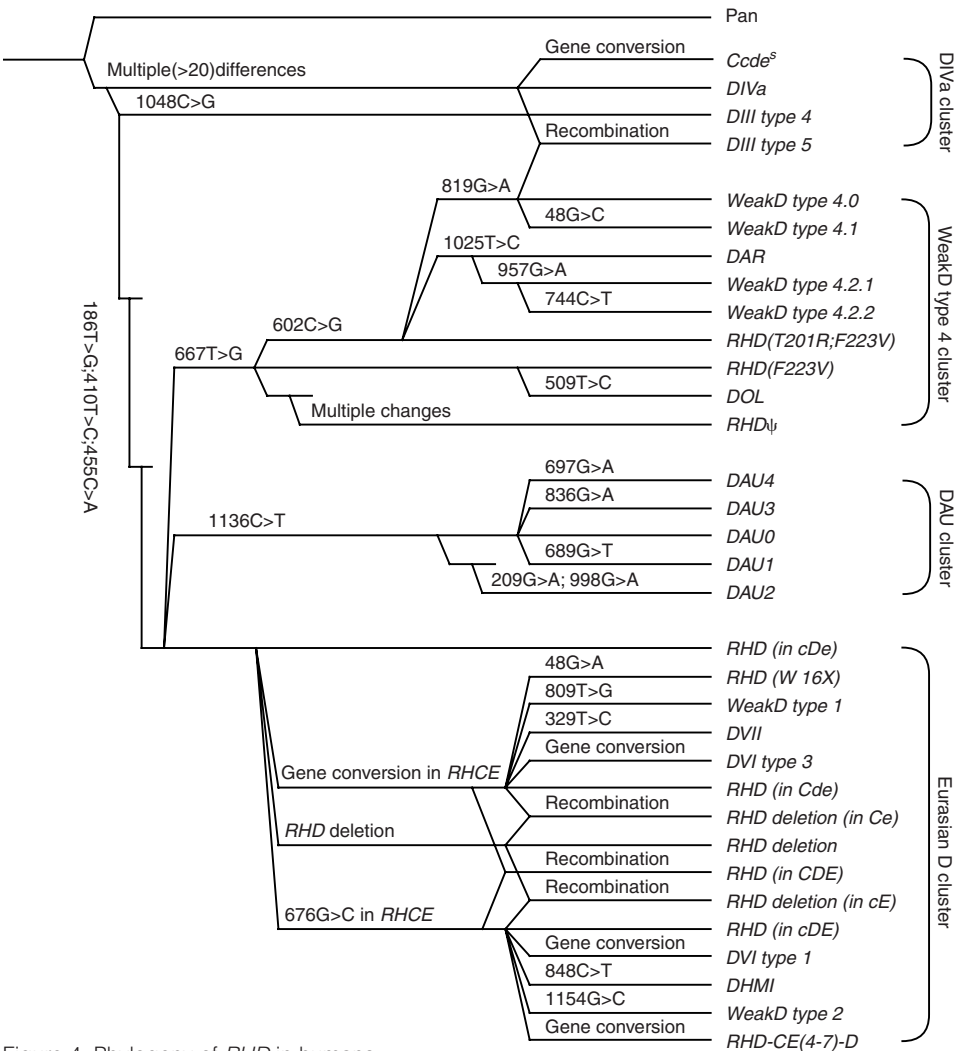

Figure 4. Phylogeny of *RHD* in humans.

1.2.2 Structure of the Rh Complex in Red Cells

The Rh blood group system antigens are part of a larger Rh complex in the red cell membrane. The RhD and RhCcEe polypeptides display an almost identical membrane organization. Both polypeptides consist of twelve membrane spanning domains

and their NH_2 and COOH domains are situated intracellular which results in six extracellular domains to which immune responses are directed (figure 5).[47,48] The RhD and RhCcEe polypeptides are encoded by the homologous *RHD* and *RHCE* genes (figure 5).[49]

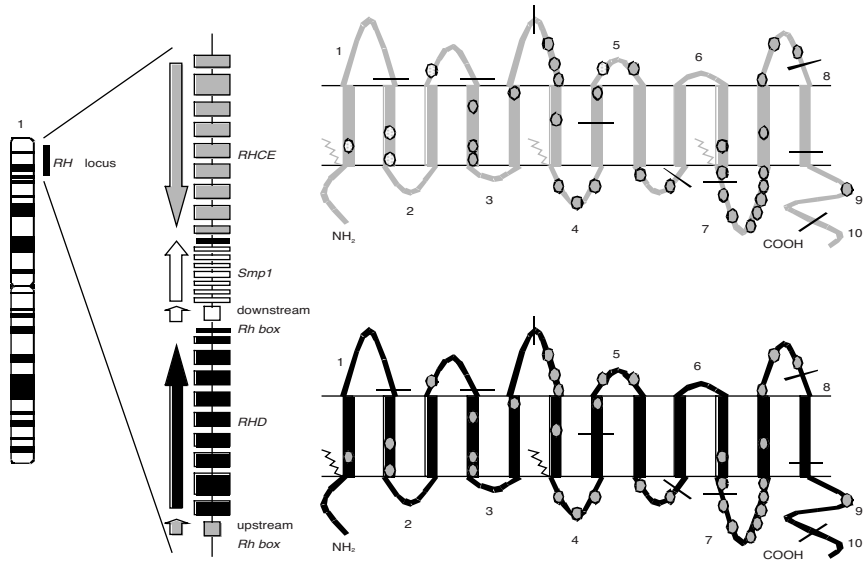

Figure 5. Schematic representation of chromosomal arrangement of the *RH* locus, the *RHD* and *RHCE* genes and the RhD and RhCE polypeptides.

In the membrane, the Rh proteins are closely associated with the Rh associated glycoprotein RhAG. Despite being genetically discrete, the Rh protein and RhAG share a 36% homology and adopt a very similar configuration in the red cell membrane. RhAG has a single N-glycan on its first extracellular loop[50] whereas Rh proteins are not glycosylated. Studies with Rh_{null} cells, a rare phenotype in which the Rh peptides are deficient, have shown the involvement of the Rh proteins with other proteins. The expression of the LW antigens is more pronounced RhD-positive cells than on RhD-negative cells. Monoclonal anti-LW coprecipitates LW glycoprotein and the Rh polypeptide.[51] This suggests a close association with the Rh proteins. The Fy5 antigen (Duffy system) is absent on Rh_{null} cells and its expression is reduced on D-- cells (cells not expressing RhCcEe proteins). Glycophorin B (GPB) expression is reduced with 60-70% on Rh_{null} cells, reflected in reduced expression of the S, s and U antigens.[52] The association between GPB and Rh complex is also reflected in the coprecipitation of GPB with anti-RhAG.[53] RhAG of GPB deficient red cells is more heavily glycosylated than normal. Due to the absence of GPB, which may facilitate transport of RhAG to the cell surface, RhAG stays in the intracellular membrane system longer, permitting more glycosylation.[54] CD47 (or integrin-associated protein IAP) expression is reduced with 75% on Rh_{null} cells compared to normal. CD47 may function as a marker of self on red cells by binding signal regulatory protein (SIRP)

on macrophages, generating a negative signal that prevents phagocytosis of the red cells.[55] Cotransfection experiments have shown an association between Band 3 (Diego antigen) and Rh in which Band 3 enhances the expression of D, c, E and endogenous RhAG.[56] The Rh proteins and their associated and accessory proteins are present as a protein cluster in the red cell membrane.[52,54] The Rh complex consists of a tetrameric core of two RhAG and two RhD/CcEc proteins, accompanied by LW, GPB and CD47. Only RhAG appears to be a requirement for the expression of Rh polypeptides.

1.2.3 Function of the Rh Complex in Red Cells

DNA sequencing revealed that the erythroïd Rh homologous RhD/RhCE and especially RhAG share a significant sequence similarity to the Mep/Amt family of polytopic proteins which span the membrane 10-12 times and function as ammonium transporters.[57] Recently, the non-erythroïd Rh homologous RhBG and RhCG have been described, which seemed to be the missing link in the ammonium transporter phylogenetic tree.[58,59] RhAG and RhCG have been shown to promote ammonium ion transport when transfected into yeast.[60] It was also shown that RhAG, transfected into Xenopus oocyte, or yeast cell, functions as an importer of ammonium.[60,61] That the Rh complex exports ammonium from human red blood cells was recently described.[62] The ammonium import was measured during the incubation of normal RBC's, Rh_{null} amorph cells (expressing 33-38% RhAG) and Rh_{null} regulator cells in a solution containing a radiolabelled analogue of NH_4Cl (^{14}C-methyl-NH_3Cl). It was shown that Rhnull regulator cells accumulated the radiolabelled methyl-ammonium ions to significantly higher levels than normal RBC's. Subsequently, Rh_{null} amorph cells accumulated an intermediate amount of methyl-ammonium ions. To show that the accumulation of methyl-ammonium was due to a decreased export, the release of intracellular methyl-ammonium ions was measured. Normal RBC's were shown to release 87% of the methyl-ammonium ions within 30 seconds, whereas Rh_{null} regulator cells released only 46% and Rh_{null} amorph cells showed an intermediate pattern.[62] The ammonium transport function of the Rh proteins could serve as a protection for the RBC in an environment with high ammonium levels, such as in the kidney during the excretion of acids.[62] It has also been postulated that Rh/RhAG proteins mediate movement of CO_2, based on the observation that expression of RH1, one of the two Rh-like proteins in blue green algea, increased when cells were grown in 3% CO_2 and decreased when these cells were shifted to air.[63] However, more than 2000 genes were induced in blue green algea when shifted to grow in CO_2, RH2 did not show this effect and no direct evidence for RH1-mediated movement of CO_2 has been demonstrated. A recent publication shows that RhAG facilitates CH(3)NH(2)/NH(3) movement across the RBC membrane and represents a potential example of a gas channel in mammalian cells.[64] It is also hypothesized that RhAG serves as an ammonium or ammonia

scavenger, keeping the total blood ammonia (NH_4^+ + NH_3) level low by trapping ammonium (NH_4^+) inside the RBC.[43] The mechanism of ammonia transport by Amt/MEP was recently demonstrated by elucidating the structure of AmtB. This study describes that the AmtB protein is the first structure of a transmembrane channel family that can conduct unhydrated molecules that in isolation would be gasseous.[65]

1.2.4 Immunogenicity of the Rh System

The Rh system comprises 49 antigens. The major Rh antigen from a clinical point of view is RhD, followed by Rhc. Eighty percent of RhD-negative individuals who receive RhD-positive blood will produce anti-D antibodies after the first encounter[66] and only 7-8% of RhD-negative individuals remain complete non-responders.[67] Antibodies against RhD are known to cause hemolytic disease of the fetus and newborn (HDFN), hemolytic transfusion reactions and are involved in autoimmune hemolytic anemia. Even after the introduction of RhD immunoglobulin, maternal sensitization occurs in 0.8-1.5% of RhD-negative mothers due to failure of prophylaxis, or during pregnancy as a result of fetomaternal hemorrhage.[68] Of the 200 cases of HDFN per year in The Netherlands, 170 cases of HDFN are due to RhD antagonism.[69] Hemolytic transfusion reactions due to anti-Rh alloantibodies (especially anti-D antibodies) are rare in countries were patient and donor are matched for the ABO and RhD antigens. Hemolytic transfusion reactions due to anti-D alloantibodies are usually severe. In autoimmune hemolytic anemia, warm autoantibodies with a broad Rh-specificity are usually found in the patients serum and eluate, causing the shortened RBC lifespan.

1.2.5 Weak D, Partial RhD and Transfusion Practice

The molecular mutations in weak D are by definition situated in those parts of the *RHD* gene that encode for the transmembrane or intracellular regions of the RhD protein. However, as described in 1.2.1.2, some of the less fequent weak D phenotypes have been described to provoke an anti-D reaction when they encounter normal RhD. Therefore, when a weak D phenotype is serologically indicated the initial transfusion policy, pending further type characterization, should be RhD-negative. When further (molecular) analysis shows the presence of weak D types 1, 2 or 3, it is advisable to convert the transfusion policy to RhD-positive since in these weak D phenotypes no immunization has ever been described and the stock of RhD-negative blood is usually limited. In all other cases RhD-negative transfusions are still recommended.[70]

Most aberrant *RHD* alleles encode RhD proteins that lack the expression of one or more RhD epitopes. Therefore, they are prone to form an anti-D against the RhD epitope(s) that they miss. For this reason, partial RhD phenotypes are always transfused with RhD-negative blood. Most partial RhD are present in non-White

populations except for the DVI phenotype which occurs with a incidence of 0.015% in the European population and is highly immunogenic.[71] Therefore, the Dutch consensus for RhD serotyping in patients recommends the use of an anti-D reagent that does not agglutinate the DVI protein so that the patient is typed RhD-negative and will therefore receive RhD-negative blood. However, in newborns, the DVI protein should be recognized as RhD-positive regarding the administration of anti-D immunoprophylaxis to the mother.[72]

1.3 Ethnic Diversity

The most recent ancestor of the human species and the chimpanzee lived approximately 5 to 6 million years ago in sub-Saharan Africa. The earliest members of the human-like (hominid) lineage, the australopithecine, remained confined to Africa. One of these australopithecines evolved into the first species of the genus *Homo* about 2.5 to 2 million years ago. The *H. ergaster/ erectus*, the first uncontested member of this genus, is believed to be the first hominid to have spread out of Africa about less than 2 million years ago. This spread is chronicled by the presence of *H. erectus*-like fossils. These fossils were found in the Middle east about 1.7 million years ago, in East Asia possibly 1.9 but certainly 1.1 million years ago and in Southeast Asia probably 1.8 million years ago. The *H. erectus*-like fossils found in Western Europe are not older than 1.0 million years. From this data a migration route could be reconstructed. Later, due to unknown reasons however a connection with the Ice Age has been postulated, a different branch of *H. erectus* evolved and spread from the Middle East to Europe. During the period of expansion, the species differentiated into distinct variants, leading to disagreement between paleoanthropologists about the identity of the *H. erectus* in different regions. What happened after the expansion is heavily disputed. The hominid fossil records (of the last 1.5 million years) have been interpreted in three different hypotheses, the candelabra hypothesis, the multiregional hypothesis and the uniregional hypothesis.[73]

1.3.1 The Candelabra Hypothesis

The Candelabra hypothesis (figure 6) was postulated by CS Coon in 1963.[74] He postulated that the different populations of H. erectus in the different regions evolved into subspecies of a new species, *H. sapiens*, independently of each other. The subspecies correspond to the four main geographical races. This model postulates an unprecedented mode of species origin. Instead of diversification in different species, isolated populations evolve towards subspecies of the same new species.

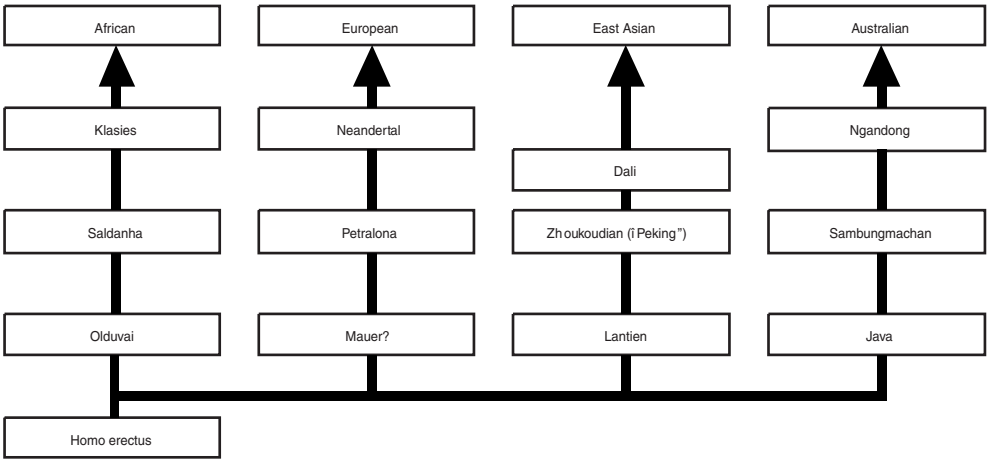

Figure 6. The Candelabra hypothesis as proposed by Coon.[73,74]
Independent emergence of *H. sapiens* from *H. erectus* in four different regions by parallel evolution.

The appearance of characteristics shared by the subspecies and specific for the new species is explained by the fact that all the mutations responsible for the appearance of these characteristics arose independently at least four times and were also fixed independently in each of the four populations. Therefore, this model implies an extraordinary form of parallel evolution.

1.3.2 The Multiregional Hypothesis

The multiregional hypothesis (figure 7) was developed by AG Thorne and MH Wolpoff in 1992.[75] This hypothesis postulates the emergence of the same species, *H. sapiens*, from the same ancestor, *H. erectus*, in the four different geographical races. It differs from the Candelabra model in that this hypothesis requires a continuous gene flow among the populations and therefore abolishes the requirement for parallel evolution. The explanation for the existence of two types of characteristics, those that differentiate the populations in the various regions from one another and those that are shared by the different populations but distinguish *H. sapiens* from *H. erectus*, is that regional differences are the result of mutations that arose locally and then remained limited to the population of each region and that the shared, *H. sapiens*-specific characters are the result of mutations that arose in one population and than spread by gene flow to all the other regional populations. Because each of the mutations arose only once, its fixation in the different populations does not represent parallel evolution. Multiregionalists believe in a evolution in which one species (*H. erectus*) evolves gradually, without splitting into a single new species (*H. sapiens*).

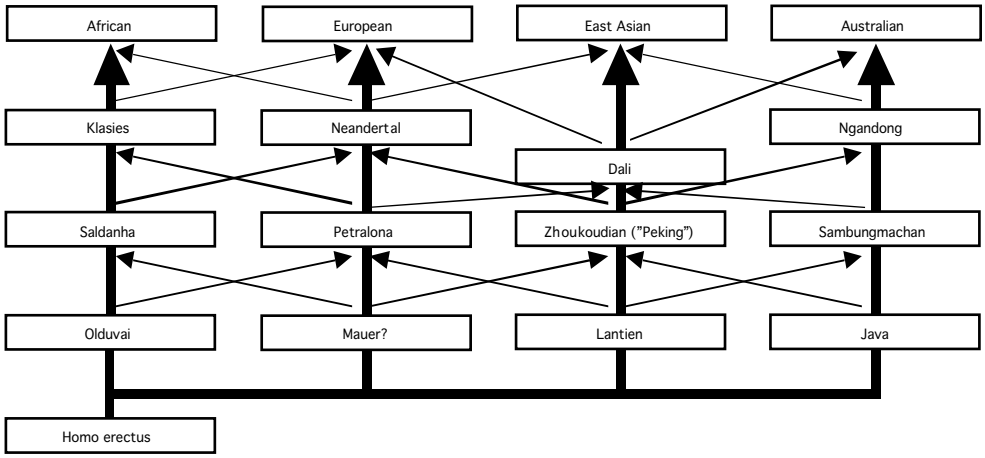

Figure 7. The multiregional hypothesis as postulated by Thorne and Wolpoff.[73,75]
The evolution of *H. sapiens* from *H. erectus* in four geographical regions by spreading of *H. sapiens*-specific characteristics from the population of their origin to all other populations. At the same time, characteristics responsible for regional continuity have remained restricted to the population of their origin.

1.3.3 The Uniregional Hypothesis

The uniregional hypothesis (figure 8) was postulated by Stringer and Andrews in 1988.[76] They divided the same sets of fossils in each of the regions into two categories, one related to *H. erectus* and one to *H. sapiens*. They interpret the first category as illustrative of the spread of *H. erectus* in the different regions, a spread that produced different forms, that all became extinct. The second category was seen as the product of a process in which the *H. ergaster* evolved in Africa into *H. sapiens* and then spread (less that 200,000 years ago) into the regions previously occupied by the descendants of *H. erectus*, as well as Australia and America. Thus, there was only one region in which the *H. Sapiens* originated and that this region was Africa. Therefore, this hypothesis is often referred to as the “Out of Africa” model.

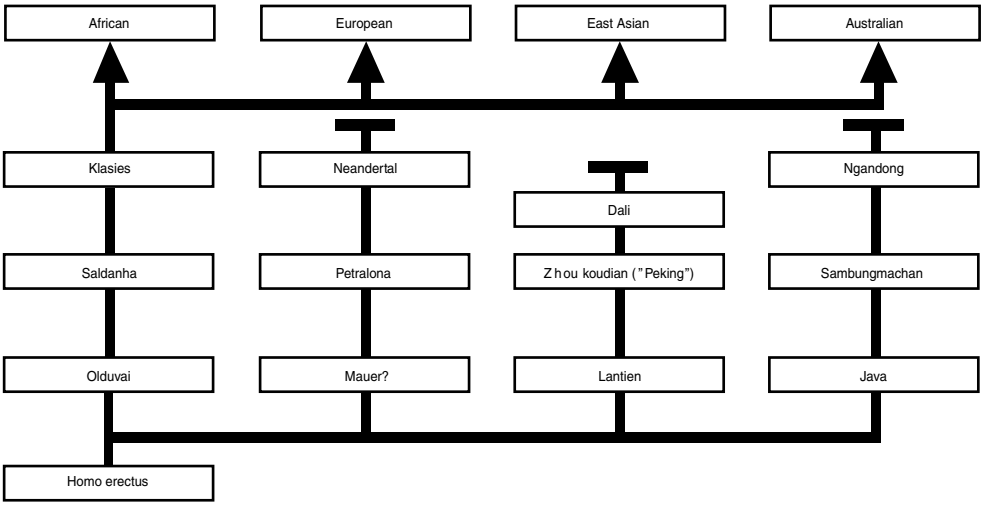

Figure 8. The uniregional (Out of Africa) hypothesis as postulated by Stringer and Andrews.[73,76]

Lineages founded by *H. erectus* became extinct in all regions except in Africa. The African lineage gave rise to *H. sapiens* which spread to all regions previously inhabited by *H. erectus* and its' descendants.

The main differences between the multiregional and the uniregional hypotheses are that the multiregional hypothesis postulates that *H. sapiens* evolved simultaneously from different populations in several regions, and that during its evolution genes flowed from one population to the other whilst the uniregional hypothesis postulates that *H. sapiens* evolved from a single population in one region and than expanded to other regions. Furthermore, the multiregional hypothesis postulates that the evolution of *H. sapiens* began less that 2 million years ago whereas the uniregional hypothesis postulates a much more recent evolution of the *H. Sapiens*, about 200,000 years ago. However, various uniregionalists differ in their opinion about what happened in Europe.

1.3.4 Phylogenetics

Since paleoanthropologists can not agree on how the fossil data should be interpreted, non-paleoanthropological data is sought that could give more insight into evolutionary pathways. Despite the fact that in the present time all lineages other than *H. sapiens* are extinct, it is still possible to gain information about the extinct ancestors by molecular analysis of especially the differentiated regions in the DNA of *H. sapiens* itself. By sequencing large stretches of DNA in different populations, mutation patterns will be found. Some mutations will be shared by different populations. The degree of sharing reflects the extend to which the DNA sequences are related just as the sharing of substitutions reflects phylogenetic relationships among

species. Therefore, the "ancestor of all ancestors" has none of the mutations that differentiate the living individuals.

The first tree of human populations was published in 1964, based on gene frequency data of five blood group systems (ABO, Rh, MN, Diego and Duffy) totalling 20 loci from 15 populations from different continents. Due to the low number of loci studied and the fact that the genes involved were all coding for blood group antigens (which probably are subject to the distorting influence of natural selection) the first tree was not perfect. However, in 1982 Nei et al. produced another tree of human populations based on gene frequencies of multiple protein encoding genes as well as blood group genes (figure 9).[73] They place the first split of *H. sapiens* approximately 110,000 years ago and the second split 41,000 years ago. Overall they conclude that all existing populations of *H. sapiens* are derived from an ancestral population that existed in Africa. Because the African population is the oldest population it had the most time to accumulate genetic differences.

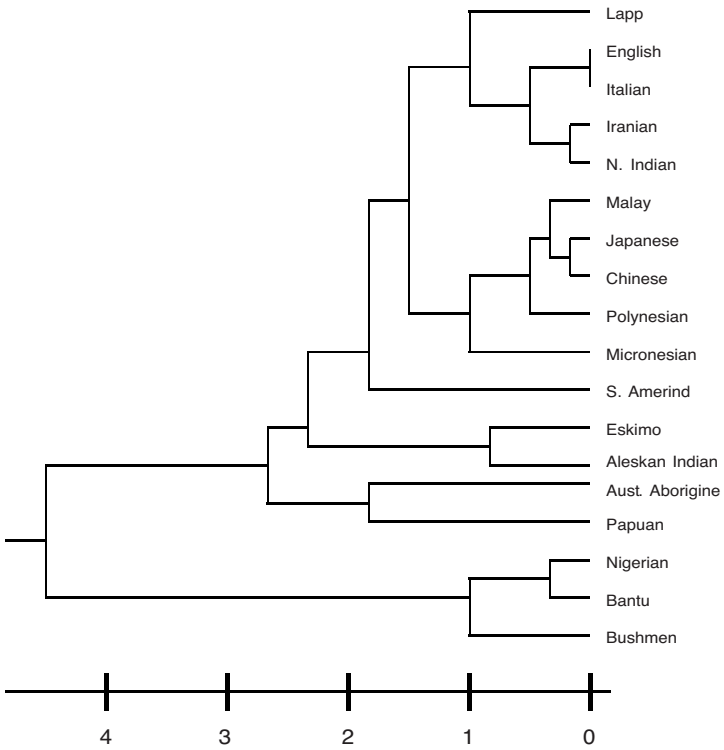

Figure 9. Tree of human populations by Nei and Roychoudhury in 1982.[73]

In 1987 a paper was published by Cann et al.[77] They conducted PCR-R(estriction) F(ragment) L(ength) P(olymorphism) analyses on mitochondrial DNA (mtDNA, does not recombine and is passed on strictly by the maternal lineage) of 147 individuals from five different populations (African, Asian, Australian, Caucasian and New

Guinean) with 12 restriction enzymes (totalling 467 sites). The tree they constructed consisted of two main branches, one with only African samples and the other with all remaining samples including some African samples. The researchers postulated that all mtDNA analysed stemmed from one woman that must have lived some 200,000 years ago, probably in Africa and that all populations, except the African population, had multiple origins. The postulated ancestral African woman is often referred to as "Black Eve", and released a genuine media spectacle. However, later studies conducted on the Y-chromosome indicate not only an African origin but also a bi-directional population movement and studies on the X-chromosome are in concordance with the multiregional hypothesis.[78,79] Studies conducted on microsatellite loci and on a different source, organic molecules or biomarkers, may give a new and deeper insight into the human evolution.

1.3.5 Hominid Speciation

To emerge as a new species, a group of individuals –the founding population– must somehow disengage itself from its parental population and begin an existence of its own (a population comprising the ancestral species is split in two). According to the founder effect hypothesis a species arisis from a pair or from very few individuals that have become isolated from the parental population. The founding period is reffered to as the bottleneck phase. The rival hypothesis interprets the transition from one species to another as a gradual proces without radical reduction in population size. It believes that it is the accumulation of small changes in the gene pool which differentiate and eventually isolate it from all other pools. According to the cladogenetic hypothesis, there are two modes of species formation, allopatric speciation (spatial) in which in order to diverge, the sisterpools must become separated by a barrier formed by long geographical distance and sympatric speciation (temporal) in which species that live in the same place diverge to form distinct species. However, the anagenetic (phyletic) hypothesis assumes a linear transformation from one population to another without a population splitting. Both modes of speciation exist.[73] Multiregionalists will argue that *H. erectus* originated by a founder effect during a bottleneck phase some 2 milione years ago. Uniregionalists will also explain the emergence of *H. sapiens* via a bottleneck, which they date much later in time. While cladogenesis in the formation of hominid species is now the preffered hypothesis, not so long ago hominid evolution was generally interpreted in terms of anagenesis. Still, multiregionalists see the transformation of *H. erectus* into *H. sapiens* from an anagenetic point of view.

1.3.6 Evolutionary Aspects of *RH*

Until the discovery of the sequence related *RHAG* homologues in vertebrates it was believed that the Rh proteins were erythroïd-specific and were only present in higher vertebrates. The *RHAG* homologues were found as 2 different *RHAG*-like genes

in *Caenorhabditis elegans* (a nematode) and as 1 *RHAG*-like gene in *Geodia cydonium* (a marine sponge), with amino acid identities of 46%, 39% and 47% respectively, as compared to human RhAG.[80,81] *RHAG* counterparts were also found in mouse and macaque.[82] The highest homology between RhAG and Rh is found in the transmembraneous parts of the protein, suggesting a conserved functional role for the RhAG protein family. *RH* orthologs were found in chimpanzees, gorillas, orang-utans, gibbons, baboons, macaques, New World monkeys, mice and cows.[83,84] The invertebrate homologues resemble *RHAG* more strongly than *RH*. Therefore it is postulated and widely accepted that the *RH* gene is a duplication of the *RHAG* gene and subsequently that this duplication event occurred during primate evolution (about 250- to 346 million years ago).[85] However it is also estimated by phylogenetic analysis that the time of coalescence of mammalian Rh cDNA sequences is 100 million years.[86] *RHCE* is believed to represent the ancestral gene while the *RHD* gene is the result of a duplication event that took place about 8 to 11 million years ago.[87]

Southern Blot experiments with human Rh cDNA probes have shown that only three species carry more than one *RH* gene; chimpanzees, gorillas and humans.[88] However, most rhesus monkeys and one among ten gorillas investigated so far were also found to carry small Rh-related 5'-fragments which correspond, most probably, to truncated *RH* genes. These truncated *RH* genes may be the result of either a partial duplication or they may be left over from an ancestral, partially deleted gene.[89,90] Recently the physical structure of the *RH* locus was revealed. *RHD* is flanked by two highly homologous sequences (98.6%) of approximately 9000 bp, dubbed upstream and downstream *Rhesus boxes* (*Rh boxes*). The downstream *Rh box* is followed by the *SMP1* gene and *RHCE*, antidromically. Deletion of *RHD* is proposed to have occurred via a mechanism of unequal crossing-over within a stretch of 1463 bp in which the Rhesus boxes share an identical sequence (identity region).[32]

The mouse orthologue of human Rh is Rhced. In mice, no sequences were found homologous to either the human *Rh boxes* or an additional *RH* gene. The mouse orthologue of *SMP1* is located antidromically at the 3'-end of the Rhced gene (the same as in humans except that in the human 3'-untranslated region both genes partially overlap).[91]

The Rh system now knows eight haplotypes which were formed by a series of duplication, mutation and recombination events (figure 10). Dce is the ancestral haplotype. Deletion or inactivation of *RHD* then created the dce haplotype. The DCe haplotype may have been the result of a non-reciprocal recombination of *RHD* exon 2 sequences into the ce allele of *RHCE* and DcE would have arisen from a point mutation in the ce allele. Then, dCe arose from a recombination between DCe and cde, dcE from recombination between DcE and dce, and DCE from recombination between DCe and DcE. The very rare dCE haplotype must have arisen from recombination between the also rare haplotypes dcE and dCe.[10,46]

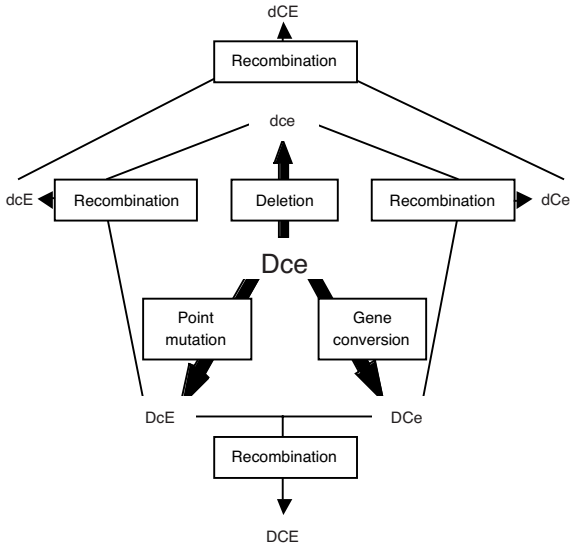

Figure 10. Schematic representation of the proposed derivation of the 7 different haplotypes from the ancestral Dce haplotype.[46]

1.4 Scope of this Thesis

The aim of the research described in this thesis was to gain insight into the ethnic variability of the Rh blood group system with the ultimate goal of developing typing methods that reliably predict the Rh blood group in a multiracial society in such a way that the effectivity of the present prevention-policy standards are guaranteed. To achieve this, we compared serological data with data obtained by molecular assays regarding *RHCc* typing and investigated the discrepancies. A new *RHC/RHc* genotyping strategy was developed that proved to generate reliable results in different ethnic groups (chapter 3).

During the course of the study the exact *RH* locus and the mechanism causing RhD-negativity in whites became known, enabling the molecular determination of *RHD* zygosity. This triggered us to study the *RHD* zygosity at the *RH* locus and the mechanism causing RhD-negativity in non-White persons (chapter 2). We also had the opportunity to investigate RhD-negativity and *RHD*-zygosity in Chinese HAN population (chapter 4).

The recently proposed phylogeny of RHD in humans shows 4 main clusters (figure 4).[45] Each of the African clusters is characterized by a specific animo acid substitution relative to the Eurasian *RHD* allele. Because of its proposed evolutionary and clinical importance we further analyzed the *RHD*(201R, 223V) cluster which includes the main part of the *weak D type 4* and *DIVa* clusters, in five ethnic groups (chapter 5).

To prevent maternal synthetization of anti-D due the maternal-fetal RhD-incompatibility IVIG-RhD is administered to all RhD-negative pregnant women in the 30th week of gestation in The Netherlands. IVIG-RhD is biological material and is becoming scarce as RhD immunization is prevented. Thus, unnecessary use of IVIG-RhD should be avoided, like administering IVIG-RhD to RhD-negative women carrying an RhD-negative fetus. Therefore, we developed a molecular assay to test for *RHD* positivity with respect to the RhD-negative *RHD* alleles (*RHD* alleles that do not give rise to an RhD protein due to various underlying molecular mechanisms) and evaluated the use of this strategy on cell-free fetal DNA derived from maternal plasma (chapter 6).

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Chapter 2

The highly variable *RH* locus in nonwhite persons hampers *RHD* zygosity determination but yields more insight into *RH*-related evolutionary events

Martine GHM Grootkerk-Tax, Petra A Maaskant-van Wijk, Judith van Drunen and C Ellen van der Schoot. The highly variable *RH* locus in nonwhite persons hampers *RHD* zygosity determination but yields more insight into *RH*-related evolutionary events. Transfusion 2005;45:327-337.

Abstract

Background: Knowledge about paternal *RHD* hemi or homozygosity is of clinical interest in alloimmunized pregnant women. D negativity in white persons is usually caused by deletion of the *RHD* gene. Recently, the physical structure of the *RH* locus and the mechanism cusing the deletion of the *RHD* gene have been explored, enabling *RHD* zygosity determination in white persons by specific detection of a hybrid *Rhesus box* characteristic for the *RHD*- locus

Study design and methods: *RHD* zygosity was determined in 402 samples from five different ethnic groups by polymerase chain reaction (PCR)-restriction fragment length polymorphism and by a newly developed real-time quantitative PCR. The *Rhesus boxes* of samples showing discrepancies between both tests were cycle sequenced.

Results: In nonwhite persons, several mutated *Rhesus boxes* exist that hamper zygosity determination by detection of the *RHD*- locus. Such mutated *Rhesus boxes* in D+ *RHD* homozygous black persons have a frequency of 0.22. In white persons, no mutated *Rhesus boxes* were encountered so far.

