Research article

RHD positive haplotypes in D negative Europeans

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Abstract

Background: Blood group genotyping is increasingly utilized for prenatal diagnosis and after recent transfusions, but still lacks the specificity of serology. In whites, the presence of antigen D is predicted, if two or more properly selected *RHD-specific* polymorphism are detected. This prediction must fail, if an antigen D negative *RHD* positive allele is encountered. Excluding *RHDψ* and *Cde*^{S} frequent only in individuals of African descent, most of these alleles are unknown and the population frequency of any such allele has not been determined.

Methods: We screened 8,442 antigen D negative blood donations by *RHD* PCR-SSP. *RHD* PCR positive samples were further characterized by *RHD* exon specific PCR-SSP or sequencing. The phenotype of the identified alleles was checked and their frequencies in Germans were determined.

Results: We detected 50 *RHD* positive samples. Fifteen samples harbored one of three new D_{el} alleles. Thirty samples were due to 14 different D negative alleles, only 5 of which were previously known. Nine of the 14 alleles may have been generated by gene conversion *in cis,* for which we proposed a mechanism triggered by hairpin formation of chromosomal DNA. The cumulative population frequency of the 14 D negative alleles was 1:1,500. Five samples represented a $D^{+/-}$ chimera, a weak D and three partial D, which had been missed by routine serology; two recipients transfused with blood of the $D^{+/-}$ chimera donor became anti-D immunized.

Conclusion: The results of this study allowed to devise an improved *RHD* genotyping strategy, the false-positive rate of which was lower than 1:10,000. The number of characterized *RHD* positive antigen D negative and D_{el} alleles was more than doubled and their population frequencies in Europe were defined.

Introduction

The antigen D encoded by the *RHD* gene is the most important blood group antigen determined by a protein. About 15% of whites are antigen D negative. Antigen D prediction by PCR was initially applied to fetus at risk for hemolytic disease of the newbom [1,2]. If serologic blood group typing cannot be performed with its usual ease, an *RHD* genotyping with a specificity and sensitivity comparable to serologic methods is of practical importance. For example, the utility of blood group genotyping in patients with recent transfusions was demonstrated by several studies [3,4,5,6].

The two *RH* genes, *RHD* and *RHCE* are about 30,000 bp apart [7], have opposite orientation [7,8] and are homologous retaining more than 90% identity [9]. The most

frequent cause for the absence of the antigen D in whites is the lack of the whole *RHD* gene [10] due to a deletion occurring in the *Rhesus box* [7]. Therefore, most methods for antigen D prediction in whites probed the presence of *RHD* specific polymorphism [1,2,11,12,13]. Two *RHD* positive antigen D negative alleles are frequent in Africans: *RHDψ* carries a 37 bp insertion at the intron 3/exon 4 boundary and also harbors a stop codon [14]; *Cde*^{s} is a *RHD-CE-D* hybrid gene [15,16,17]. In Asians, a major allele may be associated with a G314V missense mutation [18], and several other alleles may represent *RHD-CE-D* hybrid alleles [18,19,20].

In whites, *RHD* positive antigen D negative alleles were considered rare. However, the single systematic study [21] indicated a frequency of up to 22% among the rare haplotype *Cde,* which would render them the major cause of false-positive antigen D prediction by PCR in whites. The majority of *RHD* positive alleles in D negatives were reported as scattered case reports [22,23,24,25,26,27] with an often incomplete molecular work-up. The relative frequencies of these alleles and their cumulative population frequency remained unknown.

The specificity of *RHD* genotyping can be improved by a systematic characterization of *RHD* positive antigen D negative alleles. This rationale prompted us to determine the molecular causes of such alleles and their population frequencies in a random survey among European blood bank donors. We screened more than 8,000 antigen D negative blood donations by *RHD* PCR, including more than 700 rare Ccddee or ccddEe samples. Nine *RHD-CE-D* hybrid alleles, 5 other D negative and 3 D_{el} alleles were identified. Five D positive donors missed by routine serology were uncovered. Two anti-D immunizations were traced. We established frequency estimates for *RHD* positive antigen D negative haplotypes in whites, which allowed us to devise an optimized *RHD* PCR strategy with an enhanced and defined specificity.

Results

Population surveys

In a first survey, we investigated 1,068 samples of blood donors that were documented as antigen D negative according to routine serologic methods. To cover the whole length of the *RHD* gene, we tested the *RHD* promoter, intron 4, exon 7, and the 3' untranslated region of exon 10 by PCR-SSP (Table 1). As antigen D negative *RHD* gene positive alleles are known to preferentially occur in the *Cde* and *cdE* haplotypes [21,22,25], we tested 754 samples with antigen C or antigen E or both along with 314 ccddee samples. We detected 48 donors who carried the *RHD* gene. All were positive for antigen C