Conclusions: Owing to the high frequency of the mutated *Rhesus boxes*, zygosity determination by detection of the *RHD*- locus is not feasible in nonwhite persons. The cosegregation of variant *RHD* genes (*RHDψ* and *(C)cde*^{s}) with specific mutated *Rhesus boxes* yields more insight into the evolutionary events concerning variant *RHD* genes and mutated *Rhesus boxes*.

Introduction

The Rh system is a highly complex blood group system in which the Rh antigens are located on D and CE polypeptides. The polypeptides are encoded by the *RHD* and *RHCE* genes, respectively. D is involved in hemolytic transfusion reactions and hemolytic anemia. D mismatch is the major cause of hemolytic disease of the fetus and newborn (HDFN). Even after the introduction of D immunoglobulin, maternal immunization occurs in 0.8 to 1.5 percent of D- mothers owing to failure of prophylaxis or during pregnancy as a result of fetomaternal hemorrhage.1

Information about the *RHD* hemi- or homozygosity is of value for paternal testing when D antibodies are found in a D- pregnant woman. The risk of an affected child is 100 percent with a homozygously *RHD*+ father, but is only 50 percent when the father is hemizygous for *RHD*. Furthermore, *RHD* zygosity determination may be of value for preconception counseling when a previous pregnancy is complicated by severe HDFN attributed to D antibodies. The D- phenotype in white persons is mainly caused by *RHD* gene deletion, although 14 different D- *RHD* alleles have been described with low frequencies (1:1500) in Caucasian persons.[2] In the Japanese population, a D- *RHD* gene is found in 6 percent of the D- alleles, but D negativity in the Japanese population is rare (only 0.5% of the total population).[3] In other ethnic groups (especially in black persons), D negativity is frequently caused by the aberrant *RHD* genes *RHDψ* (allele frequency 0.0714)[4] and/ or (C)cde^{s} (r's, allele frequency 0.036).[5,6] In both cases, *RHD*-specific sequences are present but due to the presence of a translation termination codon (*RHDψ*) or the substitution of *RHD*-specific sequences by *RHCE*-specific sequences (*(C)cde^{s}*), no detectable D epitopes are present on the red blood cell (RBC) surface. Several methods have been described for *RHD* zygosity measurement, such as a simple guess based on phenotypic features, D antigen density and linkage disequilibrium, and associating D-negativity with polymorphisms in *RHCE*.[7,8] These approaches, however, are indirect and are therefore not very reliable. It is also possible to directly determine the extent of the *RHD* expression by quantitative polymerase chain reaction (PCR) or by classical endpoint PCR.[9,10] With these approaches, however, the aberrant *RHD* alleles should be taken into account. Recently, Wagner and Flegel[11] have elucidated the organization of the *RH* gene locus and proposed a model for the mechanism causing the *RHD*- haplotype in white persons. In this model, *RHD* negativity is caused by an unequal crossing-over event, triggered by the high homology between the upstream and downstream *Rhesus boxes* that flank the *RHD* gene (Fig. 1A). The *Rhesus boxes* have a length of approximately 9000 bp in identical orientation and share a 98.6 percent homology. The region in which the *RHD* deletion takes place (breakpoint region) is located within a stretch of 1463 bp in which both *Rhesus boxes* have an identical sequence (identity region). The *Rhesus box* resulting from the unequal crossing-over event therefore carries characteristics of both the upstream and the downstream *Rhesus boxes* (in 5'-end to 3'-end order) and is called the

hybrid *Rhesus box*. Wagner and Flegel[11] suggested two different PCR methods for *RHD* zygosity determination by specific detection of the *RHD*- genotype. One of those methods is based on the PCR-restriction fragment length polymorphism (RFLP) technology. With a consensus forward primer and a downstream specific reverse primer, both hybrid and downstream *Rhesus boxes* are amplified. The PCR is followed by enzymatic digestion that distinguishes between the hybrid *Rhesus box* and the downstream *Rhesus box* at the 5'-part of the breakpoint and/or identity region (Fig. 1B). Three recently conducted studies are known to investigate *RHD* zygosity based on the PCR-RFLP method,[12-14] of which two studies were conducted on a nonwhite population.[12,14] None of these studies, however, have made a direct comparison between zygosity determination on a quantitative level, directly on the *RHD* gene, and on the presence of the *RHD*- locus. In this study, *RHD* zygosity was determined in 402 donors from five different ethnic groups. Results of the PCR-RFLP were compared with results obtained with a newly developed real-time quantitative PCR specific for *RHD* exon 7, combined with an assay on a reference gene, for *RHD* zygosity determination. Discrepant samples were further analyzed by cycle sequencing of the *Rhesus boxes*.

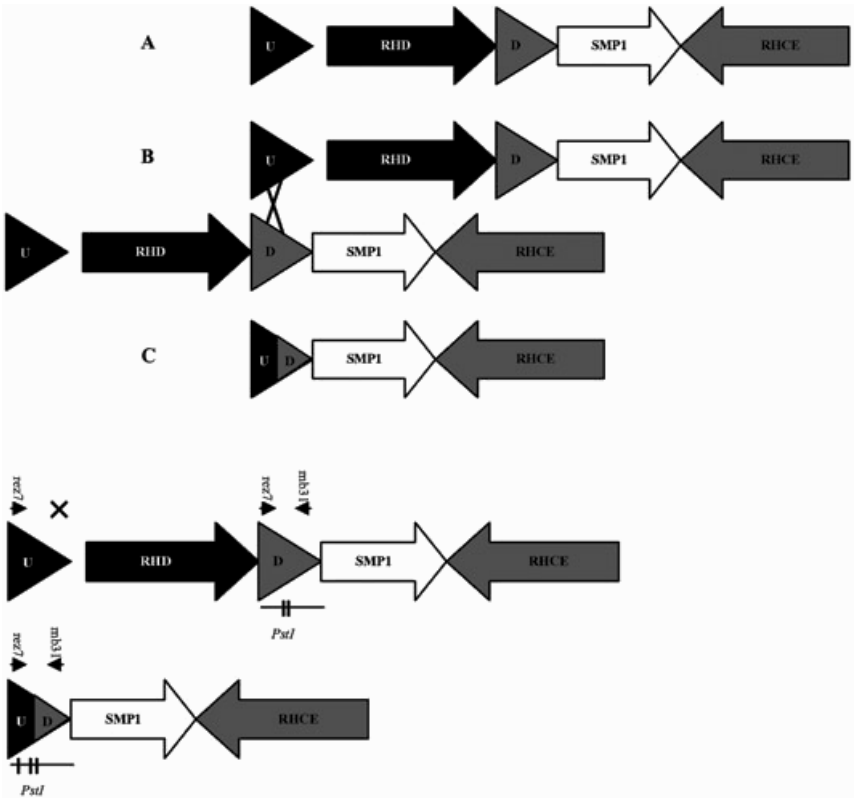


Figure 1. *RH* locus and proposed mechanism of unequal crossing-over causing the *RHD*- haplotype in white persons. (Top) Physical structure of the *RH* locus. (A) The *RHD* gene is flanked by the highly homologous upstream (U, GenBank Accession Number AJ252311) and downstream (D, GenBank

Accession Number AJ252312) *Rhesus boxes* depicted by black and gray triangles, respectively. The downstream *Rhesus box* is followed by the *SMP1* gene (white arrow) and, in the opposite direction, the *RHCE* gene (gray arrow). (B) Triggered by their high homology, the proposed unequal crossing-over takes place between the upstream and downstream *Rhesus boxes*. (C) The result of the unequal crossing-over event, the *RHD* - *RH* locus with a hybrid *Rhesus box* (UD, GenBank Accession Number AJ252313) as found in white persons.[11] (Bottom) PCR-RFLP. Both the downstream and the hybrid *Rhesus boxes* are amplified with primers rez7 (consensus) and rnb31 (downstream specific) as depicted by the line with hatched marks, indicating the *Pst* I digestion sites. Owing to the *Pst* I digestion site present in the part preceding the identity region of only the upstream *Rhesus box* (present in the amplified hybrid *Rhesus box*), the *RHD* + and *RHD* - locus are distinguished.[11]

Materials and methods

Samples

From five different ethnic groups, 263 D + and 139 D- ethylenediaminetetraacetate-anticoagulated blood samples were obtained. D + samples comprised 52 white persons from the Netherlands (9 ccDEe, 35 CcDee, 7 ccDee, and 1 CCDee phenotypes), 84 South African black persons (73 ccDee, 3 ccDEe, and 8 CcDee phenotypes), 47 South African Asian persons (2 ccDee, 8 ccDEe, 31 CcDee, 1 CcDEe, 3 CCDee, and 2 CCDEe phenotypes), 49 black persons from Ethiopia (18 ccDee, 5 ccDEe, 18 CcDee, 3 CcDEe, 4 CCDee, and 1 CCDEe phenotypes), and 31 black persons from Curaçao (13 ccDee, 4 ccDEe, 9 CcDee, 2 CcDEe, and 3 CCDee phenotypes). D- samples comprised 10 white persons from the Netherlands (8 ccdee, 1 ccdEe, and 1 Ccdee phenotypes), 10 South African black persons (6 ccdee and 4 Ccdee phenotypes), 17 South African Asian persons (16 ccdee and 1 Ccdee phenotypes), 13 black persons from Ethiopia (12 ccdee and 1 Ccdee phenotypes), and 89 black persons from Curaçao (73 ccdee, 1 ccdEe, and 15 Ccdee phenotypes). Based on their phenotypic features and the prevalence of haplotypes among the ethnic backgrounds, all nonwhite D + samples were expected to be homozygous for *RHD*.[15] Samples of the white group were selected for hemizygosity based on their phenotypic features; as a control sample, one *RHD* homozygous sample was included (CCDee).

Serology

RBCs were Rh phenotyped according to standard serologic protocols with an immunoglobulin (Ig) M monoclonal antibody (MoAb) anti-D (GAMA401, D epitope 6/ 7), a polyclonal IgG anti-D, and IgM MoAbs (from Gamma Biologicals, Houston, TX) recognizing C (MS24), c (MS33), E (GAMA402), and e (MS16, MS21, MS63 (blend)).

Genomic DNA analyses

Genomic DNA was isolated from peripheral white blood cells by a salting-out method according to standard protocol.[16] Oligonucleotide sequences of all primers (Invitrogen, Carlsbad, CA) used in this study are listed in Table 1. PCR procedures were performed in a thermal cycler (Model 9700, Applied Biosystems, Nieuwerkerk aan de IJssel, the Netherlands).

For detection of the *RHD* deletion, the PCR-RFLP method as previously described by Wagner and Flegel was performed.[11] Primers rez7 and rnb31 were used for amplification of the downstream and hybrid *Rhesus boxes* (Table 1). Primer annealing was at 66^{0}C. The enzymatic digestion pattern indicates the presence of a hybrid *Rhesus box* (*RHD* gene deleted) and/or a downstream *Rhesus box* (*RHD* gene present) (Fig. 1B).

The *RHD* multiplex (MPX) PCR was performed as described by Maaskant-van Wijk and coworkers.[17] Minor modifications were the use of high-performance liquid chromatography-purified primers and PCR master mix (AmpliTaq, Applied Biosystems). With this MPX PCR, *RHD* exons 3, 4, 5, 6, 7, and 9 are amplified simultaneously.

For the detection of *RHD*ψ, an allele-specific primer amplification was developed with primers Rex5Ws and Rex5Was (Table 1) specific for nucleotide 654C (*RHD*ψ) and nucleotide 674T (*RHD*ψ), respectively. As internal control primer set, ßACTs and ßACTas (*b-actin gene*) were used. PCR procedures were performed on 200 ng of genomic DNA in a total reaction volume of 50 uL. The reaction mixture contained 100 ng of primers Rex5Ws and Rex5Was, 50 ng of primers bACTs and bACTas, 5 mmol per uL of each dNTP, 2 U of Taq DNA polymerase (Promega, Madison, WI) in the appropriate buffer, and 2 U of *Taq* DNA polymerase antibody (Becton Dickinson, San Jose, CA), supplemented with 1.5 mmol per uL $MgCl_2$. Amplification conditions were 1-minute denaturation at 95^{0}C, 1- minute primer annealing at 60^{0}C, and 1 minute extension at 72^{0}C.

The *RHC/c/hex3* MPX PCR followed by intron 4/exon 7 MPX PCR as described by Tax and associates[18] was used for the detection of *(C)cde^s*, *DIIIa*, and *DIVa* alleles. When a hybrid exon 3 was detected by the *RHC/c/hex3* MPX PCR, both *DIIIa* and *DIVa* alleles were distinguished from a *(C)cde^s* allele by the intron 4/exon 7 MPX PCR.

Table 1. Oligonucleotide primer sequence and specificity

Primer Name	Specificity	Nucleotide sequence*
Rex5Ws	*RHDψ*	CGCCCTCTTCTTGTGGATC (F)
Rex5Was	*RHDψ*	TGGACTTCTCAGCAGAGCAA (R)
bACTs	*ß-Actin gene*	ccttcctgggcatggagtcctg (F)[17]
bACTas	*ß-Actin gene*	ggagcaatgatcttgatcttc (R)[17]
940S	*RHD* exon 7	G**G**GTGTTGTAACCGAGTGCTG (F)
1064R	*RHD* exon 7	CC**GGCTCC**GAC**G**GTAT**C** (R)
968	*RHD* exon 7	cccacagctccatcatgggctacaa (P)
Alb-F	*Albumin*	tgaaacatacgttcccaaagagttt (F)
Alb-R	*Albumin*	ctctccttctcagaaagtgtgcatat (R)
Alb-T72	*Albumin*	tgctgaaacattcaccttccatgcaga (P)
Rez7	*Consensus Rhesus box*	cctgtccccatgattcagttacc (F)[11]
Rnb31	*Downstream Rhesus box*	*cct*ttttttgtttgttttttggcggtgc (R)[11]
CBRF04	*Consensus Rhesus box*	tgcactgccctggcagagg (F)
CRBR02	*Consensus Rhesus box*	agaaatttgcatgagtaacaggag (R)
Rhbox5070	*Consensus Rhesus box*	ctacaggcccatgagagtccaaa (F)[12]
Rhbox5571	*Consensus Rhesus box*	agtgcaagccccaagccttgaca (R)[12]
CRBF06	*Consensus Rhesus box*	gttaatatgggtggctggc (F)
CRBR08	*Consensus Rhesus box*	cattaagagatacgcacagg (R)

* Uppercase and lowercase characters represent coding and noncoding sequences, respectively. Bold characters represent *RHD*-specific nucleotides, underlined characters represent downstream *Rhesus box*-specific nucleotides, and italic characters represent primer mismatches.
In parentheses: P = RQ-PCR probe; F = forward orientation of the primer; R = reverse orientation of the primer.

RHD zygosity determination by real-time quantitative PCR

For *RHD* zygosity determination a real-time quantitative PCR (RQ-PCR) was developed (Model 7700 sequence detector, Applied Biosystems).[19] In a single well, *RHD* exon 7 (primers 940S and 1064R [*RHD* specific]) and albumin (reference gene, primers Alb-F and Alb-R) were amplified. *RHD* was detected by a FAM fluorescently labeled *RHD*-specific probe (probe 968, Table 1), and *albumin* was detected simultaneously by a VIC fluorescently labeled *albumin*-specific probe (probe Alb-T72, Table 1). Primers were used in a final concentration of 900 nmol per uL for *RHD* and 300 nmol per uL for *albumin*, and both probes were used in a final concentration of 100 nmol per uL in a total volume of 25 uL of universal master mix (TaqMan, Applied Biosystems). The best results were obtained with a genomic DNA input between 50 and 100 ng. Each sample was tested in triplicate, and for each test the ratio between *RHD* and albumin was calculated by dividing the *RHD* gene quantity by the albumin gene quantity based on a standard curve. *RHD* zygosity of each sample was determined by taking the median of the three ratios. When hemizygous for *RHD*, the

median is approximately 0.5, when homozygous for *RHD* the median is approximately 0.9 (Fig. 2). With the above-described method, 149 samples were analyzed. In 114 samples, *RHD* zygosity was determined with *RHD* and albumin in separate wells because both probes carried the same (FAM) fluorescent label.

Sequencing analyses of the Rhesus boxes

The position of all primers used for *Rhesus box* sequencing and the PCR-RFLP are depicted in Fig. 3. PCR products obtained with primers rez7/rnb31 were cycle-sequenced (ABI-Prism 377, DNA sequencer, Applied Biosystems) with primers Rhbox5070, Rhbox5571, CRBF04, CRBR02, and CRBF06 with cycle sequencing chemistry (Big Dye terminator, Applied Biosystems). PCR products obtained with primers CRBF06 and CRBR08 were sequenced with primer CRBF06. Reference to nucleotide positions is given corresponding to the published downstream *Rhesus box* sequence (GenBank Accession Number AJ252312).

Results

RHD zygosity determination of D+ samples

The *RHD* zygosity of 263 D+ samples from five different ethnic groups was determined by PCR-RFLP and RQ-PCR. In white persons, no discrepancies between both methods were found. Of the 52 samples, 51 samples showed hemizygosity by PCR-RFLP and RQ-PCR and 1 sample showed homozygosity for *RHD* with both assays. In all nonwhite groups, discrepancies between PCR-RFLP and RQ-PCR were found (61 of 211). In 58 cases, the PCR-RFLP indicated the presence of a hybrid *Rhesus box*, suggestive for an *RHD* gene deletion (*RHD*+/*RHD*-), whereas by RQ-PCR 2 *RHD* genes were demonstrated (*RHD*+/*RHD*+) (Table 2). One sample showed only hybrid *Rhesus boxes* by PCR-RFLP (*RHD*-/*RHD*-) and one *RHD* gene by RQ-PCR (*RHD*+/*RHD*-) (Table 2B).

Table 2. RHD zygosity determination by PCR-RFLP versus RQ-PCR in four different ethnic groups*

	RQ-PCR	
PCR-RFLP	*RHD +/RHD +*	*RHD +/RHD-*
A. Ethiopian black persons		
RHD +/RHD +	26	0
RHD +/RHD-	**7**	16
RHD-/RHD-	0	0
B. South African black persons		
RHD +/RHD +	23	1[a]
RHD +/RHD-	**42**	17
RHD-/RHD-	0	1[b]
C. Curaçao black persons		
RHD +/RHD +	14	1[a]
RHD +/RHD-	**8**	8
RHD-/RHD-	0	0
D. South African Asian persons		
RHD +/RHD +	11	0
RHD +/RHD-	**1**	35
RHD-/RHD-	0	0

* Fifty-eight samples show a hybrid *Rhesus box* by PCR-RFLP (*RHD*+/*RHD*-) and two *RHD* genes by RQ-PCR (*RHD*+/*RHD*+) (7 Ethiopian black persons, 42 South African black persons, 8 Curaçao black persons, and 1 South African Asian person, all in bold). Two samples show two *RHD* genes by PCR-RFLP and only one *RHD* gene by RQ-PCR, both indicated by a superscript lowercase "a" (B and C). Further analysis of these samples revealed one *(C)cde*^{s} allele (South African black person) and one *DIVa* allele (Curaçao black person), both hampering zygosity determination by RQ-PCR. One South African black person showed only hybrid *Rhesus boxes* by PCR-RFLP and one *RHD* gene by RQ-PCR (B, indicated by superscript lowercase "b"). Sequencing analysis showed that this sample carried a mutated downstream *Rhesus box* as described by Matheson and Denomme.[12]

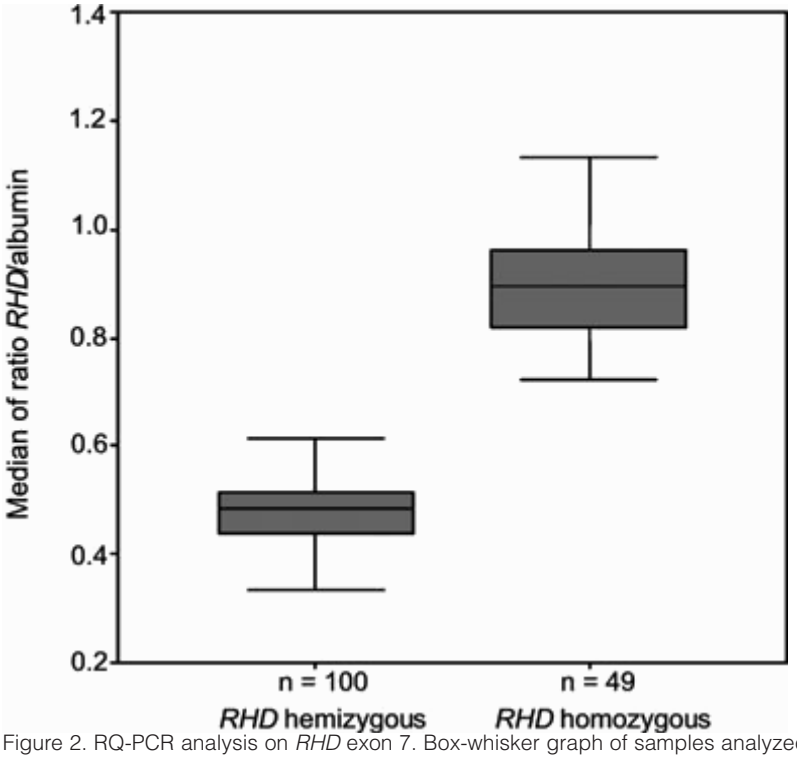


Figure 2. RQ-PCR analysis on *RHD* exon 7. Box-whisker graph of samples analyzed for *RHD* zygosity with the RQ-PCR on *RHD* exon 7 (n = 149). Of each sample the median of the ratio of *RHD* divided by albumin was calculated from triplicate samples. Of each experiment the median of the ratio of *RHD* of the samples are calculated relative to the *RHD* homozygous control. The whiskers represent the range and the boxes represent the mean ± standard deviation per group. Based on the RQ-PCR results, 100 samples were determined to be hemizygous for *RHD* and 49 samples were determined to be homozygous for the *RHD* gene.

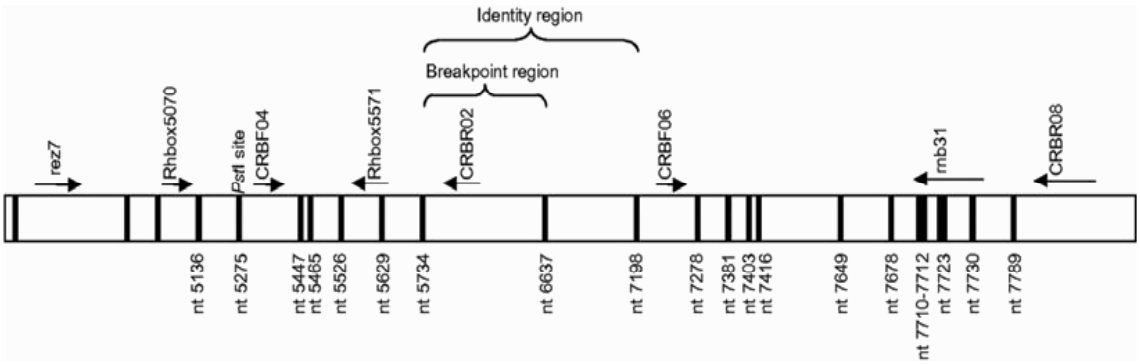
Two samples showed no hybrid *Rhesus boxes* by PCR-RFLP (*RHD*+/*RHD*+) and only 1 *RHD* gene by RQ-PCR (*RHD*+/*RHD*-) (Tables 2B and 2C). Because the RQ-PCR is specific on *RHD* exon 7 and therefore does not recognize the variant *RHD* alleles *(C)cdes* and *DIVa*, all 263 samples were analyzed for the presence of such alleles with the *RHC/c/hex3* MPX PCR followed by the intron 4/exon 7 MPX PCR. In only 2 cases, the presence of a *(C)cde*^{s} allele (South African black person) and a *DIVa* allele (Curaçao black person) was demonstrated, which explained the RQ-PCR hemizygosity, although PCR-RFLP did not indicate the presence of a hybrid *Rhesus box*.

Sequence analysis of the RH locus in samples showing a hybrid Rhesus box by PCR-RFLP and two RHD genes by RQ-PCR.

To elucidate why the PCR-RFLP indicated a deletion of the *RHD* gene, while RQ-PCR indicated the presence of two *RHD* genes, PCR-RFLP products (primers rez7 and rnb31, amplifying both the hybrid and the downstream *Rhesus boxes*) of 40 dis-

crepant samples were sequenced with forward primer Rhbox5070 and reverse primer Rhbox5571 (Rhbox primers), spanning a domain including nt. 5275, G>A (*Pst*I restriction site) and nucleotide 5447, 5465, and 5526, T>C, A>C, and A>G from upstream to downstream *Rhesus box* sequence, respectively (Fig. 4, URH and DRH). We expected to find only downstream sequences because the RQ-PCR infers the presence of two downstream *Rhesus boxes*. Six samples showed the presence of a mutated downstream box (nucleotides 5164C, 5275G, 5447C/T, 5465C, and 5526G) as described by Matheson and Denomme,[12] causing *Pst*I digestion in the PCR-RFLP (4 of 26 South African black persons, 1 of 6 Curaçao black persons, and 1 of 7 Ethiopian black persons).

Figure 3. Primer positions and specificity on the *Rhesus box*. Primer rnb31 is downstream sequence specific; all other primers are consensus (they anneal on the upstream, downstream, and hybrid *Rhesus boxes*). The upstream sequence of nucleotide (nt) 5275 forms the discriminating *Pst*I digesti-



on site.

A mutated downstream *Rhesus box* as described by Matheson and Denomme was also found in the sample showing two hybrid *Rhesus boxes* by PCR-RFLP and one *RHD* gene by RQ-PCR (Table 2B).[12] One sample (South African black person) showed a deletion-insertion in one of the sequences hampering further analyses. Of the remaining 33 samples, normal upstream (URH) and downstream (DRH) *Rhesus box* sequences were found in the 5'-part preceding the identity region, compared with an *RHD* hemizygous control sample (white person). This is in concordance with the hemizygous restriction pattern in the PCR-RFLP. Of these, 7 showed upstream and downstream sequences in a 1URH:1DRH ratio (6 of 26 South African black persons and 1 of 6 Curaçao black persons), and 26 showed the upstream and downstream sequence in a 1URH:2DRH ratio (15 of 26 South African black persons, 1 of 1 South African Asian persons, 4 of 6 Curaçao black persons, and 6 of 7 Ethiopian black persons). Because the RQ-PCR indicated the presence of two *RHD* genes, we postulated that the upstream sequence amplified in the 5'-part preceding the identity region could not be derived from a hybrid *Rhesus box* but from either a mutated upstream or a mutated downstream *Rhesus box*. To further analyze this, the

3'-part following the identity region was analyzed in 21 samples (6 1URH:1DRH and 15 1URH:2DRH) by sequencing PCR products obtained with primers CRBF06 and CRBR08 (consensus) with primer CRBF06. In five cases (all 1URH:1DRH in the 5'-part preceding the identity region), the absence of a normal upstream *Rhesus box* sequence indicated the presence of a mutated upstream *Rhesus box*. The presence of a *Pst*I digestion site in the PCR product obtained with the downstream *Rhesus box* primer rnb31 can thus be explained by the homozygous presence of a mutated upstream box causing the annealing of primer rnb31 in the 3'-part following the identity region of the mutated upstream *Rhesus box*. In four of these five samples (three South African black persons and one Curaçao black person), the mutated upstream box (further assigned as URH1, Fig. 4) showed transition from URH to DRH between nucleotide 7416 and nucleotide 7649. In one of the five samples (South African black person), a homozygous presence of a different mutated upstream *Rhesus box*, assigned URH2 (transition from URH to DRH between nucleotide 7381 and nucleotide 7416), was postulated.

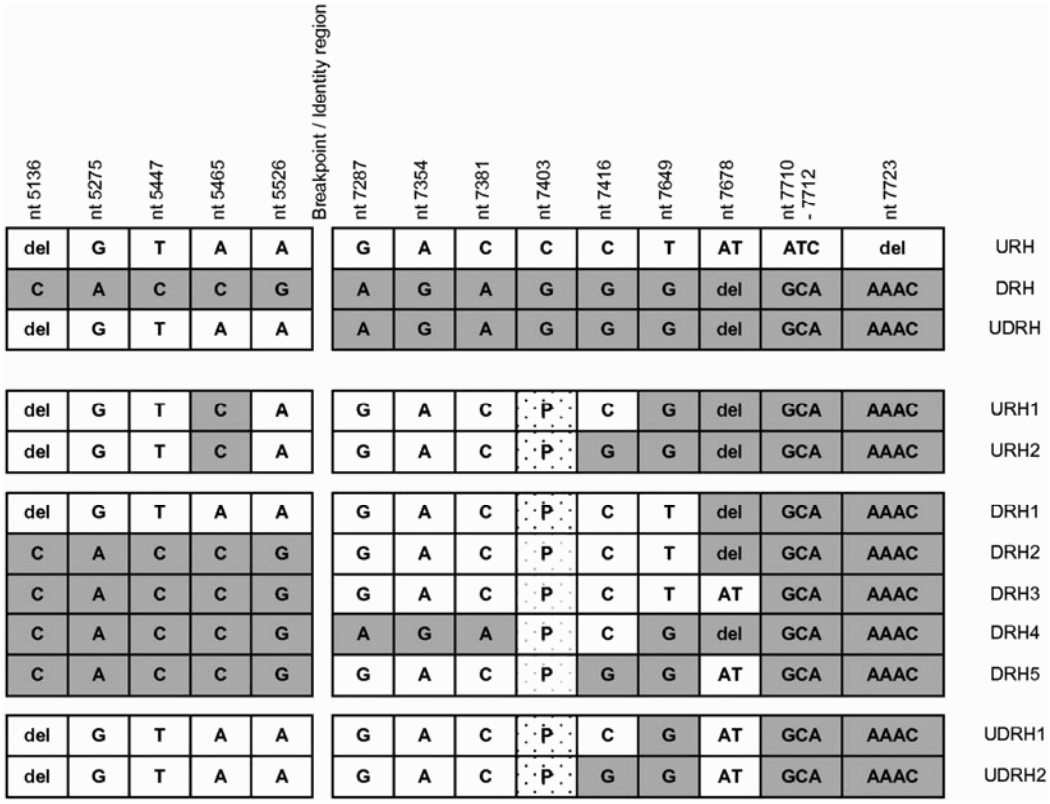


Figure 4. Sequences of mutated upstream, downstream, and hybrid *Rhesus boxes* found in black persons. The boxes URH and DRH represent the upstream (white squares) and downstream sequence (gray squares) *Rhesus boxes*, respectively, as previously described.[11] Nucleotide (nt) 7403 was found to be nonspecific for upstream or downstream sequences and is therefore denoted as P (polymorphic, dotted squares). URH1 and URH2 are mutated upstream *Rhesus boxes* and DRH1 to DRH5 are mutated downstream *Rhesus boxes* as found in nonwhite research populations. UDRH is the hybrid *Rhesus box* as previously described in white persons and in this study is predominantly found in white persons and South African Asian persons. UDRH1 and UDRH2 are the two deviant hybrid *Rhesus boxes* as found in black persons.

The sequences of the PCR products obtained with consensus primers CRBF06 and CRBR08 of the other discrepant samples were also analyzed for the possible presence of the URH1 and URH2 mutated *Rhesus boxes*. In the samples showing 1URH:2DRH in the 5'-part preceding the identity region, the presence of either URH1 (n = 11) or URH2 (n = 4) boxes was found, next to a normal upstream sequence as expected from the 1URH:2DRH ratio. The interpretation of these sequences was less clear, however, because of the presence of additional mutations in the 3'- part following the identity region (discussed later). In one sample (1URH:1DRH), sequence analyses showed that the discrepant results between RQ-PCR and PCR-RFLP could be explained by the presence of a mutated downstream *Rhesus box*, because sequencing of the consensus PCR product (primers CRBF06 and CRBR08) showed three upstream and one downstream sequence in the 5'-end

region and sequencing with the Rhbox primers (on the product obtained with primers rez7 and rnb31) showed a 1URH:1DRH ratio. The *Pst*I digestion site in the PCR-RFLP product was present in a mutated downstream *Rhesus box* assigned DRH1 (Fig. 4, transition from URH to DRH between nucleotide 7649 and nucleotide 7678). The DRH1 was found next to two normal URH and one normal DRH, causing the 1URH:1DRH ratio with Rhbox primer sequencing. As indicated previously, the sequence analysis of the consensus PCR product from the 3'-part following the identity region showed more nucleotide changes than could be explained by the assigned *Rhesus boxes* (URH, URH1, URH2, DRH, DRH1). In particular, the differences in sequence peak height suggested the presence of additional mutated *Rhesus boxes*. Therefore, sequence analysis of the 3'-part following the identity region with CRBF06 was also performed on products obtained with primers rez7 and rnb31 (downstream *Rhesus box* specific) on the 21 selected samples. All sequences could be explained by the presence of one of three newly identified mutated downstream boxes (DRH2, DRH3, DRH4, Fig. 4). These mutated downstream *Rhesus boxes* showed normal downstream- specific sequences in the 5'-part preceding the identity region and therefore do not hamper zygosity determination by PCR-RFLP. The DRH2 box was found in five samples next to a URH1, DRH3 in six samples next to a URH1, and DRH4 in one sample homozygously next to a URH1. No mutated DRH boxes were found in the five samples in which the URH2 caused the *Pst*I digestion by PCR-RFLP. In the other four samples, only normal downstream *Rhesus boxes* were found. Table 3 shows the number of discrepant samples per ethnic group and per phenotype. No association between *RHCE* haplotypes and mutated *Rhesus boxes* could be established. It has previously been suggested that a cytosine at nucleotide position 7403 was specific for the upstream *Rhesus box* sequence.[11] In most of the analyzed downstream and hybrid *Rhesus boxes*, however, and especially in black persons but also in white persons, a guanine at nucleotide position 7403 was encountered. Because of this polymorphism, it is not possible to indicate this nucleotide in the newly identified mutated *Rhesus boxes* (Fig. 4).

Table 3. Mutated Rhesus boxes, phenotype association, and occurrence of RHDψ and *(C)cde*^{s} alleles in D+ samples*

	Phenotype					
Ethnic group	ccDee	ccDEe	CcDee	CcDEe	CCDee	CCDEe
South African black persons	408 (33)	11 (2)	1 (71)	NT†	NT	NT
South African Asian persons	0 (2)	0 (8)	1 (30)	0 (1)	0 (3)	0 (2)
Ethiopian black persons	3 (15)	1 (4)	3 (15)	0 (3)	0 (4)	0 (1)
Curaçao black persons	53 (8)	1 (3)	21 (71)	0 (2)	NT	0 (3)

* The number outside the parentheses represents the number of samples carrying a mutated *Rhesus box* hampering zygosity determination by PCR-RFLP analyses per phenotype and between parentheses is the number samples tested without a mutated *Rhesus box* hampering zygosity determination by PCR-RFLP analyzes per phenotype. Superscript characters represent the number of *RHD*ψ alleles detected and subscript characters represent the number of *(C)cdes* alleles detected. † NT = not tested.

Furthermore, we found nucleotide 7354A encoding upstream and nucleotide 7354G encoding downstream sequences in all samples (also in samples without a mutated upstream *Rhesus box*) and this is therefore included as such in Fig. 4.

Analyses of the RHD gene-negative RH locus.

A total of 139 D- samples were analyzed by PCR-RFLP and by *RHD* MPX PCR (to identify the presence of the *RHD*ψ gene and/or *(C)cde*^{s} allele). PCR-RFLP showed the presence of homozygous *RHD* gene deletion sites in 105 D- samples. In 32 samples, the presence of both a hybrid and a downstream *Rhesus box* was demonstrated, indicative for the presence of one *RHD* gene. In 26 of these samples, the presence of either the nonfunctional *RHD*ψ gene (15 samples, ccdee) or the nonfunctional *(C)cde*^{s} allele (11 samples, Ccdee) was demonstrated, all in black persons from South Africa, Curaçao, and Ethiopia. In 4 samples (3 South African black persons and 1 Curaçao black person, Ccdee) both the *RHD*ψ and the *(C)cde*^{s} allele were found. In these samples, the PCR-RFLP falsely indicated the presence of a hybrid *Rhesus box*. Two samples (Ethiopian black persons, ccdee) carried the DAU2 *RHD* variant allele and were initially typed D- owing to the very low antigen density.[20] In two samples (black persons from Curaçao and South Africa, phenotype Ccdee), the presence of only downstream *Rhesus box* sequences was demonstrated, indicative for the presence of two *RHD* genes. In both samples, the presence of homozygous *(C)cde*^{s} alleles was demonstrated. Except for the four samples with *RHD*ψ and *(C)cde*^{s} alleles there was complete concordance between PCR-RFLP and *RHD* MPX PCR analyses. Because in all *RHD*- samples the hybrid *Rhesus box* was detected by PCR-RFLP, this deletion has most probably occurred in all ethnic groups by a similar mechanism. To investigate this in more detail, hybrid *Rhesus boxes* of four nonwhite groups were sequenced. To analyze the region between the *Pst*I digestion site (nucleotide 5275) in the 5'-part preceding the identi-

ty region and the identity and/or breakpoint region, PCR products obtained with primers rez7 and rnb31 of 47 homozygous *RHD*- samples (9 South African Asian persons, 9 Curaçao black persons, 10 Ethiopian black persons, 9 South African black persons, and 10 white persons) were sequenced with primers CRBF04 and CRBR02. In all cases, the 3 upstream-specific nucleotides (5447, 5465, and 5526) between the upstream-specific nucleotide 5275 (conducting the *Pst*I restriction site) and the identity and/ or breakpoint region were found. Additionally, in 2 samples the nucleotide changes 5629C>T (white person) and 5734C>T (South African black person) were found. This indicates that at least in all tested 74 ce-alleles from nonwhite persons the *RHD* deletion has not occurred upstream from the identity and/or breakpoint region. To study the hybrid Rhesus boxes in the 3'-part following the identity region, 27 homozygous *RHD*- samples were sequenced with primer CRBF06 on a PCR product obtained with primers CRBF06 and CRBR08 (8 South African Asian persons, 9 Curaçao black persons, and 10 Ethiopian black persons; no South African black persons were analyzed because in this group only samples with at least 1 nonfunctional *RHD* gene were present). All Asian samples showed downstream sequences indicating 2 normal hybrid *Rhesus boxes*. Of the black persons, 6 samples showed only downstream sequences, 3 samples showed downstream sequences with a 7403G>C change, 5 samples showed upstream and downstream sequences at nucleotides 7287, 7381, and 7403 and continued downstream, and 5 samples showed upstream and downstream sequences at nucleotides 7287, 7381, 7403, and 7416 and continued downstream. These sequence results indicate the presence of two mutated hybrid *Rhesus boxes* (Fig. 4, UDRH1 and UDRH2) in black persons. Both the UDRH1 (once) and the UDRH2 (three times) boxes were also found in D+ samples hemizygous for *RHD* (n = 6) next to a normal upstream and downstream *Rhesus box*. To study the prevalence of the UDRH1 and UDRH2 boxes in white persons, 42 *RHD*- samples were sequenced with primer CRBF06 on the PCR product obtained with primers CRBF06 and CRBR08. Thirty-eight samples showed only downstream sequences and 4 samples showed downstream sequences with a heterozygous 7403G>C polymorphism. Neither UDRH1 nor UDRH2 boxes are prevalent in a white population.

Sequence analyses of RHDψ + and/or (C)cdes+ samples.

As shown in Table 3, the *(C)cde*^{s} allele is only found in samples without a mutated *Rhesus box* that results in a discrepant result in the PCR-RFLP, whereas the *RHDψ* gene is exclusively found in samples carrying such a mutant *Rhesus box*. This suggests that the *RHDψ* cosegregates with a mutated *Rhesus box* and that *(C)cde*^{s} alleles never cosegregate with a mutated *Rhesus box* that interferes with the PCR-RFLP assay. Therefore, 30 samples (14 hemizygous *RHDψ*, 5 *RHDψ*, and *(C)cde*^{s}; 6 hemizygous *(C)cde*^{s}; and 5 homozygous *(C)cde*^{s}) were amplified with primers rez7 and rnb31 and sequenced with primer CRBF06. The sequence results showed that

the *RHD*ψ cosegregates with the URH1 (n = 13) or URH2 (n = 1) box indicating that the *RHD*ψ has originated from an *RH* locus with a mutated upstream *Rhesus box*. The *(C)cde*^{s} allele cosegregates with a normal upstream *Rhesus box* and a mutated downstream *Rhesus box* (Fig. 4, DRH5). This mutated downstream *Rhesus box* was not encountered in any of the previously sequenced samples without *(C)cde*^{s} alleles.

Hybrid *Rhesus boxes* with the exact same sequence in the downstream part (UDRH2) were frequently found (4 of 9 analyzed Curaçao black persons and 1 of 10 analyzed Ethiopian black persons).

Discussion

Despite the availability of Rh immunoprophylaxis, D mismatch is still the major cause of HDFN. In a pregnancy complicated by the presence of maternal anti-D, the *RHD* zygosity status of the father determines the chance of a fetus being either positive or negative for D. New insights into the *RH* locus and the mechanism causing the gene deletion in the *RHD*- haplotype in white persons have made it possible to discriminate *RHD*-hemizygous from *RHD*-homozygous individuals by detection of the *RHD*- *RH* locus.[11] In this study, *RHD* zygosity was determined in five different ethnic groups. Results of the PCR-RFLP were compared to results obtained by a newly developed RQ-PCR in D+ samples (n = 263). In all nonwhite ethnic groups, discrepancies were found between both methods (61 discrepancies in 211 D+ samples). Sequencing analyses of parts of the *Rhesus boxes* of 40 discrepant samples showed the presence of previously described mutated downstream *Rhesus boxes* in 6 samples.[12] These boxes, as described by Matheson and Denomme[12] in a study on 284 D+ samples (201 samples were from black descent as determined by the GATA-1 mutation at the *FY* locus), show two different sequence patterns. The most frequently found pattern (3 of 4 sequenced samples) was: nucleotide 5164C, nucleotide 5275G, nucleotide 5447C, nucleotide 5465C, and nucleotide 5526G. In this study, this sequence was found in 6 of 40 discrepant D+ samples. The other sequence pattern as found by Matheson and Denomme in 1 sample was nucleotide 5164C, nucleotide 5275G, nucleotide 5447T, nucleotide 5465C, and nucleotide 5526G and was also found once in our sequenced population. New mutated upstream and downstream *Rhesus boxes* were encountered with a very high frequency in black persons. By sequencing samples typed hemizygous for *RHD* by PCR-RFLP and homozygous for *RHD* by RQ-PCR, the physical structure of the mutated *Rhesus boxes* was elucidated. We found two mutated upstream *Rhesus boxes* (URH1 and URH2) and five mutated downstream *Rhesus boxes* (DRH1 to DRH5). Furthermore, two mutated hybrid *Rhesus boxes* were found (UDRH1 and UDRH2). Although DRH2 to DRH5 do not influence zygosity determination by PCR-RFLP, they do show that the *RH* locus is highly variable. Mutated *Rhesus boxes* affecting the PCR-RFLP assay (URH1, URH2, and DRH1) have a frequency of 0.22 in D+ homozygous black

persons, rendering zygosity determination by PCR-RFLP impossible in a nonwhite population. PCR-RFLP, however, always correctly indicates the presence of a downstream *Rhesus box*. Also, in samples known to have an *RHD- RH* locus, the presence of a hybrid *Rhesus box* was always demonstrated. This may explain why no discrepancies are found in studies concerning *RHD-* samples. A recent study conducted by Perco and coworkers[13] based on a white population consisting of 83 D+, 13 D-, and 37 weak-D samples, showed an altered downstream *Rhesus box* in a stretch of 2917 bp of a weak-D type 29 sample.[13] This result was not confirmed by this study. Mutated *Rhesus boxes* have no effect on the RQ-PCR, and given the frequency of the mutated boxes the latter assay is a better tool for *RHD* zygosity determination in nonwhite persons. The RQ-PCR has a much lower false typing rate than the PCR-RFLP. In case of an *RHD*ψ, however, the sample will be typed false-positive for D. Therefore, in nonwhite persons, the RQ-PCR should be combined with an assay indicating the presence of the *RHD*ψ, like the allele-specific primer amplification described in this study for detection of *RHD*ψ. When a D+ sample shows an *RHD*ψ, the sample carries one *RHD* gene and one *RHD*ψ and may for paternal analyses be regarded as hemizygous for *RHD*. Another option would be to use an RQ-PCR that does not amplify the *RHD*ψ (like the assay developed by Finning et al.[21]) and to use such an assay in a quantitative way. In some cases, however, it may be preferred to at least recognize the presence of an *RHD*ψ. Other *RHD* genes known with *RHD* exon 7 nucleotide changes are *D* category *IV* (nucleotide 1048G>C) and *DBT* types *I* and *II* (*RHD* exon 7 is substituted by *RHCE* exon 7). Furthermore, in Europeans *RHD*-gene-positive, Dantigen- negative variants have been described that may interfere with *RHD* zygosity determination.[2] Regarding the fact that these variants are exceedingly rare, the RQ-PCR remains the best assay for zygosity determination.

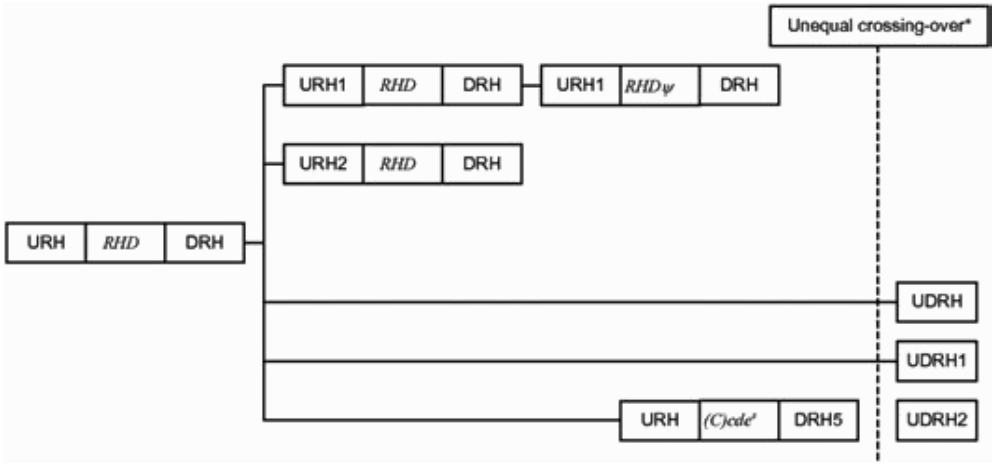


Figure 5. Schematic representation of the evolution of different *Rhesus boxes* and their cosegregation with *RHD*ψ and *(C)cde*^{s} alleles. With the acceptance that the *RH* locus originally carries URH and DRH, the *RHD*ψ has evolved in an *RH* locus already expressing URH1 (considering the existence of URH1 without *RHD*ψ but not vice versa) and the *(C)cde*^{s} allele and DRH5 have evolved in close association on the same *RH* locus.
*The UDRH and UDRH1 have evolved from an unequal crossing-over event between a URH and a DRH. The UDRH2 may have evolved from an unequal crossingover event between a URH and a DRH5 or may be the result of a gene conversion event between UDRH and DRH5.

Apart from *RHD* zygosity determination of the father to calculate the risk of the fetus being D+, it is now possible to directly determine the *RHD* status of the fetus on cell-free fetal DNA that can be derived from maternal plasma and serum.[22,23] Contamination of maternal DNA (of D- *RHD* genes or genes causing a very weak D expression), however, might lead to false-positive results, and scarcity of fetal DNA may result in a false-negative prediction of D (recently reviewed by van der Schoot et al.[24]). The *RHD*ψ cosegregates mainly with the mutated upstream *Rhesus box* URH1 but was also found once in cosegregation with URH2. Both the URH1 and the URH2 boxes are also present in samples not carrying an *RHD*ψ, indicating that the mutations in the *RHD* gene that lead to the *RHD*ψ have been introduced on an *RH* locus with a mutated upstream *Rhesus box*. The presence of one *RHD*ψ with an URH2 box may be due to recombination of the two at a later time point (Fig. 5). Owing to the cosegregation of the *RHD*ψ with a mutated URH box that causes (false) indication of a hybrid *Rhesus box* in the PCR-RFLP, the phenotypic prediction (D-) is correct. In a study concerning the *RHD* allele distribution in 58 African persons of Mali, it was also found that all D+ samples carrying an *RHD*ψ allele showed a hybrid *Rhesus box* by PCR-RFLP (n = 5).[14] These results are in concordance with the results of this study, and further molecular analyses of these samples will most probably reveal cosegregation with the URH1 or URH2 box. The *(C)cde*^{s} allele cosegregates with a DRH5 box and the DRH5 is not found in samples without the *(C)cde*^{s}. Therefore, it may be postulated that the DRH5 box and the *(C)cde*^{s} allele were formed in close association on the same *RH* locus (Fig. 5). Whether UDRH1

and UDRH2 are the result of an unequal crossing-over event that has taken place further downstream of the breakpoint and/or identity region or that the unequal crossing-over has taken place within the breakpoint region but between a URH and mutated DRH box cannot be elucidated from our data. Given the cosegregation of the *(C)cde*^{s} allele with DRH5 and the similarity between UDRH2 and DRH5, however, it is possible that either UDRH2 has evolved from a gene conversion event between DRH5 and UDRH or UDRH2 has its origin from an unequal crossing-over event involving an URH and the DRH5 box (Fig. 5). The latter possibility implies that in black persons, two *RHD* gene deletion events may have taken place, one in a normal *RH* locus and one in an *RH* locus with a *(C)cde*^{s} allele and a DRH5 box. The recent elucidation of the physical structure of the *RH* locus have made it possible to propose a model for the deletion of the *RHD* gene via a mechanism of unequal crossing-over from the *RH* locus in white persons.[11] The results of this study indicate that the *RHD* gene deletion via this same mechanism has also occurred in Asian persons from South Africa and black persons from South Africa, Curaçao, and Ethiopia.

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Chapter 3

RHC and *RHc* genotyping in different ethnic groups

Martine GHM Tax, C Ellen van der Schoot, Rene van Doorn, Lotte Douglas-Berger, Dick J van Rhenen and Petra A Maaskant-van Wijk. *RHC* and *RHc* genotyping in different ethnic groups. Transfusion 2002;42:634-644.

Abstract

Background: *RH* genotyping assays are mainly based on research in whites. These assays may not be reliable in a multiracial society because of the genetic variation in *RH* among ethnic groups.

Study design and methods: Five groups from different ethnic backgrounds were serologically typed for C and c and were genotyped on nucleotide C48 and intron 2 for *RHC* and *RHc* on nucleotides C178 and C307.

Results: *RHc* genotyping with both methods proved to be reliable. *RHC* genotyping on C48 is not reliable because of a 48G>C mutation in the *RHce* allele (false-positive prediction of C). This mutation was found in every ethnic group and does not affect c or e expression. *RHC* genotyping on intron 2 is unreliable because of r's (*(C)cde*^{s}) alleles (a false-negative prediction of C). This allele was found in whites and blacks from Curaçao and South Africa. Reactions of r's cells with anti-C are weaker, but no negative reactions with various MoAbs were found. A new method (*RHC/c/hex3*-intron 4/exon 7 multiplex PCRs) was developed based on intron 2 and r's hybrid exon 3 characteristics (*RHC*) and C307 (*RHc*).

Conclusions: Reliable *RHC* and *RHc* genotyping is possible in different ethnic groups with the *RHC/c/hex3*- intron 4/exon 7 multiplex PCR approach.

Introduction

The Rh blood group system is of clinical interest because it is involved in HDN, hemolytic transfusion reactions, and autoimmune hemolytic anemia. The most important Rh antigens are D, C, c, and E, e. These antigens are encoded by two highly homologous genes, *RHD* and *RHCE*, both located on chromosome 1p34.3-p36.1. *RHCE* encodes for both the polypeptides that carry C and c and E and e, whereas *RHD* encodes for the D polypeptide. Comparing the *RHc* and *RHC* alleles, there are six nucleotide (nt) differences, four of which result in amino acid substitutions in the corresponding polypeptides.[1,2] These substitutions are 48G>C leading to W16C encoded by exon 1 and 178 C>A, 203 A>G, and 307 C>T leading to L60I, N68S, and P103S, respectively, encoded by exon 2 (in c to C order). Studies with cells of nonhuman primates suggested that of the four amino acids that might be critical for expression of Rhc, P103, the only polymorphic site that is extracellular, plays an important role in expression of c.[3,4] Moreover, we recently described that a single point mutation 307 T>C leading to S103P in *RHD* resulted in expression of c on the D polypeptide, independent of the presence of W16, L60, and N68.[5] For C expression, the system is more complex. Although S103 is critical, C antigenicity has to be determined by other amino acids as well, as S103 is also present in the D polypeptide. K562 transfection studies clearly showed the importance of C16 in C expression.[6] It has been suggested that the interaction with the third extracellular loop is probably also required for some C epitopes. This would explain the weak expression of C by the *r's* allele, a variant *RHD* in which part of exon 3 to exon 7 of *RHD* is replaced by sequences derived from *RHCE*.[7] Several *RHC* genotyping assays have been developed. For *RHC* genotyping assays based on coding sequences, the polymorphism in exon 1 has to be used and for *RHc* genotyping the polymorphisms in exon 2 has to be used. In addition, *RHC* genotyping assays can be based on a 109-bp insert in intron 2, which is only present in *RHC*.[8] Avent[9] developed an *RHC* genotyping assay based on the 109-bp insertion in intron 2 of *RHC*, and Faas et al.[7] developed an *RHC* genotyping assay based on the C48 polymorphism in exon 1. However, the sequences of *RH* genes may vary with the ethnic background of a person. It is important to be aware of the differences in genetic sequences in order to develop genotyping methods that are reliable in a multiracial society. Therefore, in this article, the previously described *RHC* and *RHc* genotyping assays were tested on large panels of serologically typed donors from different ethnic backgrounds. For *RHc* typing, both assays (based on C178 and C307) were found to be reliable on all donors tested. Both *RHC* genotyping assays, based on C48 and the 109-bp insert in *RHC* intron 2, resulted in high numbers of discrepant results. However, by analyzing these discrepancies, we were able to develop a genotyping method for *RHC* in combination with typing for *RHc* that predicted the correct c phenotype in 100 percent and that predicted the correct C phenotype in 99.8 percent of the total research population (n = 1071).

Materials and methods

Samples

A total of 1071 EDTA blood samples were obtained from five different ethnic groups; that is, 250 whites from the Netherlands (collected by Bloodbank Rotterdam, Rotterdam, the Netherlands), 250 African blacks, and 250 African Asians (both collected by The South African Blood Transfusion Service, Johannesburg, South Africa), 170 blacks from Ethiopia (collected by The Ethiopian Red Cross Society Transfusion Service, Addis Ababa, Ethiopia), and 151 blacks from Curaçao (collected by The Red Cross Bloodbank Curaçao, Willemstad, Curaçao). All donors were chosen randomly, except for the blacks from Curaçao, which were partly selected to obtain D- samples.

Serology

RBCs of all donors were phenotyped for D, C, c, E, and e according to standard serologic protocols with an IgM anti-D MoAb (GAMA401), a polyclonal IgG anti-D reagent, and an IgM MoAb (Gamma Biologicals, Houston, TX) recognizing C (MS24), c (MS33), E (GAMA402), and e (MS16, MS21, MS63 [blend]). Expression of C was studied with MoAbs CM216-1 (Gamma Biologicals), CB250A1 (Ortho Diagnostics, Turnhout, Belgium), 8-36216 (CLB, Amsterdam, the Netherlands), C-93/44 (National Hematological Scientific Center, Moscow, Russia), 388F3 (Dominion Biologicals, Dartmouth, Nova Scotia, Canada), MS23, MS273, and MS257 (IGRI, Oslo, Norway), P3x25513G8, and DGC02 (Diagast Laboratories, Lille, France). The expression of c was studied with MS33 (IGRI), BS240 (Biotest AG, Dreieich, Germany), 951 (CRTS de Bordeaux, France), BB7.D4 (Institut National de Transfusion Sanguine, Paris, France), and POQ/3D2 (Laboratoire Immunologie Moleculaire, Toulouse, France) according to the five epitope hypothesis.[5] The expression of e and E was studied with MS16, MS21, and MS63 (IGRI), MS62, and MS69 (Serologicals Corp., Edinburgh, Scotland), and DGPE01 (Diagast Laboratories). Expression of f (RH6) was studied with a polyclonal IgG patient serum (phenotyped CcDEe).

Genomic DNA isolation

Genomic DNA was isolated from peripheral blood WBCs by a salting-out method according to standard protocol.[10] Oligonucleotide sequences of all primers used in this study are listed in Table 1.

Table 1. Primers

Primer	Sequence (from 5' to 3')*	Location †	Specificity	Reference‡
R31	CGCTGCCTGCCCCTCTGC	Exon 1 (F)	*RHC*	7
R147	TTGATAGGATGCCACGAGCCCC	Exon 1 (R)	*RHD/CE*	7
R-15	tatctagagacggacacaggATGAGC	Exon 1 (F)	*RHD/CE*	7
R159A	TCTGACCGTGATGGCGCCCC	Exon 2 (F)	*RHc*	5
R348	CTGAACAGTGTGATGACC	Exon 2 (R)	*RHD/CE*	5
R637	GCCCTCTTCTTGTGGATG	Exon 5 (F)	*RHD/CE*	5
R768	TGACCCTGAGATGGCTGT	Exon 5 (R)	*RHD/CE*	5
C/rev	gaacatgccacttcactccag	Intron 2 (R)	*RHC*	9
C/for	cagggccaccaccatttgaa	Intron 2 (F)	*RHD/CE*	9
c/rev	TGATGACCACCTTCCCAGG	Exon 2 (R)	*RHc*	9
c/for	TCGGCCAAGATCTGACCG	Exon 2 (F)	*RHD/CE*	9
R364	TCGGTGCTGATCTCAGTGGA	Exon 3 (F)	*RHD*	12
R474mmC	ACTGATGACCATCCTCATGG	Exon 3 (R)	*RHCE*	
R581	ACGGAGGATAAAGATCAGAG	Exon 4 (F)	*RHCE*	23
R667	CTCAGCAGAGCAGAGTTGAC	Exon 5 (R)	*RHCE*	23
R973	AGCTCCATCATGGGCTACAA	Exon 7 (F)	*RHCE*	
R1068mmC	ATTGCCGGCTCCGACGGCATG	Exon 7 (R)	*RHD*	
RHDin2F	tcctggctctccctctct	Intron 2 (F)	*RHD*	13
RH3R	aggtccctcctccagcac	Intron 3 (R)	*RHD/CE*	13
RHCE1F	atagacaggccagcacag	38-End promoter (F)	*RHCE*	
Rhin1R	tgggggaatctttttcctt	Intron 1 (R)	*RH*	
RHD1F	atagagaggccagcacaa	38-End promoter (F)	*RHD*	13
Rhin7DF	ctggaggctctgagaggttgag	Intron 7 (F)	*RHD*	
Rhin8R	tatgtgatcctcagggaaggag	Intron 8 (R)	*RH*	

* Uppercase letters represent coding sequences and lowercase letters represent noncoding sequences.

† (F) denotes a forward primer and (R) denotes a reverse primer.

‡ Reference number if primer sequence has been published previously.

RHC and RHc allele-specific primer amplification assays

The *RHC* and *RHc* allele-specific primer amplification (ASPA) assays have been described before.[5,7] The ASPA for *RHC* was performed with primers R31 (*RHC* specific on nt C48) and R147. To avoid false-negative results, an internal control PCR was included by adding consensus primer R-15, which would always yield a product in combination with R147. The ASPA for *RHc* was performed with primers R159A, *RHc* specific on nt C178, and R348. To avoid false-negative results, primers R637 and R768 were included, which amplify a PCR product from exon 5 of all *RH* alleles. PCR products were size separated by electrophoresis on a 12-percent acrylamide gel and were visualized by ethidium bromide staining.

RHC/c multiplex PCR

RHC typing was performed with primers C/rev, specific on the 109-bp insert in intron 2, and C/for. *RHc* typing was performed with primers c/rev (*RHc* specific on nt C307) and c/for, as developed by Avent.[9] Because both PCRs are performed in the same tube, no extra internal control PCR was included. PCR products were separated according to their size by electrophoresis on a 1-percent agarose gel.

RHC/c/hex3 multiplex PCR

A PCR was developed that amplifies the hybrid (*RHD-RHCE*) exon 3 (*hex3*) as found in an *r's* (*(C)cde*^{s}) allele. This PCR was performed with primers R364, *RHD* specific on nt A383, and R474mmC, *RHCE* specific on nt C455. The *hex3* primers were added to the original *RHC/c* multiplex (MPX) PCR. PCR products were separated by size in electrophoresis on an 8-percent acrylamide gel.

Intron 4/exon 7 MPX PCR

The previously described *hex3* primer pair is not specific for an *r's* allele but will also yield a product from other variant *RHD* (i.e., *DIIIa* and *DIVa* type 1).[11] Therefore, an MPX PCR that yields PCR products of these variant *RHD* was developed. Because the presence of a hybrid exon 3 combined with an *RHD* intron 4 in an *RHCE* environment (G602 in exon 4 and G667 in exon 5) is specific for the detection of a *DIIIa* gene, primers R581, *RHCE* specific on nt G602, and R667, *RHCE* specific on nt G667, were used. For *DIVa type 1* detection, primers R973, *RHD* specific on nt A992, and R1068mmC, *RHCE* specific on nt C1048, were included, as the presence of a hybrid exon 3 combined with 1048G>C mutation in *RHD* exon 7 is specific for the detection of this variant gene. No additional control PCR was added because primer pair R581/R667 always yields a product from *RHCE*. PCR products were separated by size in electrophoresis on a 2-percent agarose gel.

RHD-specific MPX PCR

RHD exons 3, 4, 5, 6, 7, and 9 were amplified with *RHD* sequence specific primers in a one-reaction mixture as described before.[12] Minor modifications were the use of high-performance liquid chromatography-purified primers instead of desalted and the use of Amplitaq (Perkin Elmer/Roche, Nieuwerkerk aan de IJssel, the Netherlands).

PCR conditions

All primers used in this study were desalted unless mentioned otherwise and were obtained from Invitrogen (Carlsbad, CA). All PCRs were performed in a thermal cycler (model 9700, Applied Biosystems, Nieuwerkerk aan de IJssel, the Netherlands) on 200 ng of genomic DNA in a total reaction volume of 50 mL. Reaction mixtures contained 100 ng of each primer (except for primer pairs C/for

and C/rev and R364 and R474mmC, 200 ng; R31- R147-R-15 and c/for-c/rev, 75 ng), 5 mM of each dNTP, 2 U of Taq DNA polymerase (Promega, Madison, WI) in the appropriate buffer and 2 U of Taq DNA polymerase antibody (Becton & Dickinson, San Jose, CA), supplemented with 1.5 mM of MgCl2. All PCRs started with one cycle of denaturation at 95^0C for 5 minutes and were completed with one cycle of 5 minutes at 72^0C to complete extension. The PCR conditions were 35 cycles of 1 minute at 95^0C for denaturation, 1 minute at 60^0C (*RHC* ASPA) or 53^0C (*RHc* ASPA) or 65^0C (*RHC/c* MPX PCR) or 66^0C (*RHC/c/hex3* MPX PCR) or 59^0C (intron 4/exon 7 MPX PCR) for annealing and 1 minute at 72^0C or 45 seconds at 72^0C (intron 4/exon 7 MPX PCR) for extension.

Nla IV digestion

PCR products of *RHD* exon 3 were obtained with primers RHDin2F, *RHD* specific in intron 2, and RH3R.[13] A 10-µL PCR product was digested with restriction endonuclease *Nla* IV (MBI Fermentas, GMBH, St. Leon-Rot, Germany) in a 15-µL reaction mixture containing 5 U of enzyme in the appropriate buffer supplemented with 1-percent BSA. *Nla* IV digestion is *RHCE* specific on the last polymorphic site of exon 3 (C455).

Sequence analyses

RHD exon 3 was amplified with primers RHDin2F and RH3R. *RHCE* exon 1 was amplified with primers RHCE1F, *RHCE* specific in 3'-end promoter region, and Rhin1R in intron 1. *RHD*-specific exon 1 was amplified with primers RHD1F, *RHD* specific in 3'-end promoter region, and Rhin1R. *RHD*-specific exon 8 was amplified by using primers Rhin7DF in intron 7 and Rhin8R in intron 8. PCR products were purified (concert rapid PCR purification system, Invitrogen) and cycle sequenced by using big dye terminator chemistry (ABI-PRISM 377, DNA sequencer, Applied Biosystems).

Results

RHC and RHc ASPAs and RHC/c MPX PCR analysis

Serologically determined phenotypes for C and c of 125 donors of each ethnic group were compared with results of the *RHC*-ASPA (specific on C48) and the *RHc*-ASPA (specific on C178) and with results of the *RHC/c* MPX PCR (specific on the 109-bp insert in intron 2 and on C307, respectively). For *RHc* genotyping, no discrepancies between serologically determined phenotypes and the *RHc*- ASPA nor the *RHC/c* MPX PCR were found (results not shown). For *RHC* genotyping, two kinds of discrepancies were found (Table 2). A total of 43.3 percent of serologically typed C- donors (n = 337) gave false-positive results with the C48- specific *RHC*-ASPA. No false-negative results were obtained (n = 288).

Table 2. RhC phenotyping compared with *RHC* genotyping with *RHC*-ASPA and with *RHC/c* MPX PCR approaches

		RHC genotype PCR*	
Donor origin	C+ phenotype	*RHC-ASPA* (% false positive)†	*RHC/c MPX* (% false negative)‡
Whites, Netherlands	77	80 (6.3)	76 (1.3)
Blacks, South Africa	21	91 (67.3)	14 (33.3)
Blacks, Ethiopia	54	88 (47.9)	54 (0)
Blacks, Curaçao	32	71 (41.9)	18 (43.8)
Asians, South Africa	104	105 (4.8)	104 (0)
Total population	288	435 (43.3)	267 (7.3)

* One hundred twenty-five donors of each ethnic group were tested, totaling 625 donors

† False positivity is calculated by dividing the number of false-positive typed samples by the total number of serologically negative samples times 100%.

‡ False negativity is calculated by dividing the number of false-negative typed samples by the total of number of serologically positive samples times 100%.

The presence of 48G>C in *RHc* has previously been described by Wolter et al.[14] and was stated to be the result of exon replacement. According to our results, this 48G>C mutation occurs in all ethnic groups considered here, although the prevalence is highest in blacks. These results are in concordance with the results from Faas et al.,[7] who found this mutation to be present in a *ce*-like allele, predominantly in blacks. Of the serologically typed C+ donors (n = 288), 7.3 percent gave false-negative results for *RHC* with the *RHC/c* MPX PCR. No false-positive results were obtained (n = 337). No discrepancies were found among people of Ethiopian and Asian origin. All *RHC*-discrepant samples in the *RHC/c* MPX PCR showed according to the *RHC*-ASPA the presence of a C48 (encoding Cys16) in the absence of a normal *RHC* allele. A false-negative prediction of the C phenotype by using the *RHC/c* MPX PCR may be caused by presence of *r's* (*(C)cde*^{s}), an *RHD* variant hybrid *D-CE-D* gene of which exons 1 and 2 and part of exon 3 (first three polymorphic sites) are derived from *RHD* as well as exons 8, 9, and 10 and the 3'-noncoding region. The region in between is derived from *RHCE*. The *r's* gene leads to a weak expression of C on the RBC surface but will not be detected by the *RHC/c* MPX PCR because of the absence of the *RHC* intron 2. Therefore, it was investigated in one of the discrepant samples (D- Curacao donor without *RHD*ψ according to the *RHD* MPX PCR) whether the false-negative C prediction was due to the presence of *r's*. Sequencing analyses of *RHD* exon 3 revealed that the first three polymorphic sites were derived from *RHD* (nts T361, T380, and A383), the last polymorphic site from *RHCE* (C455), and a nucleotide substitution 410 C>T leading to A137V, which all have previously been described to be present in an *r's* hybrid gene.[7,15] Sequencing analyses of *RHD* exon 8 specifically amplified with intronic primers (GenBank accession numbers AB035194 and AB035196) revealed an *RHD* exon.[16] Therefore,

we screened the 21 *RHC* discrepant samples for presence of *r's*.

Nla IV digestion and RHD MPX PCR analyses

Of the 21 C+ samples that were negative for *RHC* intron 2 with *RHC/c* MPX PCR analyses, exon 3 was amplified, followed by an *RHCE*-specific digestion of the last polymorphic site (C455) with endonuclease *Nla* IV. All 21 donors were shown to carry the hybrid exon 3, as in all cases, at least part of the PCR product was digested. Of the 14 Curacao donors who were typed false negative for *RHC*, 12 samples were completely digested, and two samples were partly digested. Samples from six out of seven South African donors who were typed false negative for *RHC* were partly digested, and one sample was completely digested. All 21 samples were screened for presence of *RHD* exons 3, 4, 5, 6, 7, and 9 by *RHD* MPX PCR analyses. In case of an *r's* gene next to an *RHD* deletion, only *RHD* exon 9 will be amplified, and in case of an *RHDψ* next to an *r's* gene, all *RHD* exons except exon 5 will be amplified. Two samples that were partly digested by *Nla* IV and were D- carried an *RHDψ* next to an *r's* gene (one Curaçao and one South Africa donor). Six samples that were partly digested and were D+ (one Curaçao and five South Africa donors) showed no deviation of *RHD* and probably carry a normal *RHD* next to an *r's* gene. Indeed, 12 samples (all D- Curaçao donors) that were completely digested showed only exon 9 by *RHD* MPX PCR analysis. One sample that was completely digested and was D+ (South Africa donor) was missing *RHD* exons 3, 4, and 5 and turned out to carry a *DIIIa* variant *RHD* by intron 4/exon 7 MPX PCR analyses next to an *r's* gene.

RHC/c/hex3 and intron 4/exon 7 MPX PCR analyses

Because all samples discrepant for *RHC* in the *RHC/c* MPX PCR showed an *r's* hybrid gene, a primer pair specific for the *RHD-RHCE* hybrid exon 3 characteristic for the *r's* variant was added to the *RHC/c* MPX PCR (*RHC/c/ hex3* MPX PCR; Fig. 1). When a product is yielded for the hybrid exon 3, there may be four possible variant genes underlying: an *r's*, a *DIIIa*,[17] a *DIVa* type 1,[18] or an R^N. Only one of the three published R^N variant *RHCE* will yield a hybrid exon 3-specific PCR product with the *RHC/c/ hex3* MPX PCR.[19] However, as R^N does encode the *RHC*-specific 109-bp insert in intron 2, it will already be typed as C+ in the *RHC/c/hex3* MPX PCR. To discriminate between different *RHD* variant genes (*r's*, *DIIIa*, and *DIVa* type 1) and, therefore, to avoid false-positive typing for *RHC*, an intron 4/exon 7 MPX PCR was developed that yields a product in case of *DIIIa* (intron 4) or *DIVa* type 1 (exon 7) (Fig. 2). If a hybrid exon 3-positive sample is also positive for *DIIIa* or *DIVa* type 1, the sample should be typed C-.

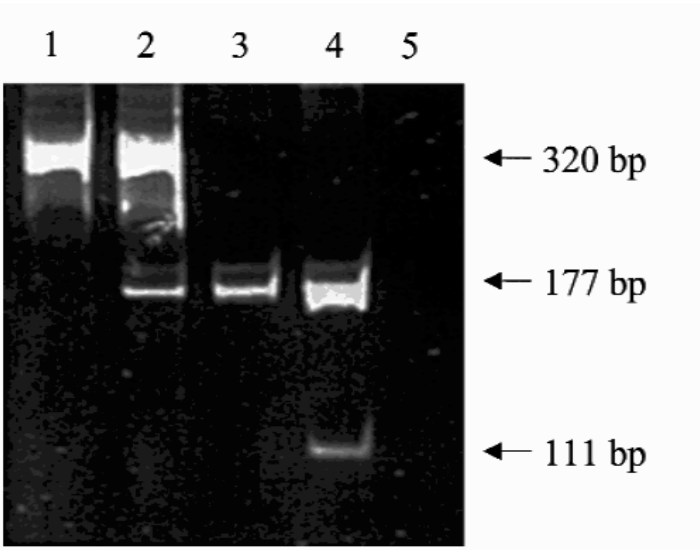

Figure 1. *RHC/c/hex3* MPX PCR. Results of the *RHC/c/hex3* MPX PCR. Lane 1, CCDee sample, an *RHC*-specific product of 320 bp is amplified; Lane 2, CcDEe sample, an *RHC*-specific product of 320 bp and an *RHc*-specific product of 177 bp are amplified; Lane 3, ccdee sample, an *RHc*-specific product of 177 bp is amplified; Lane 4, (C)cde^{s}, r's sample, both the *RHc* and the *hex3* product of 111 bp are amplified; Lane 5, water control.

The *RHC/c/hex3* MPX PCR was tested on all samples previously tested with both ASPAs, and the *RHC/c* MPX PCR and was tested on an additional 125 whites, 45 blacks from Ethiopia, 26 blacks from Curaçao, 125 blacks from South Africa, and 125 Asians from South Africa and compared with serologic results. All samples that showed a hybrid exon 3 product in absence of a normal *RHC* allele (characterized by 109-bp insert in intron 2) were also tested with the intron 4/exon 7 MPX PCR. Genotyping results obtained with the *RHC/c/hex3* MPX PCR and intron 4/exon 7 MPX PCR compared with serologic typing are shown in Table 3. The distribution of *r's*, *DIIIa*, and *DIVa type 1* genes in the groups from different ethnic backgrounds is shown in Table 4. All samples (except one) that gave false-negative results in the *RHC/c* MPX PCR are now typed correctly. The discrepant sample (South African donor, phenotype CcDee) was typed false negative for C because of the presence of both an *r's* variant gene and a *DIIIa* variant gene, as was shown by *RHD* MPX PCR analyses. One sample (Curaçao donor, phenotype ccdee) was typed false positive. This sample was false positive in the *RHC* ASPA (C48), positive for the hybrid exon 3 (*hex3*), and negative for both *DIIIa* and *DIVa type 1* variant genes, and it lacks the expression of the C protein. Sequencing analyses of *RHD* exon 3 showed the same hybrid exon 3 as in *r's* and a normal *RHD* exon 3. Because this sample shows only one (nonfunctional) *RHD* (*RHDψ*, according to *RHD* MPX PCR analyses) on the *RH* locus by *RHD* breakpoint PCR-RFLP analyses[20] (results not shown), the hybrid exon 3 is most likely located in *RHce*.

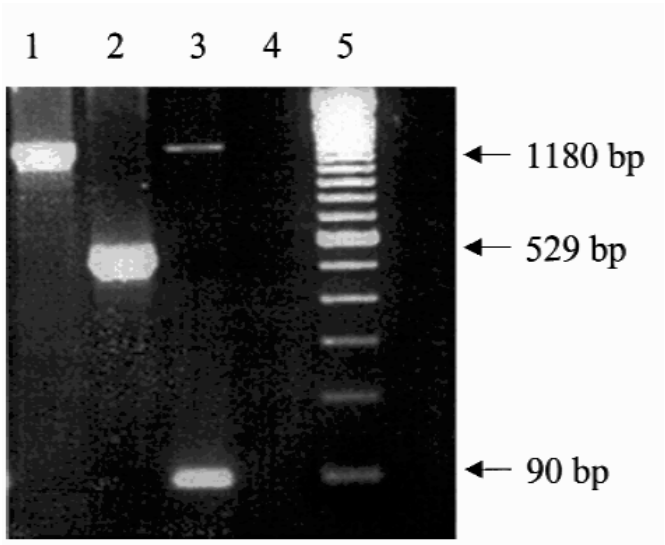

Figure 2. Intron 4/exon 7 MPX PCR. Lane 1, r's sample, the 1180-bp product of *RHCE* intron 4 is amplified; Lane 2, DIIIa sample, the 529 bp of *RHD* intron 4 is amplified with *RHCE*-specific primers; Lane 3, DIVa type 1 sample, the 90- bp product shows the presence of C1048; Lane 4, water control; Lane 5, DNA marker (100 bp). The 1180-bp product is also functioning as an internal control. The 1180-bp amplicon is not visible in Lane 2 because of competition.

The positive and negative predictive values of both the *RHC/c* MPX PCR and the *RHC/c/hex3*-intron 4/exon 7 MPX PCR analyses (Table 5) show that the new approach has a better diagnostic value for *RHC* and *RHc* genotyping in different ethnic groups.

Frequency of r's gene and 48G>C RHce alleles in different Rh phenotypes

Our results indicate that the 48G>C mutation is most likely associated with *RHce* (Table 6) and is found in both R_0 (*cDe*) and *r* (*cde*) haplotype. Of the Curaçao donors (which were partly selected to be D-), 28 of the 39 samples carrying the 48G>C mutation were found to be RhD negative. In the Ethiopian population, 3 out of 33 samples carrying the 48G>C mutation were found to be D-. Furthermore, we found that in all donors in whom the RhC expression was derived from an *r's* gene (n = 34) and that in all D- donors carrying an *RHD*ψ (as confirmed by sequencing analysis of exon 4 of all samples missing exon 5 by *RHD* MPX PCR analysis) (n = 17 in analyzed group of 1071), the 48G>C mutation was present, which suggests that the mutation is present in *cis RHce* alleles. Table 4 shows the distribution of *r's*, *DIIIa*, and *DIVa type 1* variant *RHD* genes as found in this study. We found one *r's* gene to be present among whites from the Netherlands and none of the variant genes to be present in blacks from Ethiopia. In blacks from South Africa, both *r's* and *DIIIa* genes are highly frequent. *DIVa type 1* genes are only found in blacks from in which *r's* genes are also highly frequent. One *DIIIa* gene was found among the Asians from South Africa.

Table 3. RhC phenotyping compared with *RHC* genotyping *RHC/c/hex3* MPX PCR and intron 4/exon 7 MPX PCRs

Donor origin	C+ phenotype	*RHC* + *RHC/c/hex3*	Percentage of false-negative C typing	Percentage of false-positive C typing
Whites, Netherlands (n = 250)	170	170	0	0
Blacks, South Africa (n = 250)	47	46	2.1	0
Blacks, Ethiopia (n = 170)	70	70	0	0
Blacks, Curaçao (n = 151)	37	38	0	0.9
Asians, South Africa (n = 250)	214	214	0	0
Total population (n = 1071)	538	538*	0.19	0.19

* This number includes one sample typed as false positive for C and one sample typed as false negative for C and, therefore, shows no difference from the total C+ phenotypes.

Table 4. Distribution of *r's*, *DIIIa*, and *DIVa type 1* variant genes among different ethnic groups

	Number of donors with phenotype (gene)		
Donor origin	r's *(C)cde*s**	*DIIIa**	*DIVa type 1**
Whites, Netherlands (n = 250)	1	0	0
Blacks, South Africa (n = 250)	18	30	0
Blacks, Ethiopia (n = 170)	0	0	0
Blacks, Curaçao (n = 151)	15	0	2
Asians, South Africa (n = 250)	0	1	0

* Designation of phenotypes of variant *r's*, *DIIIa*, and *DIVa type 1* was based on genomic PCR results. All samples were tested in the following assays: *RHD* MPX PCR, *RHC* ASPA, *RHc* ASPA, *RHC/c/hex3* MPX PCR, and intron 4/exon 7 MPX PCR. All samples from African blacks are also analyzed with all DAR PCRs.[22]

Table 5. Positive and negative predictive values* for *RHC* genotyping with *RHC/c* MPX PCR (*RHC* intron, 2 *RHc* C307) and *RHC/c/hex3*-intron 4/exon 7 MPX PCR analyses

	RHC/c MPX PCR†		*RHC/c/hex3*-intron 4/exon 7 MPX PCR	
	Positive predictive value	Negative predictive value	Positive predictive value	Negative predictive value
Whites, Netherlands (n = 250)	1	0.9796	1	1
Blacks, South Africa (n = 250)	1	0.9369	1	0.9951
Blacks, Ethiopia (n = 170)	1	1	1	1
Blacks, Curaçao (n = 151)	1	0.8692	0.9737	1
Asians, South Africa (n = 250)	1	1	1	1

* Positive and negative predictive values are calculated as described by Altman.[28]

† This assay was tested on only 125 donors of each ethnic group.

Table 6. Phenotype associations*

	Ccdee	CcDΨee	CcDee	ccdee	ccDΨee	ccDee	ccDEe	CcDEe
r's/48G>C (n = 1071)	14/14	2/2	14/14					4/4
DIIIa/48G>C (n = 1071)			3/3			27/23	1/1	
DIVa type 1/48G>C (n = 1071)						2/2		
48G>C+ (n = 625)				21	10	93	9	

* Number of genes/number of *RHce* alleles with 48G>C mutation associated with gene.

† Number of *RHce* alleles with 48G>C mutation not associated with *r's*, *DIIIa*, or *DIVa type 1* genes in n = 625 donors.

Serologic analysis of r's genes and 48G>C RHce alleles

Serologic analysis by titration studies of C expression encoded by *r's* on RBCs of three donors (Ccdee) with MoAbs CM216-1, CB250A1, and 8-36216 gave an identical weak expression comparable with the earlier described *r's* reaction pattern by the same laboratory (results not shown).[7] All three *r's* samples showed a titer of 4, 64, and 32, respectively (controls showed a titer of 16, 64, and 64, respectively), and showed between 60 percent and 65 percent antigen expression as compared with a CcDee cell (100%). Furthermore, to investigate whether C expression is altered when encoded by *r's*, C expression was studied with MoAbs C-93/44, 388F3, MS23, MS257, MS273, P3x25513G8, and DGC02. No differences in reactivity between control and donor cells were observed (results not shown). To investigate whether the 48G>C mutation leading to W16C on the *RHce* allele has an effect on c expression on the RBC, we amplified and cycle-sequenced *RHCE* exon 2 of all samples of the Curaçao donors and serologically tested three samples that were found to be homozygous for the 48G>C mutation (all ccDee). MoAbs MS33 (epitope 1, according to the proposed 5 epitope model for c5), BS240 (epitope 2), 951 (epitope 3), POQ/3D2 (epitope 4), and BB7.D4 (epitope 5) were used with CCDee, ccdee, and Ccdee controls (Table 7). Herewith, no altered reaction pattern between donor and control cells was observed, and therefore, the 48G>C mutation in *RHce* does not affect the expression of c. To investigate whether expression of e and f (RH6) are affected by 48G>C when encoded on the same allele, we serologically tested the previously described samples and an additional three ccDEe samples (one white and two South African donors) expressing the 48G>C mutation. To determine e expression, MoAbs as well as polyclonal antibodies were used (Table 7). No differences in reactivity were observed with MoAbs between donor and control cells. MS16 gave a very weak to no reaction with both control and donor cells. After enzyme treatment, the reactions with MS16 were stronger (2+), but again, no difference was observed between control and donor cells. For reactivity of the donor cells with anti-f, a polyclonal IgG anti-f patient serum was used with control cells ccdee, and ccdEe Titration studies showed no difference between donor and control cells; therefore, the reactivity of *RHce* carrying the 48*G*<C mutation with polyclonal anti-f is not affected.

Table 7. Reactivity of c and e with polyclonal antibodies and MoAbs and RH6 (f) reactivity with anti-f patient serum*

Donor		POQ/ BB7.Polyc													DGP	DGP	Anti-f
number	Phenotype	MS33	BS240	951	3D2‡	D4	Rhe†	MS16	MS16‡	MS62	MS69	MS69‡	MS63	MS21	E01	E01‡	serum
980717	ccDee	4	4	4	NT	1	4	vw	2	3	1	3	3	3	w	3	3
980736	ccDee	4	4	3	3	1	4	0	2	3	1	3	4	3	w	3	3
980762	ccDee	4	4	4	4	1	4	vw	2	3	1	3	3	3	1	3	3
970063	ccDEe	NT	NT	NT	NT	NT	4	0	2	3	1	3	3	3	0	2	3
980121	ccDEe	NT	NT	NT	NT	NT	4	0	2	3	1	3	3	3	0	2	3
980135	ccDEe	NT	NT	NT	NT	NT	4	0	2	3	1	2	3	3	0	2	3
Control	ccdee	4	4	4	4	1	4	w	3	3	2	3	4	3	2	3	3
Control	ccdEe	NT	NT	NT	NT	NT	4	0	2	3	w	3	3	3	vw	2	3
Control	ccDEE	NT	NT	NT	NT	NT	0	0	w	0	0	0	0	0	0	0	NT
Control	Ccdee	4	4	3	NT	1	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Control	CCDee	0	0	0	0	0	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT

* Scoring system is based on 0 (negative), vw (very weak), w (weak), to 4 (strongly positive) scale.
† Polyclonal antibody.
‡ RBCs pretreated with bromelain.

Discussion

Because *RHD* is highly variable, it is difficult to design genotyping methods for correct prediction of D expression from *RHD*. Variant *RHD* have different gene frequencies among different ethnic groups. *RHCE*, however, seems to be less variable; at least, fewer *RHCE* variants are described in literature. Because C and c cover fewer epitopes, it might be less complex to design reliable genotyping assays for *RHC* and *RHc*. In this article, the variability in *RHC* and *RHc* between different ethnic groups was studied, which may disturb reliable genotyping. Based on the observed variant genes, an *RHC/c* genotyping assay was developed that is reliable in different ethnic groups. Serologic analysis of the RBCs expressing Rh polypeptides encoded by the variant genes showed that C and c antigen expression of the cells is not affected qualitatively. In all ethnic groups, *RHce* and *RHcE* alleles seem to be strongly preserved for the mutations in exon 2 that are specific for c expression. This was expected for P103, as this amino acid is essential for c expression, but it also seems to be the case for L60. We tested 543 c+ donors for the presence of C178 and found no discrepancies. Therefore, genotyping for *RHc* is possible on both C178 and C307, but because of the recent report by Faas et al.[5] of a variant *RHD* with Rhc expression caused by a 307 T>C mutation, this position is theoretically preferred for *RHc* genotyping. *RHC/c* polymorphisms were first described by Mouro et al.[1] and by Simsek et al.[2] They both found a W at amino acid position 16 (G48) on the *RHc* allele derived from white donors. Also, in the white population in this study, *RHc* with G48 is the predominant genotype. However, 3 out of 125 white donors

were found to carry an *RHce* allele with a 48G>C mutation. Furthermore, our results show that the 48G>C mutation in exon 1 of the *RHc* allele in the absence of an *RHC* allele is highly frequent in blacks (67.3% in South Africans, 47.9% in Ethiopians, and 41.9% in Curaçao). Our results show that the 48G>C mutation is associated with the *RHce* allele and is present in both the *RHD*+ as in *RHD*- haplotypes and, therefore, is not only associated with the R_0 (*cDe*) haplotype, as suggested by Westhoff et al.,[21] but is also seen with the *r* (*cde*) haplotype, especially in black populations (2.4% in Ethiopian donors and 22.4% in Curaçao donors). Although C16 is involved in C expression,[6] clearly other conformation determining factors are also required for the expression of C. However, it is highly unlikely that this variant *RHce* is immunogenic for transfusion recipients and/or mothers lacking this gene. Serologic analysis with MoAbs directed against each of the five different c epitopes as described by Faas et al.[5] showed that the 48G>C mutation does not interfere with the expression of any of the c epitopes. The possible effect of the 48G>C mutation on e and f (RH6) expression was serologically analyzed with polyclonal antibodies and MoAbs. None of the reactions differed between the donor cells and the control cells, not even after enzyme treatment. This is in contrast with the results of Westhoff et al.,[21] who found that the 48G>C mutation induced the loss of reactivity with (among others) MoAbs MS16 and MS69, which were reported to be strongly positive (4+) on enzyme-treated control cells. In our hands, both antibodies were weakly reactive with both control cells (2+) and enzyme-treated cells of six donors carrying the 48G>C mutation (2+) (Table 7). We might have missed the decreased e expression due to low titers in our batches of MoAbs. Genotyping for *RHC* is more complicated because the only *RHC*-specific nucleotide in the coding part, C48, is not usable as described previously here. Intron 2 of *RHCE* differs between *RHC* and *RHc*. However, our results show that because of the presence of the variant *RHD* gene *r's* (*(C)de^s*), which encodes an Rh polypeptide that weakly expresses C and which lacks the *RHC* specific intron 2, this polymorphism is also not usable; the *r's* gene was found in 0.4 percent of whites, 9.9 percent of blacks from Curaçao, and 7.2 percent of blacks from South Africa. All samples that were typed false negative for C with the *RHC/c* MPX PCR, which is specific for *RHC* specific in intron 2, appeared to carry an *r's* gene. In blacks from South Africa and Curaçao in approximately 40 percent of the phenotypically C+ donors, C expression was the result of this variant *RHD*. The expression of the C polypeptide encoded by an *r's* gene was tested with MoAbs. None of the antibodies gave weaker reactions with the donor cells compared with the control cells. Therefore, it seems that *r's* genes do not give rise to altered C. However, there is one report in which an alloanti-C was found in a patient carrying an *r's* gene.[22] We first published about the *r's* gene in 1997 and suggested that *r's* genes cosegregate with an *RHce* allele with the 48G>C mutation.[7] The results of this study are in agreement with this hypothesis (Table 6). An MPX PCR was developed in which *RHc* is detected specifically on C307 and *RHC* is detected specifically on both the 109-bp

insertion in *RHC* intron 2 and on the hybrid exon 3 as found in an *r's* gene. Because this hybrid exon 3 is also present in *DIIIa* and *DIVa type 1* variant *RHD* genes, an exclusion intron 4/exon 7 MPX PCR was developed that is positive for both *DIIIa* and *DIVa type 1* genes, which can be used in a second phase when a hybrid exon 3 is detected in the absence of a normal *RHC*. If a sample is positive for either *DIIIa* and/or *DIVa type 1*, the sample should be typed negative for C. If a sample is negative for both *DIIIa* and *DIVa* type 1, the sample should be typed positive for C (Fig. 3). A false-negative prediction of the C phenotype may still be encountered because of the presence of an *r's* gene with a *DIIIa*, a *DIVa type 1*, or a *DAR*[23] gene *in trans*. This was found in one case in this study and concerned a black person from South Africa. Based on the on the frequencies of *r's*, *DIIIa*, and *DIVa type 1* genes as found in this study (Table 4), we can predict that the probability to find a black South African donor carrying an *r's* gene and a *DIIIa* or a *DIVa type 1* gene will be 0.2 percent. This will be lower in all other ethnic groups, as the frequencies of both *r's*, *DIIIa*, and *DIVa type 1* genes are much lower. The probability of carrying an *r's* gene with a *DAR* gene *in trans* in a black South African donor will be 0.18 percent, based on a gene frequency of 0.025 for the *DAR* variant gene. However, all samples from African blacks were also tested with the *DAR*-specific PCRs (results not shown). Herewith, we identified one sample that was typed *DIIIa* by *RHC/c/hex3*-intron 4/exon 7 MPX PCR analyses to be positive for a *DAR* variant gene. This sample did not carry an *RHC* and did not express C on the RBC surface. Furthermore, *r's* alleles with a *weak D type 4.0* or *4.1*[24] *in trans* will also give rise to a false-negative prediction of C. However, these alleles are rare. The other mutations found in an *r's* gene, 410 C>T and 733CG, may also be used for specific detection of an *r's* allele. Recently, Wagner et al.[25] have described an ASPA specific on G733 (V245) in an *RHCE* environment resulting in VS positivity. The presence of both the *RHD-RHCE* hybrid exon 3 (*hex3*) and G733 is indicative for an *r's* allele. However, this approach also will not be conclusive because the L245V polymorphism has been identified in VS+, C- donors without an *r's* gene.[15] The 410 C>T mutation is present in a *DIII type 4* allele and may be present in other alleles.[24] A false positive prediction of the C phenotype was made in one case. Here, a hybrid exon 3 as found in an *r's* gene is most likely present in the *RHce* allele. It could not be determined whether the *RHD*-specific mutations in this allele have influence on the expression of c or e because of the presence of a normal *RHce* allele *in trans*. Because this sample also carries the G48C mutation, analysis with the C48-ASPA (which we found never to be false-negative) is not informative.

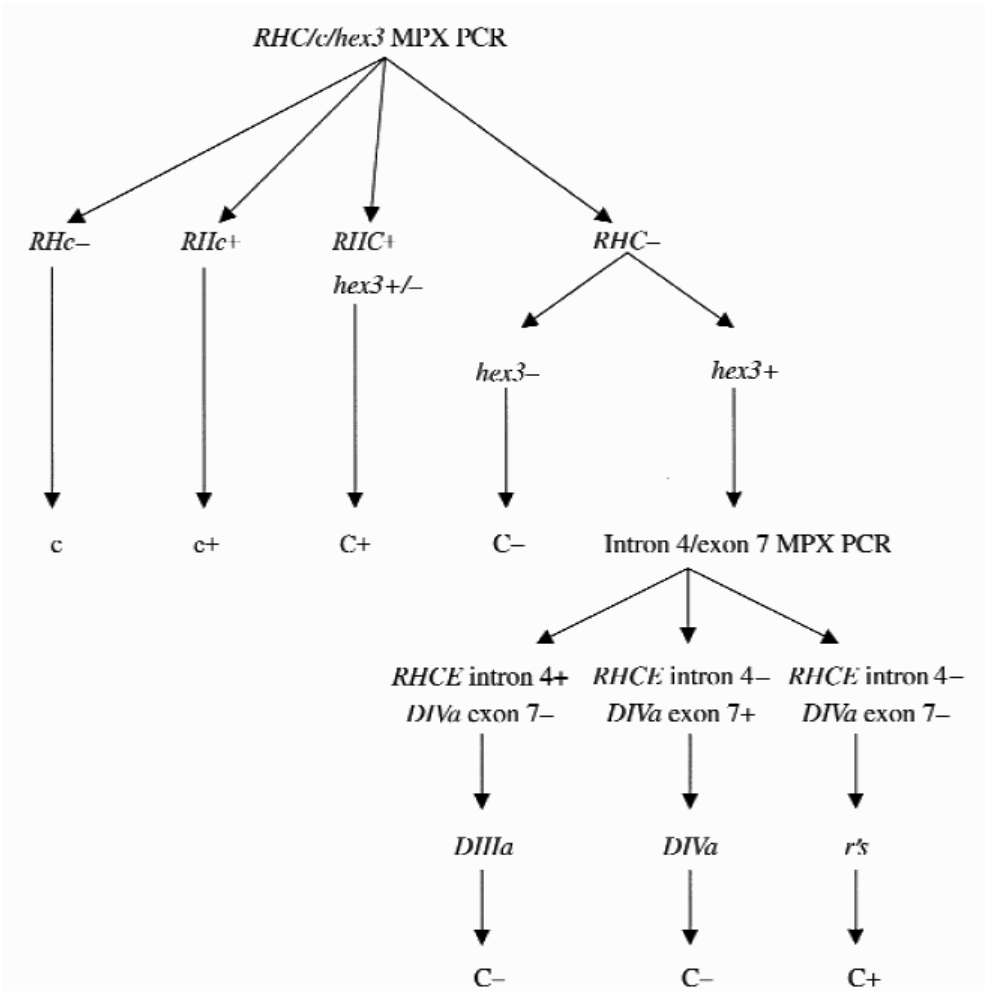

Figure 3. Flow diagram for *RHC* and *RHc* genotyping by *RHC/c/hex3*-intron 4/exon 7 MPX PCR analysis. This diagram indicates the decisions to be made when genotyping with the *RHC/c/hex3* MPX PCR. Hex3- indicates the absence of an *RHD-RHCE* hybrid exon 3, and hex3+ indicates the presence of an *RHD-RHCE* hybrid exon 3. According to the diagram, genotyping for both *RHc* and *RHC* can be done by one single assay, except when a hybrid *RHD-RHCE* exon 3 is present in an *RHC*- sample. In that case, further analyses to determine the origin of the hybrid exon 3 is necessary. When the hybrid exon 3 originates neither from *DIIIa*, nor *DIVa type 1*, the sample should be typed C+; otherwise, the sample should be typed as C-.

With the *RHC/c/hex3* genotyping method, it is now possible to type reliably for *RHC* and *RHc* in different ethnic groups. With the new approach, 1071 of the 1071 samples were correctly typed for c, and 1069 of the 1071 samples were correctly typed for C. The only positive and negative predictive values lower than 1.0 for the *RHC/c/hex3* MPX PCR in combination with the intron 4/exon 7 MPX PCR are 0.97 (blacks from Curaçao) and 0.99 (blacks from South Africa), respectively. A false-positive prediction of the phenotype can form a health hazard when typing blood transfusion recipients, as they will be transfused with antigen-positive cells and being antigen-

negative themselves, the recipient may produce allo-antibodies against the donor cells. False-negative prediction of the phenotype can form a health hazard when typing blood donors, as their cells will actually be antigen-positive and given to a antigen-negative recipient, and in prenatal diagnosis, as the fetus of an antigen-negative pregnant woman will be incorrectly typed negative and therefore the pregnancy will not be monitored while the mother may produce antibodies to antigen of the child. Genotyping may be the only diagnostic tool in cases in which direct phenotyping is not possible (e.g., prenatal diagnosis or recently transfused patients). After anti-D, anti-c is the most important RBC alloantibody that can cause HDN.[26] A correct prediction of the C phenotype is also valuable because anti-G may be responsible for HDN.[27]

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Chapter 4

Systemic analysis and zygosity determination of the *RHD* gene in a D-negative Chinese Han population reveals a novel D-negative *RHD* gene

X Qun, MGHM Grootkerk- Tax, PA Maaskant-van Wijk, CE van der Schoot. Systemic analysis and zygosity determination of the *RHD* gene in a D-negative Chinese Han population reveals a novel D-negative *RHD* gene. Vox Sanguinis 2005;88:35-40.

Abstract

Background: The aim of this study was to systemically analyse the genetic background of D negativity in a Chinese Han population.

Study design and methods: DNA of 74 D-negative samples was analysed by using an *RHD* multiplex polymerase chain reaction (MPX PCR) for the presence of *RHD* and by PCR-restriction fragment length polymorphism (PCR-RFLP) for *RHD* zygosity determination. Sixty-five samples were additionally analysed by using real-time quantitative PCR on *RHD* exon 7. *RHD* exon-specific sequencing was performed on discrepant samples.

Results: Forty-six samples (62%) showed the absence of *RHD*-specific exons by *RHD* MPX PCR and homozygous *RHD* negativity by PCR-RFLP. Twenty-two samples (30%) showed a 1227G>A mutation, characteristic for the Del phenotype. Five (7%) samples showed all characteristics of the *RHD*(1-2)-*CE*(3-9)-*D*(10) hybrid gene. One sample (1.4%) showed a novel 933C>A nonsense mutation in *RHD* exon 6, which resulted in a premature stop codon.

Conclusions: The *RHD* gene deletion, *RHD-CE-D* hybrid genes and one novel 93C>A mutation were found to be the three mechanisms that cause D negativity in our samples. The 1227G>A *Del* mutation was found to be the major cause of discrepant results between genotyping and phenotyping strategies, favouring genotyping of D-negative samples.

Introduction

The Rh blood group system is one of the most polymorphic and clinically important blood group systems in humans. In particular, the highly immunogenic RhD protein is involved in haemolytic disease of the fetus and newborn (HDFN) and transfusion reactions. The Rh antigens are encoded by two homologous genes: *RHD* and *RHCE*. It has been demonstrated that different (genetic) forms of D negativity occur in different ethnic populations. Whilst D-negative white people were almost invariably found to have a deletion of *RHD* between the upstream and downstream *Rh Boxes*,[1] the *RHDψ* gene and the *RHD* (1-3) *RHCE* (3-7) *RHD* (8-10) hybrid gene(s) were found to frequently cause D negativity in Africans.[2,3] In Asians, a very weak expression of the D antigen - the Del phenotype - was found in individuals who were apparently D negative,[4] and represents 10-33% of the Japanese or Chinese serologically typed D-negative donors.[5] Subsequent studies showed that the Del phenotype was associated with either a deletion of *RHD* intron 8 to intron 9, including whole exon 9,[6] or a missense mutation (1227G>A) in exon 9.[7] More recent studies also revealed new missense mutations and an *RHD-RHCE-RHD* hybrid gene in D-negative Chinese subjects.[8,9] Recently, the *RH* locus was explored and it was found that the *RHD* gene is flanked by an upstream *Rh box* (at its 5'-end) and a downstream *Rh box* (at its 3'-end). Both *Rh boxes* have a length of ≈ 9000 bp in identical orientation and share 98.6% homology. The region (breakpoint region) in which the *RHD* deletion occurs is located within a stretch of 1463 bp in which both *Rh boxes* have an identical sequence (identity region). The hybrid *Rh box*, only present when the *RHD* gene is deleted via the proposed mechanism of unequal crossing over, contains a sequence identical to that of the upstream *Rh box* in the 5'-part preceding the identity region, and the 3'-part following the identity region is identical to the downstream *Rh box* sequence in white people.[1] In the present study, 74 D-negative DNA samples from a Chinese Han population were analysed to explore the genetic background of the D-negative phenotype in this population.

Materials and methods

Blood samples and DNA isolation

Blood samples were collected from 74 D-negative and from 11 D-positive unrelated regular blood donors at Shandong Blood Center (Jinan, China). All donors were of Chinese Han ethnicity and lived in Shandong Province area. DNA was isolated from the samples by using a QIAamp DNA blood kit (Qiagen, Valencia, CA).

Serological methods to determine the Rh antigens

Rh antigens were serologically determined in China by using monoclonal anti-D,C,c,E,e reagents (Gamma, Houston, TX) according to the manufacturer's instructions. For all D-negative samples, the indirect antiglobulin test (IAT) was used

to retest D-antigen expression to exclude weak D or a partial D phenotype. No adsorption-elution tests were performed.

Real-time quantitative analyses of the RHD gene

The presence of *RHD* exon 7 was determined by real-time quantitative polymerase chain reaction (RQ-PCR) (7000 Sequence detector; Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). *RHD* exon 7 [primers 940S and 1064R (RHD specific)] was amplified and *RHD* was detected by using a FAM fluorescently labelled *RHD* -specific probe (probe 968; 5'-cccacagctccatcatgggctacaa-3'). Primers were used at a final concentration of 900 nM, and the probe was used at a final concentration of 100 nM , in a total volume of 25 µl of TaqMan Universal Master Mix (Applied Biosystems). Optimal results were obtained by using a final DNA concentration in the range of 2-4 ng/ml. Each sample was tested in triplicate.

Multiplex PCR analysis of RHD

As previously described, six primer pairs were used to specifically amplify *RHD* exons 3, 4, 5, 6, 7 and 9 in one reaction mixture. PCR products were analysed by electrophoresis on a 12% acrylamide gel.[10]

RHD exon-specific sequencing

RHD-specific primers were designed based on intron sequences, and PCR amplifications were performed as described previously (Table 1). Amplifications of exons 6 and 10 were performed with the use of the expand high-fidelity PCR system (Roche Molecular Biochemicals, Mannheim, Germany), as described previously (Table 1).[11] Nucleotide sequencing was performed on an automatic 377 DNA sequencer (Applied Biosystems).

PCR-restriction fragment length polymorphism (PCR-RFLP) for RHD zygosity determination

According to the method published by Wagner & Flegel,[1] a PCR amplification was performed by using the expand high-fidelity PCR system with primers rez7 (consensus, 5' of the *Rh box* identity region) and rnb31 (specific for downstream of the *Rh box*, 3' of the *Rh box* identity region). PCR products were digested with *Pst* I for 3h at 37°C, and the fragments were resolved by electrophoresis on a 1% agarose gel.

Rh box sequencing

PCR products obtained with primers rez7 and rnb31 (amplifying both the downstream and the hybrid *Rh boxes*) were sequenced with consensus primer pairs CRBF06/CRBR08 and Rhbox5070/Rhbox5571[12] (Table 1) to determine the *Rh box* sequences.

Results

RQ-PCR on RHD exon 7

All 11 D-positive samples showed the presence of *RHD* exon 7, indicating the presence of an *RHD* gene. Sixty-five of the 74 D-negative samples were analysed for the presence of *RHD* exon 7 by RQ-PCR. Twenty-three samples showed the presence of *RHD* exon 7, whereas the remaining 42 D-negative samples showed no *RHD* exon 7 (D negativity is caused by modification at the genomic level, e.g. by *RHD-CE* hybrid genes or by deletion of the whole *RHD* gene).

Table 1 Primers

Primer	Nucleotide sequence	Specificity	Position	Exon[a]	Ref.
re01	atagagaggccagcacaa	*RHD*	-149/-131	1 (F)	[11]
RHIN1R	tctgtgcccctggagaaccac	*RHD/CE*	84-64	1 (R)	[2]
RHIN2F	attgagtgagaggcatc	*RHD/CE*	-57/-40	3 (F)	
RHDIN3R	tttcaaaaccctggaaac	*RHD*	80/63	3 (R)	
RHIN3F	tccagtgagccgagatcg	*RHD/CE*	-199/-182	4 (F)	
rb12	tcctgaacctgctctgtgaagtgc	*RHD*	197/174	4 (R)	[11]
rb11	tacctttgaattaagcacttcacag	*RHD*	-267/-243	5 (F)	[11]
RHIN5R	gtggggaggggcataaat	*RHD/CE*	73/55	5 (R)	[2]
rf51	caaaaacccattcttcccg	*RHD/CE*	-332/-314	6 (F)	[11]
re71	acccagcaagctgaagttgtagcc	*RHD*	1008/985	6 (R)	[11]
rb25	agcagggaggatgttacag	*RHD/CE*	-111/-93	6 (S)	[11]
re621	catccccctttggtggcc	*RHD*	-102/-85	7 (F)	[11]
re75	aaggtaggggctggacag	*RHD*	169/152	7 (R)	[11]
RHin7DF	ctggaggctctgagaggttgag	*RHD*	-328/307	8 (F)	[12]
RHin8R	tatgtgatcctcagggaaggag	*RHD/CE*	97/76	8 (R)	[12]
RHDIN8F	gttttgacacacaatatttc	*RHD*	-82/-67	9 (F)	
RHDIN9R	cagcaagtcaacatatatact	*RHD*	82/62	9 (R)	
re91	caagagatcaagccaaaatcagt	*RHD/CE*	-40/-18	10 (F)	[11]
rr4	agcttactggatgaccacca	*RHD*	UTR 1541/1552	10 (R)	[11]
rh7	acgtacaaatgcaggcaac	*RHD/CE*	UTR 1330/1313	10 (S)	[11]
940S	gggtgttgtaaccgagtgctg	*RHD*		7 (F)	
1064R	ccggctccgacggtatc	*RHD*		7 (R)	
rez7	cctgtccccatgattcagttacc Rh box	*Rh box*	Consensus	(F)	[1]
rnb31	ccttttttttgtttgtttttggcggtgc Rh box	*Rh box*	Downstream	(R)	[1]
Rhbox5070	ctacaggcccatgagagtccaaa	*Rh box*	Consensus	(F)	[11]
Rhbox5571	agtgcaagccccaagccttgaca	*Rh box*	Consensus	(R)	[11]
CRBF06	gttaatatgggtggctggc	*Rh box*	Consensus	(F)	
CRBR08	cattaagagatacgcacagg	*Rh box*	Consensus	(R)	

[a] The number corresponds to the amplified exon, and the orientation of the primer is shown in parenthesis: F for forward; R for reverse; and S for sequence primer.

RHD multiplex PCR (MPX PCR) and PCR-RFLP for RHD zygosity determination

Of the 11 D-positive samples showing the presence of *RHD* exon 7 by RQ-PCR, eight samples were further analysed and showed all *RHD*-specific exons by *RHD* MPX PCR. Seven samples were homozygous for *RHD*, and one sample was *RHD* hemizygous by PCR-RFLP analysis. From the 74 D-negative samples, 46 (62·1%, including the 42 samples that were negative for *RHD* exon 7 by RQ- PCR) showed the absence of *RHD*-specific exons by *RHD* MPX PCR and homozygous *RHD* negativity by PCR-RFLP. Five samples (6.8%) showed no *RHD*-specific exons by *RHD* MPX PCR, but PCR-RFLP indicated the presence of one *RHD* gene. From the 23 samples negative for *RHD* exon 7 by RQ- PCR, 22 (29.7%) showed all *RHD*-specific exons by *RHD* MPX PCR, of which 19 were *RHD* gene hemizygous and three were homozygous for *RHD*. One sample (1.4%) was positive by *RHD* MPX PCR, lacking *RHD* exon 6 and the presence of an RHD gene by PCR-RFLP. Based on these results, the 74 D-negative samples were divided into groups A, B, C and D (Table 2).

RHD exon-specific sequencing

Twenty-one samples from group B (*RHD* MPX PCR positive and *RHD* gene hemizygous or homozygous) were further analysed and all showed a 1227G>A mutation in exon 9. Seven D-positive samples from Chinese subjects were sequenced as controls, and all seven showed normal *RHD* exon 9 sequences. Based on these results, the gene frequency of the *Del* 1227G>A allele is 0.17 among the serologically typed D-negative samples. The Del phenotype was mainly observed in C-positive samples, but also sporadically in c-positive samples (Table 2). From four samples of group C (*RHD* MPX PCR negative and PCR-RFLP indicating one *RHD* gene), the presence of *RHD* exons 1 and 10 was determined with *RHD*-specific PCRs (for primers sequences, see Table 1). The results indicate that the samples from group C represent hybrid *RHD*(1)-*CE*(3-9)-*D*(10) samples (the origin of *RH* exon 2 could not be determined), with a gene frequency of 0.034 among the serologically typed D-negative samples. Unfortunately, owing to the scarcity of DNA, these samples could not be analysed further. Sequencing of the *RHD* exon 6 of the single sample from group D (*RHD* MPX PCR positive, but lacking exon 6, and PCR-RFLP indicating one *RHD* gene) showed a novel 933C>A nonsense mutation (Fig. 1). *RHD*-specific sequencing of exons 1, 3-5 and 7-10 detected no difference from the Chinese control sample. The 933C>A mutation causes an unstable 3'-end primer annealing, disabling amplification of *RHD* exon 6 by *RHD* MPX PCR analyses.

Table 2 Results of *RHD* multiplex polymerase chain reaction (MPX PCR) and of PCR-restriction fragment length polymorphism (PCR-RFLP) analyses of the Chinese RhD-negative population

Group	Number (%)	*RHD* MPX PCR	PCR-RFLP	RhCcEe	Molecular background
A	46 (62·1)	Negative	All *RHD-*/*RHD-*	[a]	*RHD*-negative
B	22 (29·7)	Positive	19 *RHD+*/*RHD-*	16 Ccee 1 ccEe 1 CCee 1 NT	*RHD* 1227G>A
			3 *RHD+*/*RHD+*	1 CCee 1 ccee 1 NT	
C	5 (6·8)	Negative	All *RHD+*/*RHD-*	5 Ccee	*RHD-CE-D* hybrids
D	1 (1·4)	*RHD* exon 6 negative	*RHD+*/*RHD-*	ccee	RhD-negative *RHD* gene

[a] Unfortunately, RhCcEe phenotyping of group A was not performed.

Rh box sequencing

As the samples of group A are *RHD* negative by *RHD* MPX PCR analysis, and the PCR-RFLP indicates no *RHD* genes, it is expected that only hybrid *Rh boxes* would be found on the *RH* locus. PCR products obtained with primers rez7 and rnb31 (consensus and downstream specific, respectively, amplifying both hybrid and downstream *Rh boxes*) were sequenced to study the *RHD* breakpoint region in Chinese people. With primers CRBF06 and CRBR08 (both consensus primers, located at the 3' end of the identity region), 27 samples showed downstream sequences in the 3' part following the identity region of the hybrid *Rh box*. Thirteen samples were further sequenced with primers Rhbox5070 and Rhbox5571 (both consensus primers, located in the 5' part preceding the identity/breakpoint region) and all samples showed upstream-specific sequences in the 5' part preceding the identity/breakpoint region, indicating the presence of hybrid *Rh boxes*, as described in white people. Because the samples from group B were *RHD* MPX PCR positive and showed the presence of an *RHD* gene hemizygous or homozygously, it was expected to find no hybrid *Rh boxes* in the *RHD* homozygous samples and both a downstream and a hybrid *Rh box* in the *RHD* hemizygous samples. Eleven samples and four D-positive control samples were sequenced by using primers Rhbox5070 and Rhbox5571. Nine samples hemizygous for *RHD* showed both upstream and downstream sequences in the 5' part preceding the identity region of the *Rh box* (confirming the presence of both the downstream and the hybrid *Rh box*). The two samples homozygous for *RHD* showed only downstream *Rh box* sequences. The four D-positive samples, homozygous for *RHD*, also showed only downstream *Rh box* sequences.

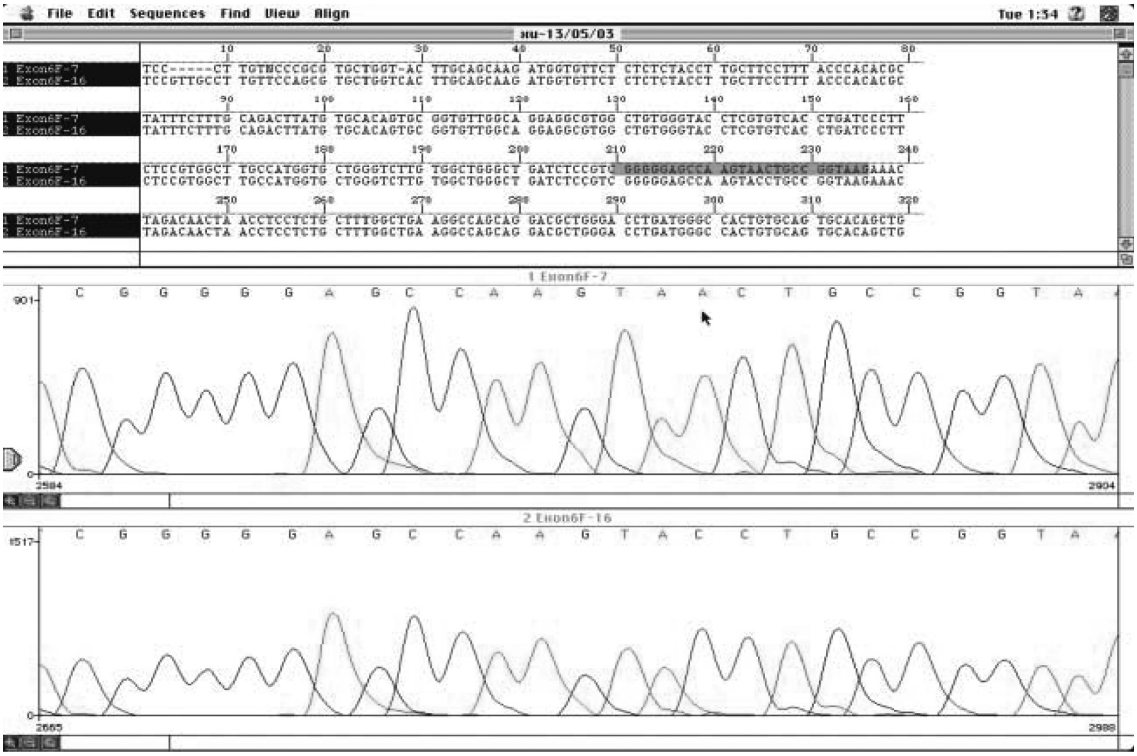

Figure 1 In one of the samples one novel 933C>A mutation (the upper sequence) in *RHD* exon 6 was found to lead to the formation of a premature stop codon.
The lower sequence is that of the Chinese RhD-positive control sample.

Discussion

The presence of an *RHD* gene in serologically D-negative individuals hampers reliable genotyping of *RHD* by introducing false-positive results. False-positive (geno)typing for the *RHD* gene may frequently occur in African populations owing to the presence of *RHDψ* and of the *RHD*(1-3)*RHCE*(3-7)*RHD*(8-10) hybrid gene(s).[9,13,14,15] In Japanese people, Okuda *et al*. found different *RHD* genes in apparent D-negative individuals.[16] Subsequent studies demonstrated all types of partial or intact *RHD* genes to be present in Chinese or Japanese D-negative blood donors.[5,6,8,9,14,17,18] It was also found that a grossly intact *RHD* gene is present in the Del phenotype (which was first described in 1984)[4] in apparent D-negative donors. The very weak D antigen of Del can only be detected by adsorption elution tests, which are not used as part of the routine testing for the D antigen. There is no consistent agreement regarding whether or not the Del phenotype should remain in the D-negative group. Chang *et al*. found a large deletion of *RHD* in all of their 21 Del phenotype individuals from Taiwan.[6] This deletion covers 1013 bp between intron 8 and intron 9, including the whole of *RHD* exon 9. However, several other groups found an intact *RHD* gene in the Del phenotype, and sequencing showed three mis-

sense mutations, of which one is the 1227G>A mutation that probably disrupts normal intron splicing.[7,8,14] More recently, two groups from Taiwan studied the Del phenotype and one group demonstrated the 1013-bp deletion in all of their Del donors (they encountered 13 Del samples in 204 RhD-negative samples),[9] while the other group only found 20% of their Del lacking exon 9, with the remaining 80% showing the presence of *RHD* exons 3, 4, 5, 7 and 9 by PCR-SSP (Sequence Specific Primer) (they encountered 34 Del samples in 156 D-negative samples).[19] Apparently, different molecular mechanisms underlie the Del phenotype. In our study, sequence analysis of 21 D-negative samples from the Chinese HAN population, which showed all *RHD* exons by *RHD* MPX PCR analysis, revealed only the 1227G>A *Del* mutation. As shown in this study, individuals with the Del phenotype frequently occur (often in association with RhCe) and are not recognized as D-positive in daily practice in blood banks in China. In fact, our study group consists of regular Chinese blood donors. Therefore, blood of the Del phenotype is frequently transfused into D-negative patients, apparently without giving rise to immunization. However, how the splicing site mutation causes the very weak expression of D antigen, and whether this weak antigen causes D immunization when transfused to D-negative patients, needs further investigation. D negativity owing to a deletion of the *RHD* gene is the most common form of D negativity in Chinese people. In 46 samples that were negative in the *RHD* MPX PCR and contained no *RHD* gene according to PCR-RFLP analysis, it is shown that D negativity is caused by a deletion of the *RHD* gene. Further sequencing of parts of the *Rh box* in this group indicates that also in Chinese people the *RHD* gene deletion has taken place within the defined breakpoint region, as previously described in white people.[1] Therefore, it is possible to determine the *RHD* zygosity by using the PCR-RFLP in a Chinese Han population. Five D-negative samples were negative when analysed by the *RHD* MPX PCR (indicating the absence of *RHD* exons 3, 4, 5, 6, 7 and 9) but PCR-RFLP analysis indicated the presence of one *RHD* gene. Further analysis of four samples revealed that they had at least *RHD* exons 1 and 10 and were associated with the DCe haplotype. This result was in concordance with previous studies in Chinese populations in which the *RHD*(1)- *RHCE*(2-9)-*RHD*(10) hybrid gene was always described in association with the DCe haplotype.[8,9,18] However, the presence of *RHCE* exon 2, and therefore the exact junction point between *RHD* and *RHCE* in our samples, could not be determined. Similar hybrid genes were also described in Europeans.[20] In these European samples the exact junction point between *RHD* and *RHCE* was not described. One novel *RHD* 933C>A nonsense mutation, which results in a stop codon, was found in one of the D-negative samples. Missense mutations are not frequently encountered in D-negative Chinese people, except for the recent cases reported by Shao et al.[8] However, *RHD* missense mutations are more frequent in Chinese people than in Europeans, stressing the advantage of their specific detection by using molecular methods. In summary, the *RHD* gene

deletion, a *RHD-RHCE-RHD* hybrid gene and one novel 933C>A mutation were found to be the three mechanisms that cause D negativity in our samples (with gene frequencies of 0.79, 0.034 and 0.006, respectively, in the Chinese Han population serologically typed as D-negative). Specific sequencing of the *Rh boxes* indicated that the physical structure of the hybrid *Rh box* on the *RHD*-negative *RH* locus in Chinese people is identical to the structure previously described in white people. Furthermore, our data support that genotyping *RHD* to predict the D phenotype is hampered by the presence of the described *RHD* genes in a Chinese population. In particular, the highly frequent 1227G>A mutation (allele frequency of 0.17 in D-negative Chinese donors), characteristic for the Del phenotype should be taken into account when typing a Chinese (Asian) population. Serologically, this phenotype will only be detected by the absorption elution technique, which is not part of routine testing and therefore results in a false-negative test result for D in donors from Asian descent.

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Chapter 5

RHD(T201R, F223V) cluster analysis in five different ethnic groups and serological characterization of a new Ethiopian variant *DARE*, the *DIII* type 6 and the *RHD*(F223V)

Martine GHM Grootkerk-Tax, Joyce D van Wintershoven, Peter C Ligthart, Dick J van Rhenen, C Ellen van der Schoot, Petra A Maaskant-van Wijk. *RHD*(T201R, F223V) cluster analysis in five different ethnic groups and serological characterization of a new Ethiopian variant *DARE*, the *DIII type 6* and the *RHD*(F223V). *Accepted for publication in Transfusion.*

Abstract

Background: The *RHD* phylogeny in humans shows 4 main clusters of which 3 are predominantly observed in (African)Blacks. Each of the African clusters is characterized by specific animo acid substitutions relative to the Eurasian *RHD* allele. *RH* phylogeny defines the framework for identification of clinically relevant aberrant alleles. This study focuses on the *weak D type 4* cluster (characterized by *RHD*(T201R, F223V)(602C>G, 667T>G)) in five ethnic groups.

Study design and methods: 1702 samples were screened for the presence of 602C>G and 667T>G by PCR-SSP. 80 Samples were assigned to the *weak D type 4* cluster and were molecularly characterized by PCR-SSP and *RHD* sequencing. Antigens of aberrant alleles were characterized with monoclonal anti-D antibodies according to the 37 epitope model when possible.

Results: Five new aberrant alleles *DIII type 6*, *DIII type 7*, *DARE*, *RHD*(T201R, F223V)(without 819G>A) and *RHD*(F223V) were identified and DIII type 6, DARE and RHD(F223V) were serologically characterized with monoclonal anti-D. Both the DARE and RHD(F223V) showed epitope loss. We postulate the 1136C>T nucleotide substitution (characterisic for the *DAU* allele cluster) to be present on the *DVa(KOU)* allele.

Conclusion: Identification of the new variant alleles refines the phylogeny of *RHD* in humans. The proposed *DVa(KOU)* allele with 1136C>T (*DVa(KOU)*T379M) is probably caused by conversion of the *DAU0* allele and the *DVa(KOU)* allele, forming a phylogenetic link between the *DV* allele and the *DAU* cluster. By describing the *RHD*(F223V)(667T>G) and *RHD*(T201R, F223V)(602C>G, 667T>G) alleles we give formal proof for the origin of the non-Eurasian cluster.

Introduction

The RhD blood group antigen is a highly immunogenic polypeptide present on the Red Blood Cell (RBC) membrane in RhD-positive individuals. In transfusion medicine, RhD incompatibility may cause hemolytic transfusion reactions and hemolytic disease of the foetus and newborn (HDFN) and anti-Rh antibodies are often involved in autoimmune hemolytic anemia. The RhD protein is encoded by the *RHD* gene (chromosome 1, p34.3-p36.1). RhD-negativity in Caucasians is mostly caused by a deletion of the *RHD* gene.[1] In blacks, RhD-negativity is frequently caused by *RHDψ* and *(C)cde*^{s} alleles (allele frequencies of 0.0714 and 0.036, respectively).[2-4] Both alleles carry *RHD* specific sequences and therefore hamper *RHD* genotyping strategies. Variant RhD-antigens encoded by aberrant *RHD* alleles, have been found with high frequencies in blacks and, with much lower frequencies, in whites.[5-7] These aberrant *RHD* alleles are mainly caused by gene rearrangements and point mutations, often between *RHD* and the highly homologous *RHCE* gene. The Rh system contains eight basic haplotypes which were formed by a series of duplication, mutation and recombination events.[8]

The ancestral *RH* haplotype was formed by the cDe haplotype which is still frequent in blacks.[8] The most recently proposed phylogeny of *RHD* in humans, based on a previously defined phylogeny,[6] shows 4 main clusters, the *DIVa*, *DAU* and *weak D type 4* clusters are predominantly observed in (African)blacks and occur in the cDe haplotype.[9] The fourth cluster of Eurasian alleles is predominantly observed in the CDe and cDE haplotypes. Each of the African clusters is characterized by a specific animo acid substitution relative to the Eurasian *RHD* allele. The amino acid substitutions are T379M (1136C>T) in the *DAU* cluster, F223V (667T>G) in the *weak D type 4* cluster and D350H (1048G>C) in the *DIVa* cluster (including *DIII type 4* and *5* and *(C)cde*^{s}). The alleles presented by the *DIVa* cluster share 3 amino acid substitutions: L62F (186G>T), A137V (410C>T) and N152T (455A>C), that are ancestral because they were also observed in chimpanzee *RH*.[10] The *weak D type 4* cluster is thought to have an ancestral allele characterized by *RHD*(F223V) (667T>G), although the existence of this allele is only postulated and has never been described. The *weak D type 4* cluster encompasses *RHD*(T201R, F223V) (602C>G, 667T>G), the *weak D types 4.0, 4.1, 4.2.1* and *4.2.2*, *DAR*, *DOL* and *RHDψ*. Both weak D types 4.2.1 and 4.2.2 are functionally equal to DAR but differ molecularly from *DAR* by 957G>A and 957G>A;744C>T respectively.[11]

This study's focus is mainly on the *RHD*(T201R, F223V) (602C>G, 667T>G) cluster in five different ethnic groups, which includes the whole of the *weak D type 4* cluster and, because of a recombination event between the *weak D type 4.0* and *DIII type 4* alleles resulting in the *DIII type 5* allele, part of the *DIVa* cluster. This focus was chosen since these clusters encompass most *RHD* alleles encoding for partial D antigens (which are clinically most relevant) and are most interesting regarding phylogenetics due to the high percentage of polymorphism.

The analysis of the alleles belonging to the *DIVa* cluster which are not included in this study (*Ccde*^{s} and *DIVa* alleles) is described by Tax et al.[12] and the *DAU* allele cluster has been intensively studied by Wagner et al.[9] In the present study we give the formal proof for the phylogenetic origin of the *weak D type 4* cluster by demonstrating the presence of the *RHD*(F223V) and *RHD*(T201R, F223V) alleles. The phylogeny of *RHD* in humans was refined by describing the new *RHD* alleles *DARE*, *DIII type 6* and *DIII type 7*. The proteins expressed by the *DARE*, *DIII type 6* and *RHD*(F223V) alleles have been serologically characterized with monoclonal anti-D. The proposed *DVa(KOU)*T379M allele may form a phylogenetic link between the *DVa(KOU)* allele and the *DAU* allele cluster, allowing speculation on the possibility of a *DV* allele cluster.

Materials and methods

Samples

Ethylenediaminetetraacetate-anticoagulated blood samples from blood bank donors were obtained from five different ethnic groups by The South African Blood Transfusion Service, Johannesburg (South African blacks and Asians), The Ethiopian Red Cross Society Trandfusion Service, Addis Ababa (Ethiopian blacks), The Red Cross Blood Bank Curacao, Willemstad (Curacao blacks) and The Sanquin Blood Bank South West Region, Rotterdam (Caucasian whites). All methods described in this study were performed at the Sanquin Blood Bank South West Region, Rotterdam and Sanquin Research at CLB, Amsterdam.

Serology

Red blood cells (RBCs) were Rh phenotyped according to standard serological protocols with a monoclonal IgM antibody anti-D (GAMA401, RhD epitope 6/7), a polyclonal IgG anti-D antibody and monoclonal IgM antibodies (from Gamma Biologicals INC., Houston, TX, USA) recognizing C (MS24), c (MS33), E (GAMA402) and e (MS16, MS21, MS63 (blend)). Extended serology was performed with monoclonal anti-D reagents as provided by the third and fourth Workshop on Monoclonal Antibodies Against Human Red Blood Cells and Related Antigens.[13-14] Extended RhD serology to obtain the 37 epitope model was performed complying to workshop protocols.[15] Samples were tested by direct (IgM) and indirect (IgG) agglutination using the antibodies listed in tabel 3.

Antibody identification of the *RHD*(F223V) (667T>G) sample was performed with untreated cells by IAGT with PEG. The ether-eluate of patient cells was also investigated with untreated cells by IAGT with PEG.

Genomic DNA analysis

Genomic DNA was isolated from peripheral white blood cells by a salting-out method according to standard protocol.[16] PCR reactions were performed in a thermal cycler (model 9700, Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands).

All DNA samples were screened for the presence of 602G in combination with 667G in *RHD* with the *RHD*(201R/223V) Sequence Specific PCR (PCR-SSP) as described before with primerset R581/R667[17] (700 whites from The Netherlands, 310 blacks from South-Africa (part of the group of 326 blacks from South-Africa previously studied by Hemker et al),[17] 319 Asians from South-Africa, 197 blacks from Curacao and 176 blacks from Ethiopia). 80 Samples carried an *RHD*-(T201R, F223V) allele and were further analyzed.

They were screened for nt. 383A (*RHD*) combined with nt. 455C (*RHCE*) (hybrid exon 3) by *RHC/c/hex3* Multiplex (MPX) PCR and for nt. 1048 C>G by intron4/exon7 MPX PCR as described before[12] for the identification of *DIII*, *DIV* or *(C)cde*^{s} (*r's*) alleles.

The *RHD* MPX PCR was performed as described by Maaskant-van Wijk et al.[18] Minor modification were the use of HPLC-purified primers and Amplitaq (Applied Biosystems). With this MPX PCR, *RHD* exons 3, 4, 5, 6, 7 and 9 are amplified in one reaction mixture.

The 1025T>C nucleotide change, specific for the *DAR* variant *RHD* allele was detected by PCR-SSP as previously described by Hemker et al. (primerset R973/R1044).[17]

The *RHD*ψ PCR-SSP was used for detection of nt. 654C and nt. 674T, characteristic for the *RHD*ψ- as described by Grootkerk-Tax et al.[19]

The 1136C>T nucleotide change (*DAU0*) was detected by PCR-SSP with primers RHin7DF (ctggaggctctgagaggttgag) and 8D1136Tmm (GACCTGTCAGGAGACCATACA).

RHD zygosity was determined by RQ-PCR as previously described by Grootkerk-Tax et al.[19]

RH-specific amplification of exon 2 (*DIII type 7* sample) was performed with primers Rex2S (ctccccaccgagcag) and rex2A (ccaccatcccaatac).

RHD-specific sequencing

The *RHD* exons were sequenced according to our previously published protocol.[20] Modifications to this protocol were the use of sequence primers RH-58in4F (ctcatccaaaaccctcgag) for exon 5, RH52in6R (gcccatcaggtcccagcg) for exon 6 and RH-193in7F (cacaatcttggaatctccgtcg) for exon 8. Additionally, *RHD* exon 2 was amplified with primers re12d and re23 and sequenced with primers re13 and RH28in2R (tccattccctctatgaccca), *RHD* exon 10 was amplified with primers re91 and rr41 and sequenced with primers re91 and rh7, the *RHD* promotor region was ampli-

fied with primers rb13 and rb11d and sequenced with only one primer, re02.[11] All samples were sequenced on an automatic 377 DNA sequencer (Applied Biosystems).
All previously known aberrant alleles were confirmed by single primer sequencing. New alleles were identified by bi-directional sequencing.

RESULTS

1702 DNA samples derived from five different ethnic groups (Caucasian whites, South-African blacks, South-African Asians, Ethiopian blacks and Curacao blacks) were screened for the presence of nt. 602G in combination with nt. 667G in *RHD* with the *RHD*(201R/223V) PCR-SSP. 80 Samples carried both nucleotide changes on the same allele (i.e. 2 whites (2.5%), 57 South-African blacks (71%), 4 South-African Asians (5%), 15 Ethiopian blacks (19%) and 2 Curacao blacks (2.5%)). Wagner et al. postulated that in the *weak D type 4* cluster at least two sub clusters can be recognized by the presence of either nt. 819A or nt. 1025C.[9] Therefore all 80 samples were tested for the presence of these mutations by *RHD*-specific sequencing of exon 6 and the 1025T>C PCR-SSP, respectively (table 1). In addition, the samples were analysed by *RHC/c/hex3* MPX PCR for nt. 383A (*RHD*) combined with nt. 455C (*RHCE*) in exon 3, a combination characteristic for the *DIII* category (table 1). None of the 80 samples in the *RHD*(T201R, F223V) cluster carried a *DIVa* allele since all samples were negative for the characteristic nt. 1048C by intron4/exon7 MPX PCR analysis. All samples were screened for the presence of an *RHD*ψ by PCR-SSP. Results of the genotyping assays are shown in table 1. Based on the reaction patterns, groups I to VII were defined.

Table 1. PCR-SSP analysis of nts 455 and 1025 and sequencing results of *RHD* exon 6 (nt 819) of 80 samples carrying *RHD*(T201R, F223V) (602C>G, 667T>G)

Group (n)	455C	1025C	819A	Phenotype (n)
I (1)	pos	pos	NT	CcDee(1)
II (23)	pos	-	pos	Table 2
III (21)	pos	-	NT	ccDee(16), CcDee(3), ccDEe(2)
IV (3)	-	pos	-	ccDee(3)
V (22)	-	pos	NT	ccDee(14), CcDee(4), ccDEe(4)
VI (9)	-	-	pos	ccDee(6), CcDee(2), CcDEe(1)
VII (1)	-	-	-	ccDee(1)

Analysis of RHD(T201R, F223V)-positive/nt. 455C-positive (groups I, II and III)

To refine identification of the alleles belonging to the *weak D type 4* cluster (*RHD*(T201R, F223V)) all groups as defined in table 1 were further analyzed. *RHD* specific sequencing analysis of exons 3, 4 and 5 of the group I sample (South-

African black) showed 410C+T and 455A+C in exon 3, 602C>G in exon 4, 667T>G and 744C>T in exon 5 and the *DAR* PCR-SSP indicated the presence of 1025C in exon 7. Therefore this sample carries both a *DAR*[17] allele and a *DIII type 5* [9] or *6* allele (see group II, subgroup B) (figure 1).

The sequencing results of group II are shown in table 2. Based on the sequencing results this group was further subdevided in 9 subgroups, A to I.

The presence of 455C in group II could either be derived from a *Ccde*^{s} allele or from alleles belonging to the *DIII* category. The presence of a *Ccde*^{s} allele could be excluded from subgroups A-E due to *RHD*-specific sequencing analysis of exons 5 and 6 which showed the presence of two exons.

The subgroup A sample (South-African black) carries an *RHD*ψ and a *DIII type 6* allele (see group II, subgroup B).

Subgroup B (2 South-African blacks and 2 South-African Asians) sequencing analysis showed 410C+T and 455A+C in exon 3, 602 C+G in exon 4, 667 G+T in exon 5 and 819 G+A in exon 8. All other *RHD* exons showed normal *RHD* sequences (including exon 2). Standard RhD serology did not indicate weak RhD expression. This group most likely carries a (normal) *RHD* allele and an allele very similar to the alleles of the *DIII* category, without the 186T in exon 2. Therefore this allele is named *DIII type 6* allele.

Subgroup C (South-African blacks) carries most likely a *DAU0* [9] allele (1136C>T) and a *DIII type 5* or *6* allele. Based on the mutation pattern this sample could also carry a *weak D type 4.0* [21] and a *DIII type 4*[11] allele. However, this is not likely due to the 1136C>T nucleotide change characteristic for the *DAU* allel cluster, which has not previously been identified outside this cluster.

The subgroup D sample (South-African black) carries a *DIII type 5* or *6* allele combined with a *DAU3* (*RHD* 835G>A and 1136C>T)[9] allele.

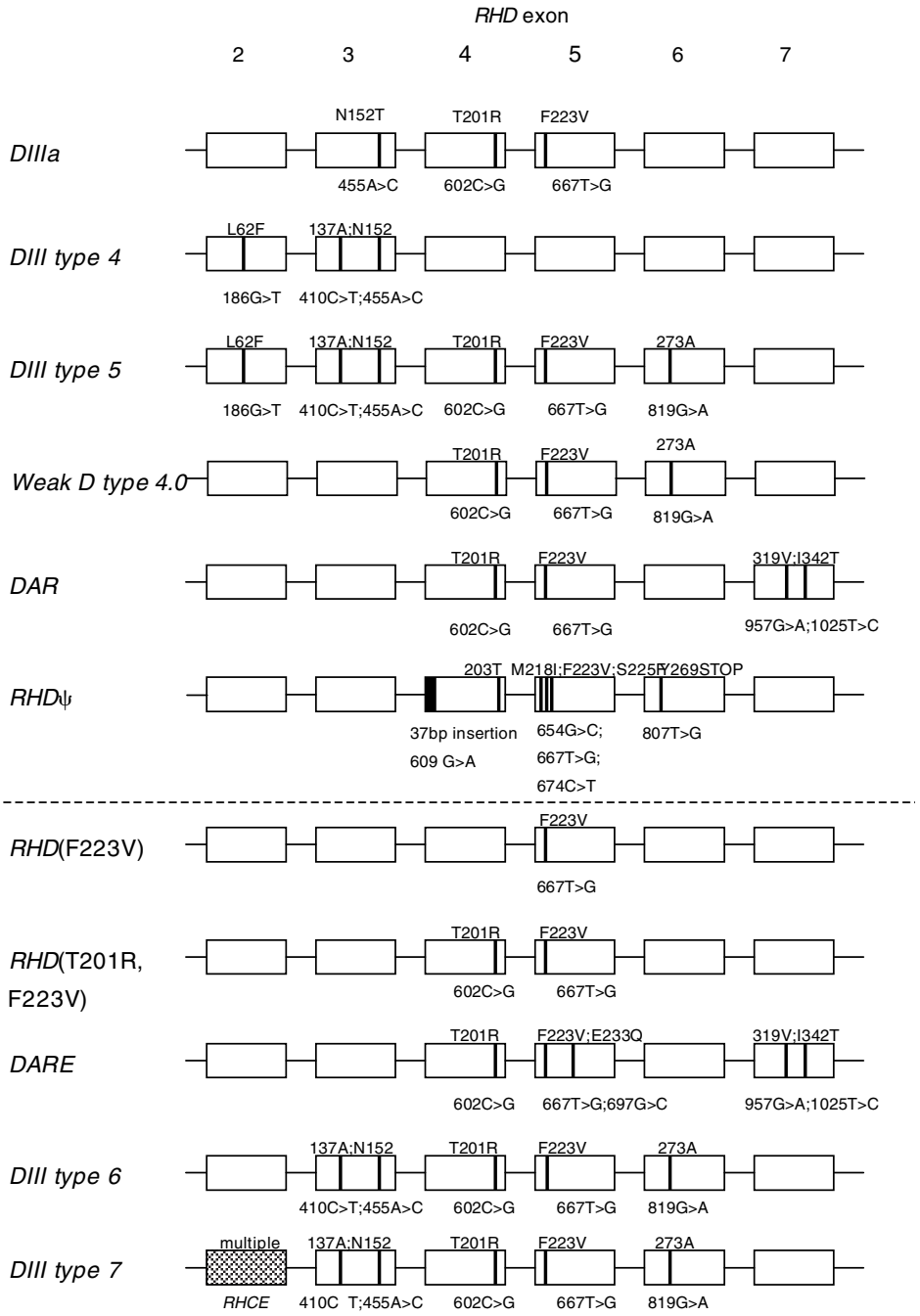

Figure 1. Previously and newly identified aberrant *RHD* alleles
Depicted are *RHD* exons 2 to 7 of multiple *RHD* alleles found in the *RHD*(T201R, F223V) analysis.
Above the dotted line, schematic representation of previously identified alleles.
Below the dotted line, schematic representation of all 5 new *RHD* alleles as identified in the present study.

Table 2. Results of *RHD*-specific sequencing analysis of group II samples (subgroups A to I)

group/subgroup/n	Phenotype(n)	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Notes
II/A/1	ccDee(1)	RHD	410C+T;455A+C	Failed*	654G+C;667T>G; 674T+C	819G+A	Exons 1, 7, 8, 9 and 10 no abnormalities
II/B/4	ccDee(2), CcDee(2)	RHD	410C+T;455A+C	602C+G	667G+T	819G+A	Exons 1, 7, 8, 9 and 10 no abnormalities
II/C/3	ccDee(3)	NT#	NT	NT	667G+T	819G+A	1136T (by PCR-SSP)
II/D/1	ccDee(1)	NT	NT	NT	667G+T	819G+A;835G+A	1136T (by PCR-SSP)
II/E/1	ccDee(1)	RHD	410C+T;455A+C	602C+G	667T>G ;697G+C	819G+A	Exons 1, 7, 8, 9 and 10 no abnormalities. 1136T (by PCR-SSP)
II/F/1	CcDee(1)	RHD	410C+T;455A+C	602C>G	667T>G	819G>A	Exons 1, 7, 8, 9 and 10 no abnormalities
II/G/8	ccDee(8)	186G>T	410C>T;455A>C	602C>G	667T>G	819G>A	
II/H/3	CcDee(3)	186G+T	410C>T;455A>C	602C>G	667T>G	819G>A	Ccdes positive by RHC/c/hex3 and intron4/exon7 MPX
II/I/1	ccDee(1)	No RHD	410C>T;455A>C	602C>G	667T>G	819G>A	

* Exon 4 sequencing analysis failed due to the presence of a 37 bp insert at the intron 3 exon 4 boundary.
#NT = not tested.

In the subgroup E sample (South-African black) we identified the presence of a *DIII type 6* and a *DVa (KOU)*[22] allele. In this sample we also found a 1136T by PCR-SSP. This nucleotide change, characteristic for the *DAU* allele cluster and subsequently not previously identified to exist outside this allele cluster, is most probably present on an allele carrying the *DVa (KOU)* characteristic nucleotide changes (see also group VI).
In the subgroup F sample (South-African black), a *Weak D type 4.0* allele combined with a *Ccde*^{s} allele was identified.
Subgroup G (7 South-African blacks and 1 South-African Asian) showed the presence of *DIII type 5* alleles either next to another *DIII type 5* allele or next to an *RHD* gene deletion.
DIII type 6 alleles were also identified in subgroup H (South-African blacks) next to a *Ccde*^{s} allele.
The subgroup I sample (South-African black) showed an almost identical sequence analysis as subgroups G and H. However, we were unable to amplify *RHD* exon 2 in the subgroup I sample. Next to the use of *RHD* specific primers located in the surrounding introns (as used for all samples) we also amplified exon 2 with consensus primers (Rex2S and Rex2A). Because the subgroup I sample was RhC-negative we were able to rule out the presence of *RHD* exon 2 (we only found sequences specific for the *c* allele of *RHCE*). *RHD* zygosity analysis by RQ-PCR showed the presence of only 1 *RHD* gene. This sample carries a new partly hybrid *RHD* allele very similar to *DIII type 5/6* alleles without *RHD* exon 2 and is named *DIII type 7* (figure 1).
All samples of group III (20 South-African blacks and 1 Ethiopian black) showed all *RHD* specific exons by *RHD* MPX PCR analysis and 455C in exon 3, 602G in exon 4 and 667G in exon 5 (amplification of all *RHD* exons by *RHD* MPX PCR in a sample with 602G in exon 4 and 667G in exon 5 indicates the presence of two *RHD* alleles). If all nucleotide changes are present on the same allele, these samples carry a *DIIIa* [23] or a *DIII type 5* (or *6*) allele next to a normal *RHD* or, if the nucleotide changes are distributed over two alleles, a *weak D type 4* and a *DIII type 4* are also possible. By genomic DNA analyses it is not possible to make a distinction between these allele combinations.

Analysis of RHD(T201R, F223V)-positive, nt. 455C-negative (groups IV, V, VI and VII)

All *RHD* specific exons and the *RHD* promotor region of the group IV samples (3 Ethiopian blacks) were sequencened revealing the nucleotide changes: 602C>G in exon 4, 667T>G and 697G>C in exon 5 and 957G>A and 1025T>C in exon 7. These samples seem to carry a *weak D type 4.2*/*DAR* allele with an additional nucleotide change in exon 5 697G>C, leading to E233Q. Therefore, this newly identified allele was named *DARE* (E for Ethiopian).

All group V samples (14 South-African blacks, 7 Ethiopian blacks and 1 Curacao black) were positive for the 1025T>C nucleotide change, indicating the presence of an *DAR* allele. 15 Samples showed amplification of all *RHD* exons by *RHD* MPX analyses, these samples carry a normal *RHD* next to the *DAR* allele. The 7 Ethiopian samples were not sequenced for exon 5, therefore more *DARE* alleles (with *RHD* alleles) might be present in this group. One sample (South-African black) lacked the *RHD* exon 5 amplicon by *RHD* MPX analysis, *RHD* specific sequencing revealed 509C+T and 602G+C in exon 4 and 667T>G in exon 5, suggesting the presence of both a *DOL* [24] and a *DAR* allele. Three samples of group V were also positive for nt. 654C and nt. 674T by *RHD*ψ PCR-SSP analysis, suggesting the presence of both a *DAR* and an *RHD*ψ allele. Of three samples of which *RHD* exons 4 and 5 failed to amplify by *RHD* MPX analysis, sequencing analysis of exons 4 and 5 showed the presence of 602C>G and 667T>G respectively, Additional sequencing of *RHD* exon 7 of two samples showed 957G>A and 1025T>C. Therefore these samples carry a *weak D type 4.2*/*DAR* allele only.

In group VI (4 Ethiopian blacks, 2 South-African blacks, 1 South-African Asian, 1 Curacao black and 1 Caucasian white), 7 samples showed amplification of all *RHD* exons by *RHD* MPX analysis. Therefore, of these samples all *RHD* specific exons and the *RHD* promotor region were sequencened revealing: 602C+G in exon 4, 667T+G in exon 5 and 819G+A in exon 6 in all samples and 48G>C in two samples, indicating the presence of a *weak D type 4.0* and *RHD* in 5 samples (2 Ethiopian blacks, 1 South-African black, 1 Curacao black and 1 South-African Asian) and a *weak D type 4.1* and *RHD* in 2 samples (both Ethiopian blacks). Of one group VI sample (Caucasian white) amplification of *RHD* exons 4 and 5 by *RHD* MPX analysis failed. Sequencing of all *RHD* exons (including the promotor region) showed: 602C>G in exon 4, 667T>G in exon 5 and 819G>A in exon 6, characteristic for a *weak D type 4.0* allele. Of one group VI sample (South-African black) the amplification of only *RHD* exon 5 failed by *RHD* MPX analysis. This sample was also completely sequenced showing: 602G+C in exon 4, 667T>G and 697C+G in exon 5, 819G+A in exon 6 and 1136C+T in exon 8, indicating the presence of a *weak D type 4.0* and a *DVa(KOU)* allele. The 1136C>T nucleotide change, characteristic for the *DAU* alleles and not previously identified to exist outside this allele cluster, is most probably present on the allele that also shows *DVa (KOU)* characteristics (see also group II, subgroup E).

The *RHD* MPX analysis of the group VII sample (Caucasian white) failed the amplification of exons 4 and 5. This sample was completely sequenced for *RHD* and showed only the 602G>C in exon 4 and 667T>G in exon 5 nucleotide changes. This sample represents the not previously identified *RHD*(T201R, F223V) allele (that is without the 819G>A nucleotide substitution characteristic for weak D type 4).

Serological characterisation of DIII type 6 and DIII type 7 antigenic proteins

The genomic organization of the sample identified in group IIA in which the *DIII type 6* is present opposite to an *RHDψ* (ccDee) allowed us to determine the 37 epitope pattern of the newly identified DIII type 6 (table 3). Like DIII type 4, only weak reactions were seen and no epitope loss was indicated.

The DIII type 7 sample identified in group II, subgroup I showed a 2+ reaction strength with monoclonal anti-D and a 1+ reaction strength with polyclonal anti-D in standard serology. Unfortunately it was not possible to perform extended serology on this sample.

Serological analysis of African DAR versus Ethiopian DARE antigenic proteins

The 697G>C nucleotide change in *RHD* (leading to E233Q) has not previously been described to be present in a DAR-like protein. Nt 697 is located on the fourth extracellular loop of the RhD protein. All 3 Ethiopian DARE samples were serologically analysed for their RhD epitope expression pattern accoding.

Table 3. Monoclonal anti-D reaction patterns of an R1r and DAR control cell and the newly identified RhD-variants DIII type 6, RHD(F223V)and DARE according to the 37 epitope model for RhD

Epitope	MoAb	R1r	DIII type 6	RHD(F223V)	DAR	DAREI	DAREII	DAREIII
1.1	IV-1-82	3	nt	3	0	0	0	0
1.2	III-1-83	2-3	1	nt	0	1	0	0
1.3	IV-1-50	**1-2**	0.5	**0**	0	0	0	0
2.1	IV-1-23	3-4	4	2	1	1	0	0
	IV-1-24	3	4	2	0	0	0	0
	IV-1-34	3-4	3	2	1	1	0	0
	III-1-51	3-4	3	nt	0	0	0	0
2.2	IV-1-60	3-4	3	3	0	0	0	0
	IV-1-51	3-4	3	3	0	0	0	0
3.1	IV-1-33	3	3	3	1	1	0	0
	IV-1-67	3-4	4	2	2	3	3	1
	IV-1-72	2-4	3	2	1	1	0.5	0.5
	IV-1-95	3-4	4	3	2	3	3	2
4.1	III-1-45	2-3	3	nt	**0**	**2**	**1**	**1**
5.1	IV-1-76	4	4	4	1	1	0	0
	III-1-116	4	4	nt	**1**	**0**	**0**	**0**
5.2	IV-1-79	3-4	4	3	1	1	0	0
	IV-1-25	2-4	3	3	1	2	0	0
5.3	IV-1-86	4	4	nt	**1**	**0**	**0**	**0**
5.4	IV-1-32	3-4	3	3	0	0	0	0
	IV-1-44	2-3	3	nt	0	0	0	0
5.5	III-1-69	3-4	3	nt	**0.5**	**0**	**0**	**0**
6.1	IV-1-87	4	4	3	3	3	3	3
	IV-1-98	4	4	4	2	2	2	1
	III-1-35	4	4	nt	1	1	1	0
	III-1-103	3	3	nt	3	2	2	0
6.2	IV-1-55	3	3	3	2	2	1	0
	IV-1-48	3-4	4	3	2	3	2	1
	IV-1-30	3-4	3	3	3	3	2	2
	IV-1-144	3-4	4	3	0	0	0	0
6.3	IV-1-52	4	4	nt	1	1	0	0
	IV-1-68	4	4	nt	2	3	0	0
6.4	IV-1-94	3	nt	3	nt	nt	nt	nt
	IV-1-62	3	nt	2	nt	nt	nt	nt
	IV-1-69	4	4	nt	1	1	0	0
	IV-1-83	3-4	4	nt	**1**	**0**	**0**	**0**
	IV-1-36	4	4	nt	**1**	**0**	**0**	**0**

	IV-1-43	4	3	nt	1	1	0	0
6.5	III-1-30	3	3	nt	2	3	2	2
	III-1-84	3-4	3	nt	1	0	0	0
	III-1-119	4	4	nt	2	3	2	2
	IV-1-100	4	4	nt	3	2	0	0
6.6	IV-1-31	3	nt	2	nt	nt	nt	nt
	IV-1-40	4	nt	4	nt	nt	nt	nt
	IV-1-37	4	4	4	1	0.5	1	0
	IV-1-85	2-4	3	3	0	0	0	0
	IV-1-77	4	4	3	0	1	0	0
6.7	IV-1-74	3-4	nt	3	1	1	0	0
	IV-1-75	3-4	nt	3	1	1	0	0
6.8	IV-1-53	3	3	3	0	0	0	0
	IV-1-49	3-4	4	2	**0.5**	**0**	**0**	**0**
8.1	IV-1-22	2-3	1	1	0	0	0	0
	III-1-74	4	3	nt	3	3	0	0
	III-1-78	4	nt	4	0	nt	nt	nt
	IV-1-145	2	nt	0	nt	nt	nt	nt
8.2	IV-1-39	4	4	nt	0	0	0	0
9.1	IV-1-88	3	3	nt	2	3	3	3
	IV-1-47	2-4	3	2	0.5	1	1	0.5
	IV-1-38	4	4	4	0	0	0	0
10.1	III-1-57	1	0	nt	0	0	0	0
12.1	III-1-38	2-4	4	nt	0	0	0	0
13.1	IV-1-21	3	3	nt	1	1	0	0
	IV-1-80	3	3	3	2	1	0	0
14.1	IV-1-63	4	nt	4	nt	nt	nt	nt
15.1	III-1-72	3	3	nt	1	2	2	1
	III-1-53	3-4	3	nt	0	0	1	0
	III-1-55	3-4	3	nt	1	1	2	1
	III-1-76	3-4	3	nt	3	3	3	3
16.1	IV-1-99	4	4	3	3	2	1	1
	III-1-117	3-4	3	nt	3	3	3	2

The scoring system is based on 0 (negative), 0,5 (weak) to 4 (strong positive) scale.

to the 37 epitope model and compared with a DAR expression pattern (table 3). The serological analysis shows a difference in epitope expression between the DAR and the DARE I reaction pattern on epitope 5.1 (MoAb III-1-116), 5.3 (MoAb IV-1-86), 5.5 (MoAb III-1-69), 6.4 (MoAbs IV-1-83 and IV-1-36), 6.5 (MoAb III-1-84) and 6.8 (MoAb IV-1-49) (table 3). In all cases the DAR sample shows a reaction strength of 1 (maximal) and the DARE samples show no reaction. Interestingly, the DARE sam-

ples gained epitope 4.1 (MoAb III-I-45) as compared with the DAR reaction pattern. The differences between the DARE I on one side and DARE II and III on the other side (table 3) are most probably due to a weaker expression of the RhD antigen of the DARE II and III samples which was also observed by standard serology.

Serological and RHD sequencing analysis of RHD(F223V)

A blood sample of an apparently RhD-positive patient (Rh phenotype CcDee) of Indian descent was referred to Sanquin Diagnostics (Amsterdam) because of an allo anti-D formation following an RhD-positive transfusion. In the patient's serum, the anti-D was confirmed besides the presence of other antibodies. No antibodies were detectable in the patient's eluate. To exclude the possibility of an auto anti-D, the patient's plasma and cells were incubated for 1h. at 37^0C and a new eluate was prepared. In this eluate no anti-D was detectable by PEG-IAT or direct agglutination with papain treated cells. Sequencing analysis of all *RHD* exons including the *RHD* promotor region showed only a 667T>G nucleotide change, leading to F223V. Amino acid 223 is located at the end of the seventh transmembrane domain (towards the fourth exofacial loop) of the RhD protein. Extensive serology was performed according to the 37 epitope RhD model of which the results are shown in table 3. The *RHD*(F223V) sample showed loss of RhD epitopes 1.3 and 8.1 (with MoAb IV-1-145) as compared with a R1r control sample. Unfortunately, the alloanti-D of the patient was too weak to prove the epitope loss on RBCs carrying the 667T>G nucleotide change.

Discussion

The present study describes the analysis of the *RHD*(T201R, F223V) or *weak D type 4* allele cluster.[9] As expected we found all aberrant alleles (except the *RHD*(T201R, F223V) allele and a *weak D type 4.0* allele) in non-Caucasian individuals and in association with the ancestral cDe haplotype. Surprisingly, the *RHD*(T201R, F223V) allele was identified in a Caucasian white individual and in a cDe haplotype. However, the familial ethnic background of this individual is unknown. The *RHD*(F223V) allele was identified in a individual from India in most probably a CDe haplotype. The familial ethnic background of this individual is also unknown. The *RHD*(F223V) allele was the unidentified allele on which the non-eurasian phylogeny is based.[6, 9] By the identification of this allele we now give the formal proof for the line of evolution of the non-eurasian abberant *RHD* alleles (figure 2).

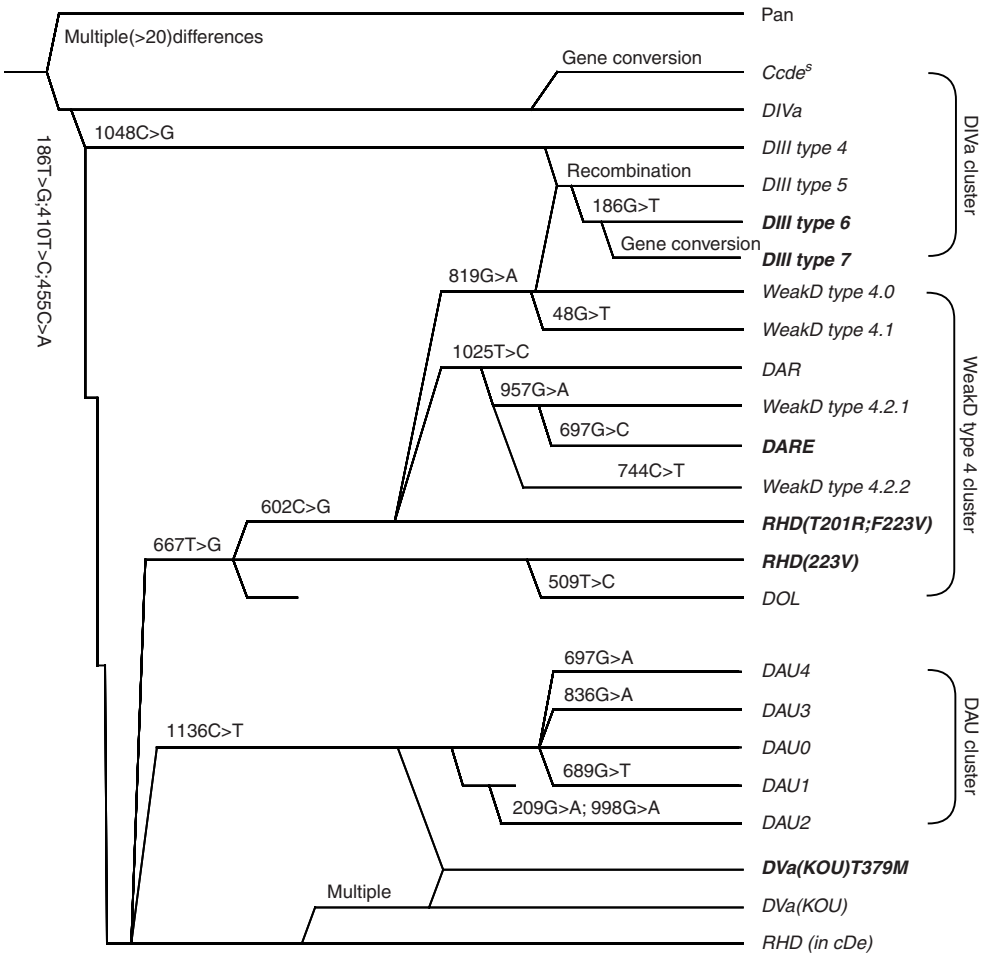

Figure 2. Refined phylogeny of *RHD* in humans
The extended phylogeny of *RHD* in humans is described by Wagner et al.[9] This figure shows the *DIVa*, *weak D type 4* and *DAU* clusters only. The *DIVa* cluster now includes the *DIII type 6* and *7* alleles. The *weak D type 4* cluster now includes the *DARE* allele. The *DVa(KOU)*T379M allele is now depicted as the phylogenetic link between the *DVa(KOU)* allele and the *DAU* allele cluster.

Within the non-eurasian allele cluster we identified 3 new *RHD* alleles (figure 2). The *DIII type 6* allele carries the *DIII type 5* characteristics 410C>T and 455C>A in exon 3, 602C>G in exon 4, 667T>G in exon 5 and 819G>A in exon 6 but lacks the 186G>T nucleotide change in exon 2 (figure 1). The *DIII type 6* allele has an estimated allele frequency of 0,031 in South-African blacks and 0,003 in South-African Asians (assuming an equal distribution of types 5 and 6 in the undetermined samples) and was not found in the other populations. Extended serology of the newly identified DIII type 6 showed many weaker reactions with the MoAbs as compared to the control sample but no evidence of epitope loss (table 3), as expected from the results of the previously identified *DIII type 4* allele.[23]

The *DIII type 7* allele is also very similar to the *DIII type 5* allele. The only difference is that the *DIII type 7* allele as found in this study has no *RHD* specific sequences in exon 2 and no *RHD* exon 2 could be amplified with intron primers specific for *RHD* and the *C* allele of *RHCE* (figure 1). The *DIII type 7* allele was only found once in a South-African black population of 310 donors. The absence of *RHD* exon 2 suggests the presence of *RHCE* exon 2 since this allele is most likely the result of a gene conversion in *cis* during hairpin formation. The exon conversion has slightly affected the standard RhD serology (a 2+ reaction strength with monoclonal anti-D and a 1+ reaction strength with polyclonal anti-D). The whole of loop 2 of the RhD expression model as proposed by Avent et al. consists of exon 2.[25] However, the sequences of *RHD* and the *C* allele of *RHCE* are identical in this exon. Loop 2 is proposed to play a role in the expression of epitope 8. DIIIb also lacks *RHD* exon 2 and lacks expression of the G antigen. Unfortunately it was not possible to perform extended serology of the DIII type 7.

In the black Ethiopian population we identified a new variant *RHD* allele, very similar to the *DAR* allele with the additional 697G>C nucleotide change, leading to E233Q on the fourth extracellular loop of the RhD protein (figure 1). The *DARE* allele has an estimated frequency of at least 0,0085 in Ethiopian blacks. The serological analysis shows a difference in epitope expression between the DAR and all three DARE reaction patterns on epitope 5.1 (MoAb III-1-116), 5.3 (MoAb IV-1-86), 5.5 (MoAb III-1-69), 6.4 (MoAbs IV-1-83 and IV-1-36), 6.5 (MoAb III-1-84) and 6.8 (MoAb IV-1-49) (table 3). These observations are in concordance with the model proposing six different RhD epitope clusters.[25] This model predicts that the expression of epitopes 5 and 6/7 is dependent on a combination of loops 3 and 4 which may explain why only 2 of 8 epitope 5 monoclonals and 4 of 32 epitope 6 monoclonals show a complete loss of reactivity. However, the DAR control sample showed a maximal reaction strength of 1+ on these epitopes. Interestingly, the DARE samples seem to have gained the expression of epitope 4.1 (MoAb III-1-45) as compared to DAR.

The *DAR* allele was identified in blacks from South-Africa, Ethiopia and Curacao with estimated allele frequencies of 0,024, 0,020 and 0,0025 respectively. The *weak D type 4.0* alleles were found in blacks from South-Africa (estimated allele frequency 0,005) and Ethiopia (estimated allele frequency 0,006) and the Asian population from South-Africa (estimated allele frequency 0,0016). *Weak D type 4.1* was only seen in Ethiopians (estimated allele frequency 0,006). Compared to the previously published *weak D type 4* allele frequency in whites (0,001) we can conclude that the *weak D type 4* allele is more frequent in black populations. The higher frequency of *weak D type 4* in black populations was expected since the highly related *DAR* allele is also found in black populations. Surprisingly, the *weak D type 4* allele has been found in white populations whereas the *DAR* allele has not been identified in white populations.

The primordial *DAU* type, *DAU0* is characterized by 1136C>T nucleotide change.

Based on this primordial *DAU* type, the *DAU* alleles *0* to *4* form a separate allele cluster. All enumerated *DAU* alleles occurred in a cDe haplotype and were predominantly found in Africans.[9] In this study, the 1136C>T change was found in two samples, one carried a *DIII type 6* and a *DVa(KOU)* allele (group IIE) and one carried a *weak D type 4.0* and a *DVa(KOU)* allele (group VI). Since both samples show an allele carrying the *DVa(KOU)* characteristics, we postulate that the 1136C>T nucleotide change is present on the DVa(KOU) allele and is dubbed *DVa(KOU)T379M* because this allele shows more molecular similarity to the *DVa(KOU)* allele than the *DAU0* allele. Both samples were drawn from phenotypic ccDee (genomic ccDDee), South-African black donors. The *DVa(KOU)T379M* allele probably represents a conversion between the *DAU0* and *DVa(KOU)* allele, which would create a phylogenetic link between the proposed fifth allele cluster of *DV* alleles and the *DAU* allele cluster (figure 3). The *DV* cluster is characterized by the *DVa-associated SM* allele or *RHD233E>Q*.

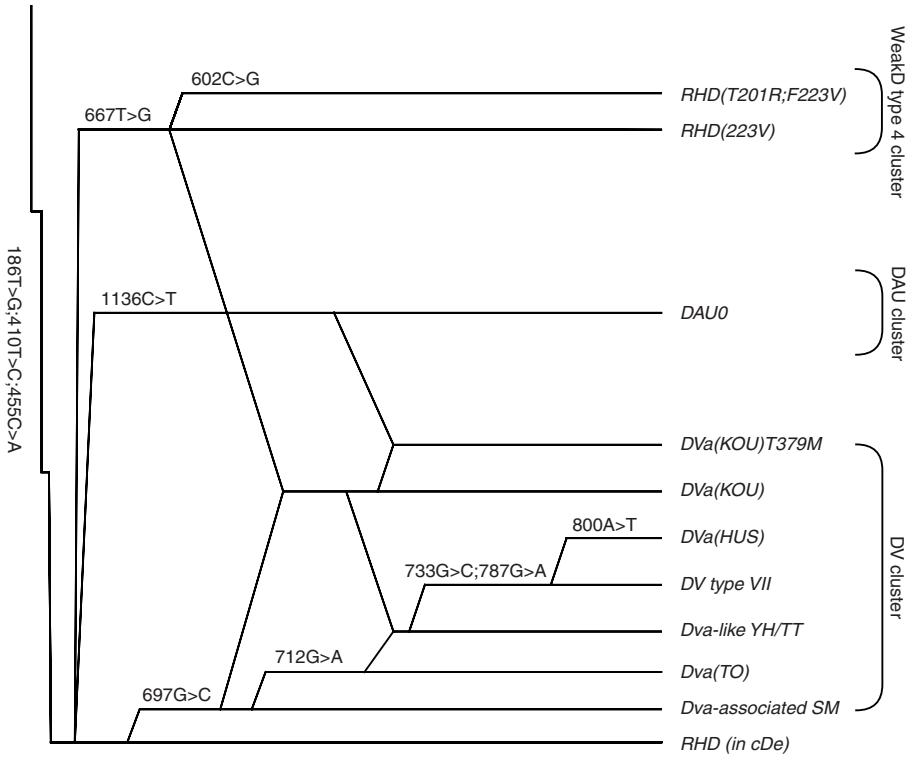

Figure 3. Proposed fifth allele cluster of *DV* alleles
This figure shows the extended phylogeny of *RHD* in humans concerning the *DV* alleles. The *DV* allele cluster is characterized by *RHD*(233E>Q) (nt 697G>C), relative to the eurasian *RHD* allele and shows interaction with both the *weak D type 4* cluster (by means of the *RHD*(F223V)allele) and the *DAU* cluster (by means of the *DAU0* allele).

Knowledge about the phylogeny of *RHD* in humans enables the prediction of potential clinically important RhD variants in any population. It may be postulated that interactions (e.g. by gene conversions) between the defined clusters will eventually change this classification into one (non-eurasian) cluster. New aberrant *RHD* alleles are identified, refining our understanding of the phylogeny of *RHD* in humans.

After the reviewing process of the present manuscript a manuscript was published by Chen and Flegel in which the presence of the 1136C>T nucleotide change in a *DVa(KOU)* characteristic allele was also demonstrated (Q. Chen and W.A. Flegel. Random survey for RHD alleles among D+ European persons. Transfusion 2005;45:1183-1191). Chen and Flegel however dubbed this allele *DAU5* and assigned this allele to the *DAU* cluster whereas in the present manuscript this allele is dubbed *DVa(KOU)T379M* and is assigned to the proposed *DV* cluster.

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Chapter 6

Fragmented cell-free fetal DNA in the maternal circulation may hamper prenatal genotyping strategies; evaluation of a prenatal *RHD* genotyping strategy on cell-free plasma DNA

Martine GHM Grootkerk-Tax, Aicha Ait Soussan, Masja de Haas, Petra A Maaskant-van Wijk, C Ellen van der Schoot. Fragmented cell-free fetal DNA in the maternal circulation may hamper prenatal genotyping strategies; evaluation of a prenatal *RHD* genotyping strategy on cell-free plasma DNA. *Submitted.*

Abstract

Background: The discovery of cell-free fetal DNA in maternal plasma led to the development of assays to predict the fetal D-status using RHD-specific sequences. Few assays are designed in such a way that the fetus can be typed in *RHDψ* (present in the majority of D-negative African blacks) mothers, and that *RHDψ* fetuses are correctly typed. Due to the limited knowledge about the mechanism responsible for the presence of fetal DNA in maternal plasma, precautions in developing prenatal genotyping strategies have to be made.

Study design and methods: Real-time PCR (RQ-PCR) assays were developed for prenatal diagnostic use with cell-free plasma DNA. An RQ-PCR assay on *RHD* exon-5 (amplicon 361 bp), negative on *RHDψ* was developed with gDNA and evaluated with cell-free plasma DNA. Furthermore, a previously published *RHD* exon-5 RQ-PCR was duplexed with an in-house developed *RHD* exon-7 RQ-PCR and evaluated with cell-free plasma DNA samples from pregnant D-negative *RHDψ*-positive women.

Results: The in-house, with gDNA developed *RHD* exon-5 assay showed on cell-free plasma DNA samples from D-negative women pregnant with a D-positive fetus, too low amplification levels, resulting in high Ct values and false negative results. Apparently, due to fragmentation of cell-free plasma DNA, too few *RHD*exon-5 stretches of sufficient length (>360 bp) are present. The *RHD* exon-5 and -7 RQ-PCR duplex was evaluated with *RHDψ*-positive cell-free plasma DNA samples and showed complete specificity and maximal sensitivity.

Conclusion: Assays designed for prenatal genotyping should be developed and evaluated on cell-free plasma DNA. Prenatal *RHD*-genotyping is accurate with the *RHD* exon-5 and -7 duplex strategy.

Introduction

Once a clinically relevant antibody with the potential to cause hemolytic disease of the fetus and/or newborn (HDFN) is found in the plasma of pregnant women (such as anti-D) it becomes important to determine the phenotype of the fetus, especially when laboratory parameters such as the antibody titer or tests on the biological activity are indicative of significant fetal hemolysis. The elucidation of the molecular basis of various blood group systems has allowed the development of genotyping methods, mostly by PCR, for fetal blood group phenotype prediction. Molecular assays can be performed on fetal DNA obtained by invasive means, such as amniocentesis or chorionic villus sampling. These procedures however are associated with a pregnancy loss rate of about 0.3%.[1] The procedures themselves may in fact further sensitize the mother against fetal red cell antigens.[2] More recently, it has become clear that invasive sampling can be avoided by the use of fetal cells or cell-free fetal DNA present in the maternal circulation, leading to a virtually risk-free means of prenatal blood group testing in women negative for the target antigen.[3,4] Fetal DNA can already be detected at the 5th week of gestation.[4] The fetal DNA concentration increases with gestational age with a sharp increase during the last 8 weeks of pregnancy.[4] On average, 25 fetal genome equivalents/ml maternal plasma can be detected in the first trimester, increasing to 100 genome equivalents in the third trimester.[5] Furthermore, because of the rapid clearing of DNA, fetal DNA is not detectable in plasma of women outside pregnancy.[6,7,8] Because of the high number of maternal DNA copies compared to the low number of fetal DNA copies (fetal DNA makes up an average of 3,4% and 6,2% of total DNA in maternal circulation in early and late gestation, respectively),[4] a genotyping strategy using fetal cell-free plasma DNA should be more specific (amplification of the desired DNA sequence only) and sensitive (able to detect a very low DNA copy number) than a strategy developed for use on gDNA.

The fetal D-status can be predicted by *RHD* genotyping on cell-free fetal DNA obtained from maternal plasma.[9,10,11] To accurately predict the fetal D-status it is important to distinguish functional *RHD* from non-functional *RHD* sequences. A deletion of the *RHD* gene is the most common form of D-negativity in Caucasian populations. However, the majority of D-negative black persons carry either an *RHDψ* (allele frequency 0.0714)[12] or an *(C)cde*^{s} allele (allele frequency 0.036).[13,14] In both alleles, *RHD*-specific sequences are present but due to the presence of a translation termination codon (*RHDψ*) or the substitution of *RHD*-specific coding sequences by *RHCE*-specific coding sequences (*(C)cde*^{s}), no detectable D epitopes are present on the RBC surface. Finning et al. developed a fetal *RHD* genotyping strategy that detects only *RHD* (and not *RHDψ* or *(C)cde*^{s}) using fetal DNA in maternal plasma.[15] With this strategy, *RHD* exon 4, 5 or 6 is amplified in Real-Time and compared with the control amplicon of *RHD* exon 10. When either an *RHDψ* or an *(C)cde*^{s} allele is present, exon 4, 5 or 6 will not be amplified whereas exon 10 will

be amplified.
In this paper, we demonstrate the importance of specificity and sensitivity for genotyping strategies using fetal cell-free plasma DNA and we describe the evaluation of a duplex combination of an in-house developed assay with a previously published method[15] on (the clinically relevant) *RHDψ*-positive cell-free plasma DNA samples.

Material and methods

Control samples

Red blood cells (RBCs) were Rh phenotyped according to standard serological protocols with a monoclonal IgM antibody anti-D (GAMA401, RhD epitope 6/7), a polyclonal IgG anti-D antibody and monoclonal IgM antibodies (from Gamma Biologicals INC., Houston, TX, USA) recognizing C (MS24), c (MS33), E (GAMA402) and e (MS16, MS21, MS63 (blend)).
Donor blood samples were obtained with informed consent, according to the rules of Sanquin. Genomic DNA was isolated from EDTA-anticoagulated blood samples from black blood bank donors from Curacao (*RHDψ* controls) and from Caucasian blood bank donors from The Netherlands according to a standard protocol.[16]

Maternal and fetal blood samples

EDTA-anticoagulated blood samples of D-negative pregnant women (around 28-30 weeks of gestation) were sent to Sanquin Diagnostics for routine D antibody screening. D blood group typing was performed either on a WADIANA system (Diamed, Cressier sur Morat, Switzerland) or Autovue system (Ortho Clinical Diagnostics, Tilburg, The Netherlands), using the appropriate manufacturer's card systems. The D status of the fetuses were confirmed by standard serology protocols.
All blood samples were centrifuged 1200 g for 10 minutes. The plasma was centrifuged again at 2400 g for 20 min, and the supernatant was collected in fresh tubes and stored at -20°C until it was used for DNA isolation. 200 ul of buffy coat was removed and DNA was isolated using MagNA Pure LC (Roche Biochemicals, Basel, Switerland) with the MagNA Pure LC DNA Isolation Kit 1 according to the "DNA I Blood_Cells Fast" protocol (Roche Biochemicals). DNA was eluted in 100 µl H_2O.

Fetal cell-free DNA extraction from maternal plasma.

Fetal cell-free DNA was extracted from 1 ml maternal plasma using the MagNA Pure LC (Roche Biochemicals) with the "Total Nucleic Acid Large Volume" reagent set (Roche Biochemicals) according to manufacturer's instructions. For a control on the fetal DNA-isolation, in each run a plasma pool of D-negative women, pregnant of a D-positive fetus was included. The isolated DNA was eluted in 55ul. Within 8 hrs after isolation the DNA was automatically dispersed into a 96 wells plate by the MagNA Pure LC for real-time quantitative (RQ)-PCR analysis. Samples 5539797

and 5539929 were obtained from a large cohort of D-negative pregnant women tested for the presence of fetal RHD sequences, and were initially recognized because of the extremely high amount of *RHD* exon-7 sequences.

PCR assays

The *RHD* status of the control samples and samples 5539797 and 5539929 was determined by serology and by *RHD* multiplex (MPX) PCR analysis as described before.[17]

The presence of an *RHDψ* was confirmed by PCR-SSP with forward primer RexWs, specific on nt 654C (*RHDψ*) and reverse primer Rex5Was, specific on nt 674T (*RHDψ*) and an internal control on the *ß-actin* gene as previously published.[18]

RQ-PCR assays

All primers used are listed in table 1. Oligonucleotides were purchased from Invitrogen (Carlsbad, CA) and fluorescent probes were purchased from Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands). All RQ-PCRs were developed based on the Taqman technology by using a 7000 Sequence detector (Applied Biosystems).

Table 1. Name, type (primer or probe), nucleotide sequence, location and specificity of all oligonucleotides used in this study.

Name/type	Sequence	Location	Specificity
Rex5Ws/primer	CGCCCTCTTCTTGTGGATC	exon 5	*RHDψ*
Rex5Was/primer	TGGACTTCTCAGCAGAGCAA	exon5	*RHDψ*
FDTin4-A/primer	gtgtctgaagcccttccatc	intron 4	*RH*
RDTex5-Aprimer	gagttgaaacttggcctgaac	exon5	*RHD+RHCE*
RHDin4/VIC labelled probe	agcacttcacagagcag	intron 4	*RHD/ RHDψ*
EX5F/primer	CGCCCTCTTCTTGTGGATG	exon 5	*RHD/RHCE*
EX5R/primer	GAACACGGCATTCTTCCTTTC	exon 5	*RHD/ RHDψ*
EX5P/VIC labelled probe	TCTGGCCAAGTTTCAACTCTGCTCGCT	exon 5	*RHD*
940S/primer	GGGTGTTGTAACCGAGTGCTG	exon 7	*RHD*
1064R/primer	CCGGCTCCGACGGTATC	exon 7	*RHD*
968/FAM labelled probe	cccacagctccatcatgggctacaa	exon 7	*RHD*

All primers have been previously published, see Materials and Methods section for the appropriate references.

For the RQ-PCR on *RHD* exon 5-361 bp the consensus forward primer (FDTin4-A) is located in intron 4 before the 650 bp insertion present in *RHCE*. The reverse primer (RDTex5-A) is specific on nt 654 G (negative on *RHDψ* due to nt 654 G>C in *RHDψ*) and therefore only hybridizes with *RHD* and *RHCE* specific DNA. With these

primers, only *RHD* will be amplified since the *RHCE* product is too large to be amplified (*RHD* product is 361 bp whereas the *RHCE* product would be 1011 bp). The FAM fluorescent labeled probe (RHDin4) is located in intron 4, covering the junction point of the *RHCE* 650 bp insertion and will therefore only hybridize with *RHD* specific DNA. Variable concentrations of DNA were analyzed in a mixture of 25 ul of Universal Mastermix (Applied Biosystems), 300nM of each primer and 100nM probe. PCR was started with 2 minutes at 50^0C and 10 minutes at 95^0C followed by 50 cycles of denaturation for 15 seconds at 95^0C, primer annealing 1 minute at 58^0C and primer extension 1 minute at 72^0C. All samples were tested in triplicate. To develop the assay, samples from donors with the following phenotypes were used: R_1R_1 (n=2), R_2R_2 (n=1), R1r (n=2) and rr (n=2); all Caucasian, and 4 rr samples positive for the *RHD*ψ (all blacks from Curacao) by PCR-SSP.
The *RHD* exon-5 RQ PCR from Finning et al. is also based on detection of *RHD* and not *RHD*ψ exon 5 but is generating an 82-bp product (primers EX5F and EX5R and a VIC-labeled, probe EX5P).[15] EX5F amplifies *RHD* and *RHCE* but not *RHD*ψ, EX5R amplifies *RHD* and *RHD*ψ but not *RHCE* (the PCR amplicon is *RHD*) and EX5P hybridizes with *RHD* only. This PCR was combined with a previously developed RQ-PCR for *RHD* exon-7 (primers 940S and 1064R and a FAM-labeled probe 968 (generating a 122 bp product),[9] into an *RHD* exon-5 and -7 duplex PCR. If cell-free plasma DNA was analysed the RHD exon-5 and -7 duplex PCR was used, but if genomic DNA was tested the assays were not combined. For both assays the reaction mixtures contained 25 ul Universal Mastermix, 300nM of each primer and 100nM probe. PCR was started with 2 minutes at 50^0C and 10 minutes at 95^0C followed by 50 cycles of denaturation for 15 seconds at 95^0C and primer annealing and elongation for 1 minute at 60^0C. All RQ-PCR data were compared to an *RHD* homozygous standard.[18]

Results

RHD exon 5-361 bp RQ-PCR

The *RHD* exon 5-361 bp RQ-PCR was developed using genomic DNA (gDNA) with known Rh phenotypes and *RHD* genotypes as described in the Materials and Methods section. gDNA of an *RHD*ψ gene positive sample and heterozygous *RHD* gene positive sample were tested both in concentrations ranging from 0 to 10000 pg. The RQ-PCR was completely specific as no signal of the *RHD*ψ sample was detected. Furthermore, the RQ-PCR was sensitive enough to detect gDNA derived from one heterozygous *RHD*-positive cell (median Ct value of 39.43). Serial dilutions of the *RHD* heterozygous sample in a constant concentration of 1000 pg of *RHD*ψ gene positive gDNA or in H_2O demonstrated identical results.
The sensitivity and specificity for fetal DNA of the *RHD* exon 5-361 bp RQ-PCR was tested with 45 plasma samples from D-negative pregnant women. These samples

were also tested with the RQ-PCR on *RHD* exon 7, that has previously been shown to have maximal sensitivity for detection of fetal *RHD* sequences.[6] In 26 of the 45 cases the *RHD* exon-7 RQ-PCR showed Ct values in the expected range for the presence of fetal *RHD* (all triplicates positive, all samples Ct values between 33 and 37, figure 1A). The D-positivity of these 26 fetuses was confimed by cord blood serology. The remaining samples were all *RHD*-negative (data not shown). The *RHD* exon 5-361 bp RQ-PCR however, showed a much lower sensitivity (Ct values of 40 and higher), and was negative in 11 of the 26 exon 7 positive plasma samples (figure 1B). Although the *RHD* exon 5-361 bp RQ-PCR showed to be a specific and sensitive assay when used on gDNA, this assay, when performed on cell-free fetal DNA derived from maternal plasma, it is not applicable because of a far too low sensitivity.

RHD genotyping by RQ-PCR on RHD exon-5 and -7 in duplex on cell-free fetal DNA derived from maternal plasma.

The RQ-PCR on *RHD* exon 5, as developed by Finning et al.[15] was tested with cell-free fetal DNA derived from maternal plasma. A total cell-free plasma DNA (isolated from a heterozygous D-positive donor) input of 100 pg generates Ct values between 33 and 36 for all triplicates and 10 pg cell-free plasma DNA input generates a Ct value of about 36 in one of the triplicates. The specificity of the assay was tested on gDNA of an *RHD*ψ sample in serial dilutions ranging from 0 to 100 ng and showed no amplification of the *RHD*ψ DNA. The RQ-PCR on *RHD* exon-5 and the RQ-PCR on *RHD* exon-7 assays were duplexed by using a different fluorochrome label for each probe (a VIC labeled exon-5 probe and a FAM labeled exon-7 probe) and subsequently tested on cell-free fetal DNA from maternal plasma. RQ-PCR results of 55 duplexed cell-free plasma DNA samples are shown in figure 1C (*RHD exon-7*) and figure 1D (*RHD exon-5*). In 35 of the 55 cases both *RHD* exon-7 and *RHD* exon-5 specific products were amplified, indicating D-positivity. Of the remaining 20 samples, neither *RHD* exon-5 nor *RHD* exon-7 amplicons were obtained, indicating D-negativity. The median Ct values of the *RHD* exon-7 RQ-PCR performed as single assay (median Ct 36.4) and in duplex with *RHD* exon-5 (median Ct 36.0) show that both approaches are (equally) sensitive.

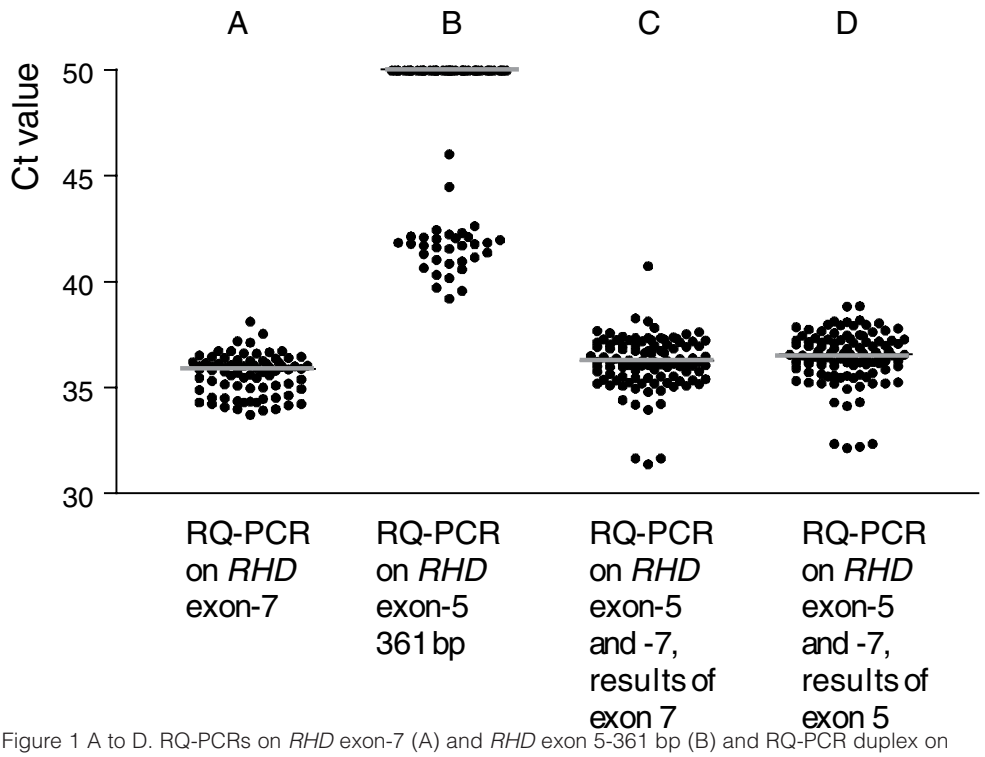

Figure 1 A to D. RQ-PCRs on *RHD* exon-7 (A) and *RHD* exon 5-361 bp (B) and RQ-PCR duplex on *RHD* exon-5 (D) and-7 (C) on cell-free fetal DNA derived from maternal plasma
The horizontal lines depict the median Ct value of each RQ-PCR assay.
Figures 1A and 1B show the Ct values from fetal cell-free plasma DNA of 26 (*RHD* exon-7) or 11 (*RHD* exon-5) D-positive fetusses of 45 D-negative pregnant women, tested in triplicate. Figure 1A shows the Ct values from RQ-PCR on *RHD* exon-7, performed on the same samples as used for 1B. Figure 1B clearly shows the low sensitivity by high Ct values obtained with the RQ-PCR on *RHD* exon 5-361 bp.
Figures 1C and 1D show the Ct values of *RHD* exon-5 (D) and-7 (C) tested in duplex from fetal cell-free plasma DNA of 35 D-positive fetusses of 55 D-negative pregnant women, tested in triplicate. Figure 1C shows the Ct values obtained with the *RHD* exon -7 when performed in duplex with *RHD* exon-5. Figure 1D shows that the Ct values of the RQ-PCR on *RHD* exon-5 (as developed by Finning et al.)[15] are within a reliable area and is much more sensitive than 1B.

The diagnostic value of the *RHD* exon-5 and -7 duplex genotyping method was evaluated with two special samples. Sample no. 5539797 represents a D-negative *RHDψ*-positive pregnant woman, carrying a D-negative fetus. Sample no. 5539929 represents a D-negative *RHDψ*-positive pregnant woman carrying a D-positive fetus. Both samples were subjected to the RQ-PCR duplex on *RHD* exon-5 and -7. Of both maternal samples, gDNA was isolated from WBCs and subjected to the RQ-PCR on *RHD* exon 5-361 bp, the RQ-PCR on *RHD* exon-7 and the *RHD* Multiplex (MPX) PCR. In this PCR assay, exons 3, 4, 5, 6, 7 and 9 are amplified with *RHD* specific primers in one reaction mixture. In case of an *RHDψ*, *RHD* exon 5 will not be amplified by the *RHD* MPX PCR. The amplification plots of samples 5539797 and 5539929 by RQ-PCR duplex on *RHD* exon-5 and -7 are shown in figures 2 and 3, respectively.

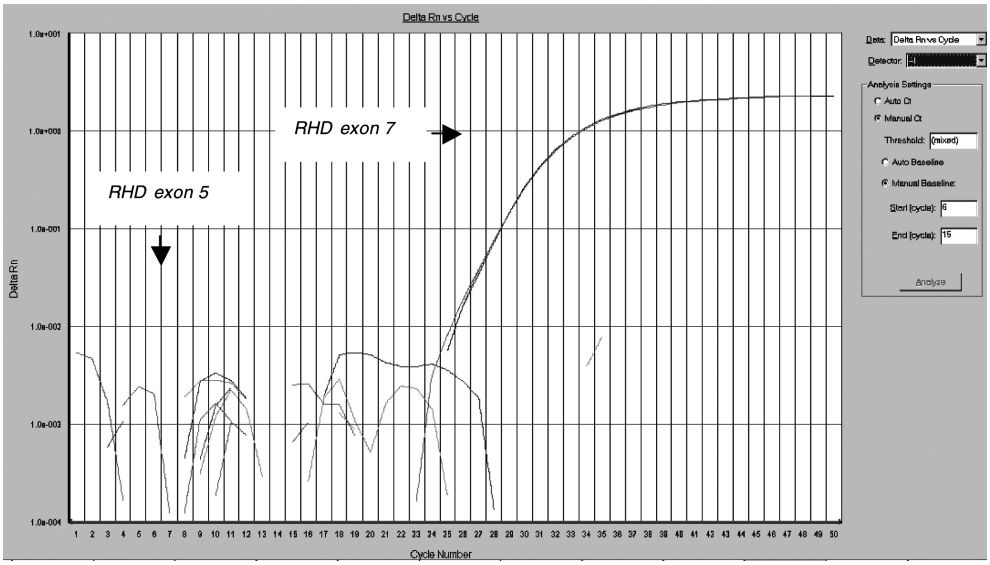

Fig. 2. RQ-PCR MPX on *RHD* exon-5 and -7 amplification plot of sample no. 5539797
The amplification plot shows the real time amplification of *RHD* exon-7 (Ct values of 28.33, 28.36 and 28.36, threshold set at 0.10), the exon-5 fluorescent signal does not cross the threshold (Ct values of 50).

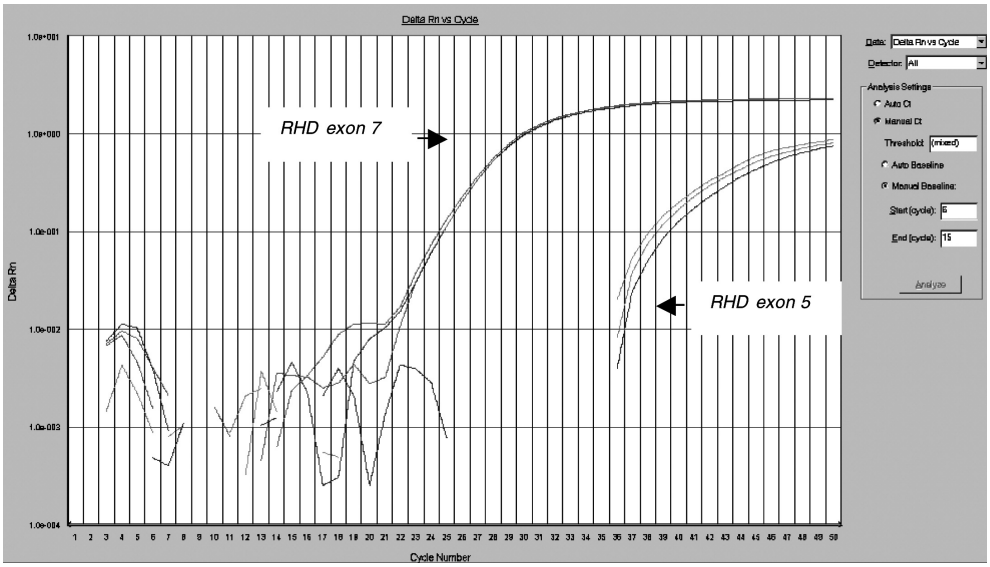

Figure 3. RQ-PCR MPX on *RHD* exon-5 and -7 amplification plot of sample no. 5539929
The amplification plot shows the real time amplification of *RHD* exon-7 (Ct values of 24.45, 24.75 and 24.74, threshold set at 0.10) and *RHD* exon-5 (Ct values of 36.44, 37.41 and 36.89, threshold set at 0.03).

The amplification plot of sample 5539797 shows amplification of *RHD* exon 7 and not of *RHD* exon 5, indicating the presence of an *RHDψ*. Furthermore, the low Ct

value (28.35, corresponding with about 54ng DNA/ml plasma) of the exon 7 RQ-PCR suggests that maternal DNA is amplified, hence, the D-negative mother is carrying an *RHDψ*. Indeed, the maternal origin of the *RHDψ* is confirmed by analysis of maternal leukocyte-derived gDNA, again showing negativity of the RQ-PCR on RHD exon 5-361 bp assay (Ct value of 50) and positivity of the RQ-PCR on *RHD* exon-7 assay (Ct value of 23.71). The *RHD* MPX PCR, amplifying exons 3, 4, 5, 6, 7 and 9, shows only the absence of the *RHD* exon 5 amplicon, which is also indicative for the presence of an *RHDψ*. The cord blood serology was indeed D-negative.
The amplification plot of sample 5539929 shows early amplification of *RHD* exon-7 (Ct 24.64 corresponding with about 570ng DNA/ml) and late amplifcation of *RHD* exon-5 (Ct 36.91). These results point to the presence of a maternal *RHDψ* gene (*RHD* exon-5 negative and exon-7 positive) and an *RHD*-positive fetus (*RHD* exon-5 and -7 positive). Again, the maternal origin of the *RHDψ* was shown upon analysis of maternal gDNA with the *RHD* exon 5 and 7 RQ PCRs and the *RHD* MPX PCR. After delivery, cord blood serology showed that the fetus was indeed D-positive.
The results indicate that the *RHD* exon-5 and-7 duplex can be used for fetal *RHD* typing in the presence of a maternal *RHDψ* gene on fetal cell-free DNA derived from maternal plasma in a diagnostic setting.

Discussion

Despite the rapid introduction of diagnostic assays that use fetal DNA isolated from maternal plasma in clinical settings, the molecular characteristics of cell-free plasma DNA in pregnant women are not completely known. In the present study we found that a newly developed RQ-PCR on *RHD* exon 5, generating a 361 bp product, showed high specificity and sensitivity on genomic DNA, but not on cell-free fetal DNA isolated from maternal plasma (figure 1). The same cell-free plasma DNA samples showed normal Ct values in the RQ-PCR on *RHD* exon 7. Recently, Chan et al. analyzed plasma DNA samples from 21 pregnant women, who were carrying male fetuses, for the size distribution of DNA fragments encoding the *SRY* gene.[19] They reported that the median relative concentration of the *SRY* gene determined with primers producing amplicons longer than 313 bps was <1%. They also suggest that most of the circulating DNA molecules are in the range of 145-201 bp. Several studies have demonstrated a correlation between plasma DNA concentrations and apoptosis of tumor cells in cancer patients,[20,21] and several studies have also shown that the lengths of plasma DNA molecules in cancer patients are very short and are in multiples of nucleosomal DNA.[20,22] Whether the DNA fragmentation is random is not known. The presence of only small sized fetal DNA in maternal plasma caused the RQ-PCR on *RHD* exon 5-361 bp to fail when performed on cell-free fetal DNA isolated from maternal plasma. The RQ-PCR's on *RHD* exon 5 developed by Finning et al., and the in-house developed *RHD* exon 7 RQ PCR have amplicons of 82 bps and 122 bps, respectively. However, not only the size of the DNA fragments may be important, because if the cell-free fetal DNA is not randomly fragmented, it is possible that the digestion site is located within the chosen stretch of DNA for amplification and the PCR will fail or will be less sensitive independent of the amplicon size.

This study also presents an RQ-PCR duplex on *RHD* exon-5 (as developed by Finning et al)[15] and -7 for prenatal genotyping on cell-free plasma DNA and has been evaluated with two samples carrying a maternal *RHDψ* gene. In the original publication of Finning et al. no maternal *RHDψ* positive samples were tested for the presence of D-negative or D-positive fetal cell-free plasma DNA. However, during routine screening, one fetal *RHDψ* gene was detected in an *RHD*-negative woman.[15] The present study however, shows the importance of including maternal *RHDψ* samples in the evaluation phase of assays developed for diagnostic purposes. In The Netherlands, the frequency of serologically D-negative pregnant women carrying an *RHDψ* gene is 0.25% (unpublished results). Therefore, these samples will be encountered if the fetal *RHD* status will be determined routinely to direct the gift of antenatal anti-D prophylaxis.[23] The combination of *RHD* exon-5 and -7 will prevent amplification of *RHD* specific sequences (false positive results) when an *(C)cde*^{s} allele is present (*RHD*1-3, *CE*3-7, D hybrid allele without D-antigen expression). More importantly, the accuracy of D-negative typing by the absence of both ampli-

cons (neither exon 5 nor exon 7) of this strategy has been reported to be 100% [15], and was confirmed by this study. However, some aberrant *RHD* alleles may give results similar to *RHDψ* by RQ-PCR on *RHD* exon-5 and -7 duplex (e.g. *Dva*) and therefore complicate interpretation of the *RHD* genotyping assay. Therefore, when a fetal sample negative for *RHD* exon 5 and positive for RHD exon 7 is identified, further (molecular) analysis of the fetus or the father may be desired. The *DIVa* variant will cause an amplicon of exon 5 only, and those fetuses should be regarded as D-positive. Also D-positive haplotypes have been described to be present in a D-negative European population which may hamper genotyping with this assay due to the possible presence of such an allele in the mother, and might lead to false positive results.[24] However, all other known D-variant haplotypes will be (correctly) recognized as D-positive by the described strategy.

Many fundamental questions remain concerning the characteristics of the circulating DNA in maternal plasma and the release and elimination of plasma DNA. The present study shows that it is very important to develop and evaluate a diagnostic assay on the relevant cell-free fetal DNA, especially since it is not known whether *RHD* is randomly fragmented.

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Chapter 7

Discussion

Rh is the most complex blood group system. The Rh blood group system comprises 46 antigens. Of the RhD antigen alone, more than 50 partial RhD and 40 weak D types have been described, all caused by molecular alteration(s). The *RHD* and *RHCE* genes, which encode for the RhD and RhCE proteins respectively, are evidently prone to molecular changes. Especially the high homology between the two genes and the proposed hairpin formation during RNA synthesis cause a great number of aberrant *RHD* alleles carrying *RHCE* nucleotides or *RHCE* exon(s). Other causes of molecular alterations that give rise to an altered RhD protein include missense mutations, nonsense mutations and nucleotide deletions causing a frameshift and premature stopcodon.[1]

Most aberrant *RHD* alleles encode an abnormal (or partial) RhD protein. Carriers of such partial RhD may form alloantibodies against epitopes of the normal RhD antigen that they lack. Many of the partial RhD phenotypes react weakly with anti-D reagents. Some aberrant *RHD* alleles may encode an RhD protein with only a reduced expression of RhD on the red cell surface (the so-called *weak D* alleles or, when RhD is only detectable by adsorption elution techniques, *Del* alleles). Weak D phenotypes are caused by amino acid substitutions in the intracellular (cytoplasmic) or transmembrane-spanning domains of the protein and do not produce alloanti-D. Weak D phenotypes are treated as regular RhD-positive phenotypes in transfusion medicine.

The frequency of partial RhD depends on the population. Most aberrant *RHD* alleles caused by multiple missense mutations are more frequently found in (African) black populations than in Eurasian populations, probably because the alleles that evolved in blacks have a longer phylogenetic distance from standard RhD (introduction, figure 4 and chapter 5).

Ethnic variability of RhD-negativity

The RhD-negative phenotype is found in 15% of the Caucasian population, whereas only about 5% of the black population is RhD-negative.[2] In the Asian population, RhD-negativity is even more rare (about 0,5%(including Del)).[3] The frequency of the *RHD* deletion (RhD-negative haplotype) in a white English population is about 0.39.[2] The *RHD* gene deletion is caused by an unequal crossing over event, triggered by the high homology of the upstream and downstream Rhesus boxes that flank the *RHD* gene, resulting in an *RH* locus without an *RHD* gene but with a newly created hybrid *Rhesus box*.[4]

Homozygosity for the *RHD* gene deletion is the main cause of the RhD-negative phenotype in Caucasians. In non-Caucasian populations, RhD-negativity is associated with a grossly intact *RHD* gene. In RhD-negative African blacks, both the *(C)cde*^{s} (or *r's*) allele and *RHD*ψ allele are frequently present.[5,6] In RhD-negative Asians, the *Del* allele is often present.[7,8] Due to the extreme low RhD-antigen expression, which can only be seen by adsorption elution techniques, these pheno-

types are often classified as RhD-negative. Because of the presence of RhD-negative *RHD* genes in non-Caucasian RhD-negative individuals, the true *RHD* gene-negative haplotype has a low frequency in non-Caucasians.
Sequencing analysis of the hybrid *Rhesus box* of the *RH* locus in RhD-negative blacks, Asians and Caucasians showed that the hybrid *Rhesus box* is present in all populations and that only in blacks, a second hybrid *Rhesus box* is present (chapter 2). The presence of an identical hybrid *Rhesus box* indicates that the mechanism causing the *RHD* gene deletion is most probably the same for all ethnic groups studied.

Ethnic variability of partial RhD, weak D and Del

The most frequently occurring aberrant *RH* alleles have been fully explored in black and Caucasian populations. Studies exploring the *RH* alleles of Asian populations and the development of matching *RH* diagnostic strategies have been published to a lesser extent.[9,10] Between 82% and 88% of Europeans and around 95% of black Africans are RhD-positive. Among Europeans, the frequency of all (known) partial RhD combined is less than 1%.[11,12] In Africans, the occurrence of aberrant *RHD* alleles and anti-D immunizations in RhD-positive individuals is much higher. For example, 11% of anti-D in pregnancies in the Cape Town area (South-Africa) occurred in RhD-positive women.[13] Appendix I shows the molecular and ethnic characteristics of partial RhDs. Although the number of different aberrant *RHD* alleles is higher in Caucasians than in blacks, the population frequencies (or the number of probands) of the aberrant alleles are considerably higher in blacks. Only a few aberrant *RHD* alleles as found in blacks are also identified in Caucasians (like *weak D type 4* and *DAU 0*) (chapter 5). It is currently unknown whether these alleles were present during the primary bottleneck (the migration out of Africa) or entered the Caucasian allele pool by secondary migrations. Many of the Caucasian aberrant alleles have only been identified in single individuals. The current knowledge of aberrant *RHD* alleles in non-Caucasian populations may be distorted by the limited amount of research in this area. Extensive knowledge of the aberrant *RHD* alleles enables the reconstruction of the evolutionary pathway of *RHD* by means of *RH* phylogeny. Knowledge about the *RH* phylogenetics in humans is of practical importance because it defines the framework for determining clinically relevant *RH* alleles in any population.[14]
Weak D red cells are considered to have all epitopes of RhD expressed weakly, are not able to produce anti-D and are caused by amino acid substitutions in the intracellular (cytoplasmic) or membrane-spanning domains of RhD. Currently, 41 different *weak D* alleles have been identified.[15] However, some individuals classified as weak D have developed anti-D and are therefore now considered partial RhD (as recently described for weak D types 4.2, 15 and 26).[16,17] Interestingly, we recently found that the weak D phenotypes 1, 2 and 3, most frequently occurring in

Caucasians, are not present in blacks from Ethiopia, Curacao and South-Africa or in Asians from South-Africa.[18] The absence of weak D in these groups implies that the *weak D* allele is, from an evolutionary point of view, a relatively young type of allele, which has its origin in Caucasians and has not spread out to other populations yet. In contrast, the *weak D type 4* allele was identified more frequently in blacks than in Caucasians and Asians.[18] The *weak D type 4* allele shares two nucleotide substitutions with the partial RhDs *DAR* and *DOL* (602C>G, 667T>G) and together these alleles form the phylogenetical African *weak D type 4* cluster. While analyzing this *weak D type 4* cluster we identified an *RHD* allele with the 667T>G only in a patient who made an anti-D after a RhD-positive transfusion (chapter 5). Because of the molecular similarities and serological consequences, it is debatable whether the weak D type 4 alleles should be classified as weak D.

RhD is a high incidence antigen in the Far-East, even reaching 100% in some populations. RhD-positivity has been reported to reach 99,7% in Hong-Kong Chinese and Japanese populations.[3] However, a substantial proportion of those classified as RhD-negative express the Del phenotype. The molecular background for the Del phenotype is variable (appendix III). Del phenotypes were initially proposed to be non-immunogenic because the amount of RhD was supposed to be too low to trigger an immune response. However, recently a case was described wherein anti-D was produced by an RhD-negative patient after transfusion with red cells carrying the Del phenotype, strongly suggesting that the Del phenotype is immunogenic.[19] This finding has great implications for transfusion medicine in those geographical regions where Del is a frequently occurring phenotype, like most Asian countries.[20] The recent identification of anti-D formation by weak D indicates that the original classification of aberrant Rh proteins in “weak D” and “partial RhD” based on the location of the amino acid substitution(s) and the presence of anti-D (at the time of molecular identification) is not very accurate anymore. Maybe all RhD phenotypes encoded by aberrant *RHD* alleles should rather be initially regarded as having the potential to generate anti-D antibodies (partial D) unless proven otherwise.

Ethnic variability of RhCcEe

The RhC and Rhc antigens have Caucasian allele frequencies of 0.44 and 0.56, respectively. In blacks, the frequency of Rhc is much higher and the frequency of RhC is lower, whereas in Asians RhC and Rhc expression approaches an allele frequency of 0.75 and 0.25, respectively (table 1).[2]

Table 1. Haplotype distributions (in % occurrence) in Caucasian, black and Asian populations.

Haplotype	Caucasians	Blacks	Asians
CDe (R_1)	42	17	70
Cde (r')	2	2	2
cDE (R_2)	14	11	21
cdE (r")	1	0	0
cDe (R_0)	4	44	3
cde (r)	37	26	3
CDE (R_z)	0	0	1
CdE (r^y)	0	0	0

RHC differs from *RHc* at only 6 nucleotide positions, 4 of which lead to an actual amino acid substitution. This means that variant RhC and Rhc proteins are rare and aberrant *RHC* and *RHc* alleles are also less frequent. *RHC* differs from *RHD* at only 1 coding nucleotide position (nt 48).

The most frequently occurring aberrant *RHc* allele is caused by a single nucleotide substitution (48G>C), hampering *RHC* genotyping.[21] The *RHce* 48G>C allele is highly frequent in black populations (identified in 67%, 48% and 42% of RhC-positive blacks from South-Africa, Ethiopia and Curacao, respectively) but does not affect the expression of Rhc (chapter 3). Most deviant RhC and Rhc antigens are caused by nucleotide substitutions in the *RHD* allele rather than in the *RHCE* alleles (e.g. D^c and *r's*).[5,22] The very rare D-- phenotype, in which the red cells express RhD but not RhC, Rhc, RhE or Rhe, has a heterogeneous molecular genetic background but is mostly seen in combination with *RHD-CE-D* hybrid alleles.[23]

Serological identification of partial RhD and weak D and clinical aspects

Partial RhDs usually lack the expression of one or more RhD epitopes. Therefore, human monoclonal antibodies can be used for the classification of partial RhD in terms of expressed epitopes. The current model consists of 37 different epitopes.[24] Partial RhD phenotypes differ in their ability to form alloanti-D against the lacking epitopes. Many partial RhDs give weak reactions with anti-D reagents and can only be classified by 37 epitope model analysis. The application of this model however, is dependent on the availability of monoclonal antibodies and the analysis is very complicated and laborious.

In populations with little African admixture (like Caucasians, Asians and Arabs) partial RhD phenotypes are likely to be rare and to derive from the limited and serologically well-defined set of alleles of the Eurasian allele cluster. Typing strategies for African populations and those with African admixture may take account of the various frequently occurring alleles of the "African" clusters (chapter 5).[14] Several of these alleles are difficult to discern by serological means and may in the future

warrant genotyping approaches for detection in patients and donors.
Some partial RhD phenotypes are associated with Low Frequency Antigens (LFA).[25] These antigens are expressed due to novel structures on the RBC surface and can be useful markers for the identification of partial RhD.
The predictive value of a method used for medical diagnostics should be as accurate as possible. The raw outcome of a test can only be correctly interpreted when the subject is well defined. When an altered RhD protein is identified in a blood donor, its products must be labelled RhD-positive. When RBCs with an altered RhD protein are administered to an RhD-negative recipient, the chance of an immune response (primary immunization) is present. However, if an altered RhD protein is identified in a recipient, this recipient must be regarded as RhD-negative. If this recipient receives RhD-positive blood the chance that antibodies against the missing RhD epitopes are produced is present.
Weak D blood products should only be administered to RhD-positive patients. Ongoing research shows that some of the non-frequently occurring phenotypes primarily identified as weak D can develop anti-D when transfused with RhD-positive red blood cells.[16,17] Therefore, recipients identified with weak D phenotypes type 4 and higher, should be regarded as RhD-negative.
The multi-ethnic character of our (Dutch) society is reflected in the population of blood recipients but unfortunately not in the blood donor population. This may cause problems with the availability of RhD-negative blood products for people with altered Rh proteins or antigen combinations that are rare in the donor population. Moreover, the reliability of standard typing assays is influenced by the ethnic origin of the donor/recipient.

Molecular identification of RHD

Blood group identification using molecular rather than serological methods is most often seen when serological typing is difficult. When patients have received multiple transfusions, it can be very hard to determine the patient's original blood group due to the presence of donor red cells in the patient's circulation. The blood group of a multiple transfused patient can be obtained by molecular methods. When the Direct Agglutination Test (DAT) is found positive with the patient's red blood cells, it is difficult to serologically type for blood groups by direct agglutination. Molecular tests can also be of use for donor selection when serological typing reagents are not available or of poor quality. Currently, the most reliable method for the determination of *RHD* zygosity is molecular analysis (chapter 2).[26] Blood group genotype analysis can also be used to improve the quality of red blood cells to be used as reagent[27] or in cases where no red blood cells are available. Currently, fetal molecular *RHD* typing (and in lesser extent *K1* and *RHc* and occasionally other blood groups) is used for the prediction of the risk of the fetus for Hemolytic Disease of the Fetus and Newborn (HDFN) in RhD-negative women.[28,29] Cell-free fetal DNA can be obtained

from maternal plasma, eliminating the need for invasive procedures such as amniocentesis or chorionic villus sampling for fetal DNA extraction.[30,31] The chances of a (potential) fetus for being *RHD*-positive and therefore being at risk for HDFN when the RhD-negative mother-to-be has an anti-D can be elucidated from the *RHD* zygosity of the father. Therefore, *RHD* zygosity determination can be used as a diagnostic tool in prenatal counseling.

High throughput molecular typing methods can be applied to large scale donor typing for many blood groups.[32,33,34] With a large donor population typed for many blood groups it becomes easier to find a matching blood product for patients with uncommon antibodies. If blood products are matched for more than just the standard blood groups the formation of additional antibodies will decrease as well. Furthermore, large scale donor typing will identify donors that are negative for high frequency antigens (HFA) which is important for transfusion of patients with anti-HFA antibodies due to prior transfusion(s) or pregnancy.

Molecular methods are increasingly used in patient diagnostics. For instance, when an antibody is identified in a recently transfused patient, the origin (allo or auto) of the antibody can be determined by molecular analysis of the corresponding antigen. The use of molecular typing methods in patient diagnostics to supplement serological testing has been reported to increase the overall accuracy.[35]

The research presented in this thesis has provided a deeper insight into the ethnic variability of the *RH* genes, enabling the development of molecular typing methods that are reliable in multi-ethnic (heterogeneous) societies. Knowledge of the ethnic variability of the *RH* genes is important for the development of molecular screening methods, like a DNA array (DNA chip technology). However, the research presented in this thesis is limited by the number of populations studied. It can be anticipated that more research among populations residing in Africa (like Ghana) and Asia will show even more variability concerning *RH*. Mediterranean populations are also relatively unexplored concerning *RH*.

The molecular typing methods available for *RH* are complex. Therefore, application of these methods by blood group identification laboratories is a difficult task when no in house experts are available. For the accurate development of *RH* genotyping strategies it is of great importance that genetic variation is well documented.

The importance of accurate and reliable genotyping by *RH* variant identification is dependent on the patient population. In this thesis we have described several molecular typing assays which take into account the most frequently occurring variant *RH* genes in blacks. Like for *RHC* and *RHc* genotyping on genomic DNA (chapter 3) and RhD phenotype prediction on cell-free plasma DNA (fetal DNA) (chapter 6). We also analyzed the molecular background of RhD-negativity in a Chinese Han population (chapter 4) in order to facilitate the development of reliable genotyping strategies. Molecular analysis of the *Rhesus boxes* flanking the *RHD* gene in different ethnic groups showed a high variability of the *RH* locus in nonwhite persons,

hampering *RHD* zygosity determination by *Rhesus box* analysis. Therefore, we developed a real-time quantitative PCR method on *RHD* exon 7 to reliably determine *RHD* zygosity in different ethnic groups (chapter 2).
In Dutch society, the different ethnicities are now emerging in the patient population because the first generation of different ethnic groups within Dutch society are now reaching old age which often comes with ailment and also because pregnancies with parents from different ethnic backgrounds are becoming more common. Therefore, the coming years will clarify the clinical importance of accurate *RH* typing. So far, the general feeling has been that the variant *RH* genes were only rarely involved in immunization events. However, only systematic review of unresolved anti-D immunization in combination with the availability of the typing assays as described in this thesis will resolve this issue.[17,36] The recent observations of alloanti-D in weak D type 4 recipients and the induction of alloanti-D by the Del phenotype indicate that this problem might be underestimated.

Evolutionary aspects

The frequency of the aberrant *RHD* alleles is high in black populations when compared to Asian and Caucasian populations (appendix I). Some aberrant alleles are only present in a single population whereas other aberrant alleles may be present in several populations, with different frequencies. This implies that many aberrant *RHD* alleles have evolved from the same template. As a template *RHD* allele is present in all populations this can be regarded as an *H. Sapiens*-specific characteristic that has propagated from its population of origin to all other populations. The population difference (or ethnic variability) in aberrant alleles can be regarded as part of the characteristics responsible for regional continuity that have remained restricted to the population of their origin. The presence of identical aberrant *RHD* alleles in different ethnic populations may indicate that these alleles were present in *H. sapiens* before the expansion of *H. sapiens* to the diferent regions, strongly supporting the plausibility of the uniregional hypothesis (see chapter 1, introduction, page 16).
The above also implies that there must be some evolutionary advantage in changing the RhD protein. It has been postulated that an altered amino acid sequence might prevent parasites, like Plasmodia that causes malaria, from using the transmembrane Rh proteins for invasion of the red cell. However, a causal relationship has not been demonstrated. Recently, Rh-related proteins (including RhAG) have been shown to export ammonia.[37,38] It may therefore be speculated that amino acid substitutions located in transmembraneous Rh protein segments may affect the function of the Rh protein.

Developments

Large scale (molecular) typing methods are now being developed.[32,33,34] For the identification of aberrant *RH* alleles so many different areas of the *RH* genes should

be investigated, that the DNA microarray seems to be the best format. The aim of large scale donor typing is to have a fully typed donor population in order to be able to match for more antigens and subsequently diminish (additional) antibody formation. Avoiding antibody formation is especially important for women of child bearing age and patients that regularly need transfusions, like people who suffer from hemoglobinopathies (like sickle cell anaemia and thalassemia, not uncommon in non-Caucasian populations) and patients who suffer from hemolytic anaemias. Most blood groups other than ABO and Rh are encoded by Single Nucleotide Polymorphisms (or SNPs). Because these blood group systems have no homologue genes present on the same chromosome (except the MNSs blood group system) they are less prone to genetic variation within the coding allele. SNP-based blood groups may be suitable candidates to include in large scale molecular typing methods. The *RH* system however, is very complex and not fully explored (yet). Even in Caucasian/European populations, RhD-negative *RHD* alleles and new variant alleles are continuously identified.[17,39] With the current knowledge of the *RH* system most aberrant alleles can be identified. It is, however, virtually impossible to map all low frequency aberrant alleles because the emerging of aberrant alleles is infinite (intrinsic to evolution).

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Appendix I

Characteristics of partial RhDs.

Name	Nucleotide Change	Protein Sequence	Membrane Localization	Molecular Basis	Associated Haplotype	Number of Probands	Ethnic Origin*
DII	1060C>G	A354D	EC		Ce	One	C
	1061C>A	A354D	EC				
DIIIa	455A>C	N152T	TM		ce, G+	Many	B
	602C>G	T201R	IC				
	667T>G	F223V	TM				
DIIIb	90T>C	30F			ce, G-	Few	B
	178A>C	I60L	TM				
	203G>A	S68N	TM				
	307T>C	S103P	EC				
DIIIc	361T>A	L121M	TM		Ce, G+	Many	C
	380T>C	V127A	TM				
	383A>G	D128G	TM				
	455A>C	N152T	TM				
DIII Type 4	186G>T	L62F	TM		-	Few	C
	410C>T	137A					
	455A>C	N152T	TM				
DIII Type 5	186G>T	L62F	TM		ce	One	
	410C>T	137A					
	455A>C	N152T	TM				
	602C>G	T201R	IC				
	667T>G	F223V	TM				
	819G>A	273A					
DIII type 6	410C>T	137A					B
	455A>C	N152T	TM				
	602C>G	T201R	IC				
	667T>G	F223V	TM				
	819G>A	273A					

Name	Nucleotide Change	Protein Sequence	Membrane Localization	Molecular Basis	Associated Haplotype	Number of Probands	Ethnic Origin*
DIII	exon 2D>C		EC				B
type 7	410C>T	137A					
	455A>C	152N>T	TM				
	602C>G	201T>R	IC				
	667T>G	223F>V	TM				
	819G>A	273A					
DIVa	186G>T	L62F	TM		ce	Many	B
	455A>C	N152T	TM				
	1048G>C	D350H	EC				
DIVb	1048G>C	D350H	EC				
type 2	1053C>T	351T					
	1057G>T	G353T	EC				
	1059A>G	G353T	EC				
	1060G>A	A354N	EC				
	1061C>A	A354N	EC				
	1170T>C	389L					
	1193A>T	E398V	IC				
DIVb	DIVb type 2	DIVb type 2			Ce	One	C
type 3	916G>A	V306I	TM				
	932A>G	Y311C	IC				
	941G>T	G314V	IC				
	968C>A	P323H	IC				
	974G>T	S325I	IC				
	979A>G	I327V	IC				
	985G>C	G329H	IC				
	986G>A	G329H	IC				
	989A>C	Y330S	IC				
	992A>T	N331I	IC				
	1025T>C	I342T	TM				
DIVb	1048G>C	D350H	EC		Ce	Several	
type 4	1057G>T	G353T	EC				
	1059A>G	G353T	EC				
	1060G>A	A354N	EC				
	1061C>A	A354N	EC				

Name	Nucleotide Change	Protein Sequence	Membrane Localization	Molecular Basis	Associated Haplotype	Number of Probands	Ethnic Origin*
DVI	Dva(HUS)	Dva(HUS)	EC/TM/IC		cE	Many	C
type 1	505A>C	M169L	EC				
	509T>G	M170R	EC				
	514A>T	I172F	EC				
	544T>A	S182T	TM				
	577G>A	E193K	IC				
	594A>T	K298N	IC				
	602C>G	T201R	IC				
DVI	DVI type 1	DVI type 1	EC/TM/IC		Ce	Many	C,
type 2	916G>A	V306I	IC				J
	932A>G	Y311C	IC				
DVI	DVI type 2	DVI type 2	EC/TM/IC		Ce	Few	C
type 3	361T>A	L121M	TM				
	380T>C	V127A	TM				
	383A>G	D128G	TM				
	455A>C	N152T	TM				
DVII	329T>C	L110P	EC		Ce	Many	C
DAR	602C>G	T201R	IC		ce	Many	B
	667T>G	F223V	TM				
	1025T>C	I342T	TM				
DAU-0	1136C>T	T379M	TM		ce	Few	C
DAU-1	DAU-0	DAU-0	TM		ce	One	B
	689G>T	S230I	EC				
DAU-2	DAU-0	DAU-0	TM		ce	One	B
	209G>A	R70Q	TM				
	998G>A	S333N	IC				
DAU-3	DAU-0	DAU-0	TM		ce	One	B
	835G>A	V279M	TM				
DAU-4	DAU-0	DAU-0	TM		ce	One	B
	697G>A	E233K	EC				

Name	Nucleotide Change	Protein Sequence	Membrane Localization	Molecular Basis	Associated Haplotype	Number of Probands	Ethnic Origin*
DBT	DVa (HUS)	DVa (HUS)			EC/TM/IC		
Ce>	Several	C,					
type 1	916G>A	V306I	TM		(C)		B,
	932A>G	Y311C	IC		(e)		J
	941G>T	G314V	IC		and		
	968C>A	P323H	IC		ce		
	974G>T	S325I	IC				
	979A>G	I327V	IC				
	985G>C	G329H	IC				
	986G>A	G329H	IC				
	989A>C	Y330S	IC				
	992A>T	N331I	IC				
	1025T>C	I342T	TM				
	1048G>C	D350H	EC				
	1053C>T	351T	EC				
	1057G>T	G353T	EC				
	1059A>G	G353T	EC				
	1060G>A	A354N	EC				
	1061C>A	A354N	EC				
DBT	DBT type 1	DBT type 1	EC/TM/IC		Ce	Several	J
type 2	1170T>C	389L					
	1193A>T	E398V	IC				
DFR	505A>C	M169L	EC		Ce>	Many	C
	509T>G	M170R	EC		cE		
	514A>T	I172F	EC				
DFW	497A>C	H166P	EC		Ce	One	C
DHMi	848C>T	T283I	TM		cE	Several	C
DHO	704A>C	K235T			Ce		C
DHR	686G>A	R229K			cE		C
DIM	854G>A	C285Y			cE	One	C
DMH	161T>C	L54P			ce		C
DNB	1063G>A	G355S			Ce	Many	C
DNU	1057G>A	G353R			Ce	Few	C
DOL	509T>G	M170T	EC		ce	Several	B
	667T>G	F223V	TM				
							

Name	Nucleotide Change	Protein Sequence	Membrane Localization	Molecular Basis	Associated Haplotype	Number of Probands	Ethnic Origin*
DHAR/ R0HAR	RHCE-RHD exon5-RHCE	multiple			c(e) G-	Many	C

* B=blacks, C=Caucasian and J=Japanese.

Appendix II

Characteristics of the RhD category V phenotype

Name	Nucleotide Change	Protein Sequence	Membrane Localization	Molecular Basis RHD exon 5
DVa (KOU)/FH	667T>G	223F>V	TM	
	697G>C	233E>Q	EC	
DVa (HUS)	667T>G	223F>V	TM	
	697G>C	233E>Q	EC	
	712G>A	238V>M	EC	or
	733G>C	245V>L	TM	
	787G>A	263G>R	IC	
	800A>T	267K>M	TM	
DVa (TO)	697G>C	233E>Q	EC	
	712G>A	238V>M	EC	
DVa-like YH/TT	667T>G	223F>V	TM	
	697G>C	233E>Q	EC	
	712G>A	238V>M	EC	
DV type VII	667T>G	223F>V	TM	
	697G>C	233E>Q	EC	
	712G>A	238V>M	EC	
	733G>C	245V>L	TM	
	787G>A	263G>R	IC	
Dva-associated HK/DYO	697G>A	233E>K	EC	
Dva-associated SM	697G>C	233E>Q	EC	

Appendix III

Characteristics of the Del phenotype

Name	Nucleotide Change	Protein Sequence	Associated Haplotype	Number of Probands	Ethnic Origin*
Del	IVS3+1G>A	-	Ce	Few	C
Del	IVS8+1G>A	-	Ce	One	C
Del	885G>T	295M>I	Ce	Few	C
Del	1227G>A	-	Ce	many	A
Del	1013 bp deletion, including exon 9	-	-	Few	A

* A=Asian and C=Caucasian

Summary

The RhD bloodgroup was first described by Levine en Stetson in 1939 after the manifestation of a hemolytic transfusion reaction in a woman who recently gave birth, after transfusion with her husbands red cells. The RhD-negative woman produced antibodies against the RhD present on the red cells of her child. The child inherited the antigenic character from her father. Nowadays, the initial antibody production in pregnant women is prevented by means of anti-D prophylaxis because this antibody may cause severe hemolytic disease of the newborn in a next pregnancy. Also, the presence of anti-D in a patient who is transfused with RhD-positive red cells may give rise to a severe hemolytic transfusion reaction. The Rh phenotype can be determined by means of serological techniques based on agglutination of a known antibody (anti-D in this case) with the erythrocytes. In cases where no red cells are available (like in prenatal diagnostics) or when serological testing is hampered (like after a recent transfusion or when it is difficult to obtain the antibody) the Rh phenotype can be predicted from DNA analysis. To be able to make a reliable prediction of the Rh phenotype, it is important to genotype on those DNA stretches that are the least prone to changes and that are equal among different ethnic groups. The research described in this thesis has tried to map the ethnic differences in *RH* genes and to develop reliable genotyping methods that can be applied in a multi-ethnic society.

The discovery of the physical structure of the *RH* locus and the mechanism causing the *RHD* gene deletion in RhD-negative white populations has made it possible to genotype for the absence of the *RHD* gene (RhD-negativity) by means of the hybrid *Rhesus box* (PCR-RFLP). Knowledge about *RHD* zygosity is of clinical interest for prenatal counselling when a woman has developed anti-D and the father is RhD-positive. The chance of a RhD-positive child is 50% when the father carries one *RHD* gene (hemizygous for *RHD*) and 100% when the father carries two *RHD* genes (homozygous for *RHD*). Chapter 2 describes the analysis of the *RH* locus among different ethnic populations (blacks from South-Africa, Curaçao and Ethiopia, Asians fom South-Africa and Caucasians from The Netherlands). Apart from the confirmation of the known fact that RhD-negativity in non-Caucasians is frequently caused by the *RHD* and/or the *r's* gene, we showed that the DNA sequence of *Rhesus boxes* is highly variable in non-Caucasians (aberrant sequences of *Rhesus boxes* were found with a frequency of 0.22 in RhD-positive *RHD* homozygous black popu-

lations). These aberrant Rhesus boxes cause a false negative prediction for the presence of an *RHD* gene by PCR-RFLP. To be able to reliably predict the *RHD* zygosity in a multi-ethnic society, a real time quantitative PCR method on *RHD* exon 7 was developed and evaluated. The real time quantitative PCR method gives more reliable results than the PCR-RFLP method and is the preferred method for *RHD* zygosity determination.

In chapter 3 the reliability of the prediction of the RhC and Rhc phenotypes by genotyping among different ethnic groups was studied. Blood samples of people from five different ethnic groups (blacks from South-Africa, Curaçao and Ethiopia, Asians fom South-Africa and Caucasians from The Netherlands) were genotyped for *RHC* and *RHc* and the genotyping results were compared with the results from serological analysis. *RHc* genotyping was performed on two different coding nucleotide positions, namely nt 178C (*RHc* ASPA) and 307C (*RHC/c* MPX PCR). No discrepancies were found between genotyping and serology. Therefore, both nucleotide positions are a reliable predictor for the Rhc phenotype. However, because of the existence of the D^c allele (variant *RHD* gene with Rhc expression because of a 307T>C mutation) genotyping on 307C is the preferred method. *RHC* genotyping was performed on the *RHC* coding nt 48C (*RHC* ASPA) and on a *RHC* specific 109 bp insert in intron 2 of the *RHC* allele (*RHC/c*MPX PCR). When compared with serology, 43.3% of the serological RhC-negative samples showed a false positive prediction of the RhC phenotype (false positive for RhC) with the nt 48C method. This discrepancy was caused by the presence of an *RHce* allele with a nt 48G>C substitution which was identified in all ethnic groups that were analyzed. The nt 48G>C substitution does not influence the expression of Rhc. False negative predictions of the *RHC* phenotype were not made with this method. The *RHC* genotyping method on the 109 bp insert in *RHC* intron 2 generated a false negative prediction of the RhC phenotype in 7.3% of the RhC-positive samples. This discrepancy was caused by the presence of an *r's* allele that was only identified in blacks from Curaçao and South-Africa and in one Caucasian blood donor. To improve the reliability of the present *RHC* genotyping techniques, the existing *RHC/c* MPX PCR method was extended with specific PCRs for the detection of the *r's* allele (*RHC/c/hex3* method, possibly in combination of the intron4/exon7 MPX PCR) and validated on the same samples. This method resulted in a correct predictive value of 100% for the Rhc phenotype and 99.8% for the RhC phenotype.

Chapter 4 describes the *RHD* gene analysis and *RHD* zygosity determination in a selected group of RhD-negative Chinese Han blood donors (n=74). No specific *RHD* sequences were identified in 46 RhD-negative samples (62%) that also showed the hybrid *Rhesus box* only (indicative for the *RHD* gene deletion). 22 Samples (30%) showed the presence of the *Del* genotype (nt 1227G>A) which causes a very weak

expression of RhD. In 5 samples (7%), a hybrid *RHD-RHCE-RHD* gene was identified. In 1 sample (1.4%) a new nonsense mutation was identified (933C>A). RhD-negativity in the presence of an *RHD* specific sequences is often found in the Chinese Han population. The main cause for the discrepancy between serology and genotyping was caused by the Del phenotype which is typed RhD-negative by standard serological methods. Therefore, in a RhD-negative Chinese Han population, genotyping is preferable to serological typing.

Chapter 5 describes the analysis of the weak D type 4 cluster (characterized by nts 602G and 667G) from a phylogenetic perspective. For this purpose, 80 samples were selected (from a total of 1702 samples from 5 different ethnic groups) for the presence of nt 602G in combination with 667G and were analyzed further. Apart from the known and expected variant *RHD* alleles, three new variant *RHD* alleles were identified using molecular methods, and where possible serological methods. By identifying new variant *RHD* alleles it is possible to refine the existing *RH* phylogeny. In this analysis we also identified the *RHD*(F223V)(602C>G) and *RHD*(T201R, F223V)(602C>G, 667T>G) alleles which gave us formal proof for the origin of the *weak D type 4* cluster. Furthermore, we identified the 1136C>T nucleotide substitution (characteristic for the *DAU* allele cluster) to be present on two *DVa(KOU)* alleles. This “new” allele is probably caused by gene conversion of the *DAU0* allele and the *DVa(KOU)* allele, most probably forming a phylogenetic link between the *DAU* cluster and the *DV* allele cluster, proposed in this chapter.

Knowledge about the RhD-status of a fetus is important for the prevention of RhD immunization in RhD-negative women. Also the administration of anti-D prophylaxis can be based on whether the fetus is positive or negative for RhD. This prevents the administration of anti-D to those mothers that carry an RhD-negative fetus. The discovery of cell-free fetal DNA in maternal plasma led to the development of several assays to predict the fetal D-status using *RHD*-specific sequences. Few assays are designed in such a way that RhD-negative *RHD* genes are taken into account (like the *RHD*ψ, present in the majority of D-negative African blacks) hampering correct genotyping of the fetus in *RHD*ψ-positive mothers and leading to a false positive prediction of the RhD-status when the fetus carries an *RHD*ψ. Chapter 6 describes the development of a reliable prenatal genotyping method on genomic DNA and the evaluation of this method on cell-free plasma DNA. During the course of events, the importance of the use of the proper material (in this case cell-free plasma DNA) during the development of a genotyping method on cell-free plasma DNA was emphasized because of the different characteristics of genomic DNA and cell-free plasma DNA and due to the limited knowledge in this area. Chapter 6 describes an *RHD* genotyping method that generates a product of 361 base pairs (bp) and shows a high specificity and sensitivity on genomic DNA but does not generate a product

when used with cell-free plasma DNA. During this research, it was published that the majority of cell-free plasma DNA present in the maternal circulation has a size distribution of 145 to 201 bp, which explained why no 361 bp product was amplified. Eventually, a previously published *RHD* genotyping method was evaluated on cell-free plasma DNA of RhD-negative *RHD*ψ-positive pregnant women and showed maximal specificity and sensitivity.

In chapter 7, the discussion, the findings as described in the previous chapters are shown in a broader perspective based on the present knowledge about the (ethnic) variability of the Rh blood group system. Also, attention is given to the advantages and disadvantages of possible future genotyping methods for both blood donors and blood recipients.

Samenvatting

De RhD bloedgroep werd voor het eerst beschreven door Levine en Stetson in 1939 nadat een hemolytische transfusie reactie was opgetreden in een pas bevallen vrouw na transfusie met bloed van haar man. De RhD-negatieve vrouw bleek antistoffen gemaakt te hebben tegen het RhD dat aanwezig was op de cellen van haar kind. Het kind had deze antigene eigenschap geërfd van de vader. Tegenwoordig wordt geprobeerd deze initiële antistofvorming in zwangere vrouwen te voorkómen door middel van anti-D prophylaxe omdat deze antistofvorming aanleiding kan geven tot ernstige hemolytische ziekte van de pasgeborene bij een volgende zwangerschap. Ook kan de aanwezigheid van anti-D in een patiënt die getransfundeerd wordt met RhD-positief bloed leiden tot een ernstige hemolytische transfusiereactie. Het Rh fenotype kan worden vastgesteld met behulp van serologische technieken op basis van agglutinatie van een bekende antistof (in dit geval anti-D) met de te typeren erytrocyten. In die gevallen waarin geen rode cellen voorhanden zijn (bijvoorbeeld in prenatale diagnostiek) of wanneer serologische bepalingen worden bemoeilijkt (bijvoorbeeld na recente transfusie of wanneer een antistof moeilijk verkrijgbaar is) kan het Rh fenotype worden voorspeld uit DNA analyse. Om een goede voorspelling van het Rh fenotype te kunnen doen, is het van belang om te genotyperen op die delen van het DNA die zo min mogelijk onderhevig zijn aan veranderingen en die gelijk zijn binnen verschillende etnische groepen. Het onderzoek zoals beschreven in dit proefschrift heeft getracht om de etnische verschillen tussen de *RH* genen in kaart te brengen en betrouwbare genotypeer methoden te ontwikkelen die kunnen worden toegepast in een multi-etnische samenleving.

Door de ontdekking van de fysieke structuur van de *RH* locus en het mechanisme dat de *RHD* gen deletie veroorzaakt in Kaukasische RhD-negatieven is het mogelijk geworden om te genotyperen voor de afwezigheid van het *RHD*-gen (RhD-negativiteit) met behulp van de hybride *Rhesus box* (PCR-RFLP). Kennis over *RHD* zygositeit is van klinisch belang voor prenatale counseling bij een vrouw die anti-D heeft ontwikkeld en de vader RhD-positief is. De kans op een RhD-positief kind is 50% wanneer de vader één *RHD* gen draagt (hemizigoot voor *RHD*) en 100% wanneer de vader twee *RHD* genen draagt (homozygoot voor *RHD*). Hoofdstuk 2 beschrijft de analyse van de *RH* locus in verschillende etnische populaties (negroïden uit Zuid-Afrika, Curaçao en Ethiopië, aziaten uit Zuid-Afrika en kaukasiërs uit Nederland). Naast het bevestigen van het reeds bekende feit dat in niet-kaukasische

populaties RhD-negativiteit vaak wordt veroorzaakt door het *RHDψ* en/of het *r's* gen, hebben we aangetoond dat de DNA sequentie van de *Rhesus boxen* in niet-kaukasiërs zeer variabel is (afwijkende sequenties in de *Rhesus boxen* werden gevonden met een frequentie van 0.22 in RhD-positieve *RHD* homozygote negroïde populaties). Deze afwijkende *Rhesus boxen* geven een vals negatieve voorspelling voor de aanwezigheid van een *RHD* gen met de PCR-RFLP methode. Om toch de *RHD* zygositeit betrouwbaar te kunnen bepalen in een multi-etnische samenleving is een kwantitatieve real-time PCR methode op *RHD* exon 7 ontwikkeld en geëvalueerd. De kwantitatieve real-time PCR methode geeft beter betrouwbare resultaten dan de PCR-RFLP en is de aanbevolen methode voor het bepalen van de *RHD* zygositeit.

In hoofdstuk 3 is de betrouwbaarheid van de voorspelling van het Rhc en RhC fenotype middels genotypering in verschillende etnische groepen bestudeerd. Bloedmonsters afkomstig van mensen uit 5 verschillende etnische groepen (negroïden uit Zuid-Afrika, Curaçao en Ethiopië, aziaten uit Zuid-Afrika en kaukasiërs uit Nederland) zijn gegenotypeerd voor *RHc* en *RHC* en deze resultaten zijn vergeleken met de resultaten van de serologische analyse. Voor *RHc* genotypering zijn twee verschillende coderende nucleotiden (nt) bekeken, namelijk nt. 178C (*RHc* ASPA) en nt. 307C (*RHC/c* MPX PCR). Er werden geen discrepanties gevonden tussen het genotyperen en de serologie, daarom zijn beide *RHc* specifieke nucleotiden een betrouwbare voorspeller voor het Rhc fenotype. Vanwege het voorkomen van een D^c allel (variant *RHD* allel met Rhc expressie als gevolg van een 307T>C mutatie) geniet genotyperen van *RHc* op nt 307C de voorkeur. De *RHC* genotypering is uitgevoerd op basis van het *RHC* specifieke nt. 48C (*RHC* ASPA) en op basis van een *RHC* specifiek 109 bp insert in intron 2 van het *RHC* allel (*RHC/c* MPX PCR). Vergeleken met de serologie gaf 43.3% van de serologisch RhC-negatieven een vals positieve voorspelling van het fenotype (onterecht RhC-positief) met de nt. 48C methode. Deze discrepantie werd veroorzaakt door de aanwezigheid van een *RHce* allel met een nt. 48G>C substitutie welke voorkomt in alle bestudeerde etnische groepen. De 48G>C substitutie heeft geen invloed op de expressie van Rhc. Vals negatieve voorspellingen kwamen met deze assay niet voor. Met de assay gebaseerd op het 109 bp insert in *RHC* intron 2 gaf 7.3% van de serologisch RhC-positieven een vals negatieve voorspelling van het fenotype. Deze discrepantie werd veroorzaakt door de aanwezigheid van een *r's* allel welke alleen werd geïdentificeerd in negroïden uit Curaçao en Zuid-Afrika en in één kaukasische bloeddonor. Om de betrouwbaarheid van de huidige *RHCc* genotypeertechniek te verhogen, is de *RHC/c* MPX PCR uitgebreid met specifieke PCRs welke tevens het *r's* allel aantonen (*RHC/c/hex3* methode, wel of niet in combinatie met *intron4/exon* 7 MPX PCR) en gevalideerd op dezelfde monsters. Dit heeft geresulteerd in een juiste voorspellingswaarde van 100% voor het Rhc en 99.8% voor het RhC fenotype.

Hoofdstuk 4 beschrijft de *RHD* gen analyse en *RHD* zygositeitsbepaling in een geselecteerde RhD-negatieve Chinese Han populatie van bloeddonoren (n=74). In 46 RhD-negatieve monsters (62%) werden geen *RHD*-specifieke exonen gevonden en werd ook alleen de hybride *Rhesus box* aangetoond (karakteristiek voor *RHD* gen deletie). In 22 monsters (30%) werd het *Del* genotype dat gepaard gaat met een zeer zwakke RhD expressie (nt 1227G>A) vastgesteld. In 5 monsters (7%) werd een hybride *RHD-RHCE-RHD* gen geïdentificeerd. In 1 monster (1.4%) werd een nieuwe nonsense mutatie geïdentificeerd (933C>A). RhD-negativiteit in de aanwezigheid van *RHD* specifieke sequenties komt veel voor binnen de Chinese Han populatie. De grootste discrepantie tussen serologie en genotypering werd veroorzaakt door het Del fenotype welke met de standaard serologische technieken als RhD-negatief wordt getypeerd. Daarom verdient in de RhD-negatieve Chinese Han populatie het genotyperen de voorkeur boven serologische typering.

Hoofdstuk 5 beschrijft de analyse van het zwak D type 4 cluster (gekenmerkt door nts 602G en 667G) vanuit fylogenetisch perspectief. Hiertoe zijn 80 monsters (uit 1702 monsters afkomstig van vijf etnische groepen) geselecteerd op de aanwezigheid van nt 602G in combinatie met nt 667G en moleculair gekarakteriseerd. Naast de bekende variant *RHD* allelen werden ook drie nieuwe variant *RHD* allelen moleculair, en wanneer mogelijk tevens serologisch, geïdentificeerd. Middels de identificatie van nieuwe variant *RHD* allelen is het mogelijk om de fylogenetische boom voor *RH* te verfijnen. Tevens werden in deze analyse de twee allelen geïdentificeerd die de grondslag leggen voor het gehele *zwak D type 4* cluster (*RHD*(F223V)(602C>G) en *RHD*(T201R, F223V)(602C>G, 667T>G), de hoofdtakken). In twee *DVa(KOU)* variant allelen werd een mutatie gevonden die voorheen exclusief werd toegeschreven aan het *DAU0* allel. Dit “nieuwe” allel is zeer waarschijnlijk ontstaan door een gen conversie van het *DAU0* allel en het *DVa(KOU)* allel waardoor deze zeer waarschijnlijk een fylogenetische link vormt tussen het *DAU* allel cluster en het in dit hoofdstuk voorgestelde *DV* allel cluster.

Kennis omtrent de RhD-status van een foetus is van belang voor de preventie van RhD-immunizatie bij RhD-negatieve vrouwen. Tevens kan, wanneer bekend is of de foetus RhD-positief danwel negatief is, het geven van anti-D profylaxe hierop afgestemd worden. Dit voorkomt onnodige toedienning van anti-D in geval van een RhD-negatieve foetus. Sinds de ontdekking van foetaal DNA in de maternale circulatie zijn meerdere *RHD* genotypeerstrategieën ontwikkeld op cel-vrij plasma (foetaal) DNA dat wordt geïsoleerd uit maternaal plasma. Slechts weinig strategieën houden rekening met RhD-negatieve *RHD* genen (zoals het in negroïden zeer frequent voorkomende *RHD*ψ waardoor foetale genotypering bij *RHD*-positieve aanstaande moeders wordt bemoeilijkt en de foetus alsnog ten onrechte als RhD-positief kan worden beschouwd wanneer deze zelf een *RHD* draagt. Hoofdstuk 6

beschrijft het opzetten van een betrouwbare prenatale *RHD* genotypeerassay op genomisch DNA en de evaluatie van deze test op cel vrij plasma DNA. Hierbij kwam het belang naar voren van het gebruik van het eigenlijke uitgangsmateriaal (in dit geval cel-vrij plasma DNA) bij het opzetten van een genotypeer assay op cel-vrij plasma DNA omdat gebleken is dat genomisch DNA en cel-vrij plasma DNA verschillende eigenschappen hebben en de kennis op dit gebied beperkt is. Hoofdstuk 6 beschrijft een *RHD* genotypeerassay die een produkt genereert van 361 baseparen (bp) en een hoge specificiteit en sensitiviteit kent op genomisch DNA maar geen product genereert wanneer cel-vrij plasma DNA wordt gebruikt. Ten tijde van deze bevinding werd door anderen gepubliceerd dat het meeste in maternaal plasma circulerende DNA een grootte heeft tussen 145-201 bp hetgeen verklaarde waarom geen 361 bp product werd gegenereerd. Een elders gepubliceerde *RHD* genotypeermethode werd uiteindelijk geëvalueerd op het celvrij plasma DNA van RhD-negatieve *RHD*ψ-positieve zwangere vrouwen en liet een maximale specificiteit en sensitiviteit zien.

In hoofdstuk 7, de discussie, worden de bevindingen zoals beschreven in de voorgaande hoofdstukken van dit proefschrift in een breder daglicht geplaatst aan de hand van de huidige kennis op het gebied van de (etnische) vaiabiliteit van het Rh bloedgroepsysteem. Daarnaast wordt ook aandacht besteed aan de voor- en nadelen van mogelijk toekomstige genotypeermethoden voor zowel de donors als ontvangers van bloed.

Dankwoord

Reik niet naar de hemel, maar haal 'm naar je toe.
(Uit: "Lef", J. van Dongen)

Daar is 'tie dan. Eindelijk. Ondanks alle hulp die tijdens het onderzoek is geboden heb ik mijn promotie aardig kunnen uitstellen. Het was dan ook een zeer bijzondere tijd. Veel mensen hebben op vele fronten een bijdrage aan dit proefschrift geleverd, en hier is de plek om al deze mensen te bedanken.

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In “vakantiepark” Sanquin Research te Amsterdam, zijn de kwantitatieve testen opgezet en geëvalueerd en waren de wekelijkse werkbesprekingen. De kern van het Amsterdamse “Rhesus groepje” werd gevormd door Serge, Mirte, Onno, Goedele en op de valreep Nigel. We hebben samen veel werk besproken en cursussen gevolgd. Naast de inhoudelijke discussies hebben we ook veel lol gehad wat de onvermijdelijke dagen vol (kloneer) mislukkingen wat dragelijker maakte. Serge, ik vind het heel fijn dat jij mijn paranimf wil zijn, nu durf ik wel. Bernadette en Aicha bedankt voor jullie hulp met het opzetten van de kwantitatieve en prenatale TaqMan testen. Eloise bedankt voor je (voor)raad bij het sub-kloneren. Martin bedankt voor het delen van je kennis omtrent sequentie analyse. Natuurlijk wil ik ook de andere mensen van IHE en D bedanken voor de gezelligheid in o.a. de bureauruimte en koffiekamer. Peter L. (de Serologie-Piet) bedankt voor de serologische analyses (en bijbehorende uitleg) van de vele monsters, ik heb veel van je geleerd. Dear Xu, you came to Amsterdam for 9 months to study D-negativity in the Chinese Han population on the samples you brought with you, both in Amsterdam and in “Doltekt”. I think we both learned a lot.

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Martine

Curriculum Vitae

Martine Tax werd geboren op 10 oktober 1974 te Haarlem. Ze groeide op in Heemstede en Aerdenhout en behaalde in 1994 het diploma VWO te Leiden.

In datzelfde jaar begon zij met het propedeuse jaar van de studie Gezondheidswetenschappen aan de Universiteit Maastricht. Na dit propedeutisch jaar stapte zij over naar de interfacultaire bovenbouw studie milieugezondheidkunde gedoceerd aan de faculteit der Gezondheidswetenschappen en de faculteit der Geneeskunde, aan dezelfde universiteit. Deze studie werd afgerond in het jaar 2000 na een buitenland stage van 6 maanden bij de World Health Organization, European Center for environment and health, Regional office for Europe, Rome division (Italie), onder leiding van Prof. dr. M. Maroni (Universiteit van Milaan), en een wetenschappelijke stage van 3 maanden bij Sanquin Research te Amsterdam onder leiding van Prof. dr. E. Hack en Prof. dr. L.A. Aarden.

Aansluitend is Martine begonnen aan haar promotieonderzoek als Junior Onderzoeksmedewerker bij Sanquin Research te Amsterdam binnen de afdeling Experimentele Immunohematologie en bij de Sanquin Bloedbank regio Zuidwest binnen de afdeling Onderzoek en Ontwikkeling met promotor Prof. dr. D.J. van Rhenen (Sanquin Bloedbank regio Zuidwest). De dagelijkse begeleiding van het promotieonderzoek was in handen van dr. C. Ellen van der Schoot (Sanquin Research) en dr. Petra A. Maaskant-van Wijk (Sanquin Bloedbank regio Zuidwest).

Sinds 1 februari 2004 werkt zij als hoofd van het Referentielaboratorium Erytrocytenserologie op de afdeling Immuun Hematologische Diagnostiek van de Sanquin Bloedbank regio Zuidwest.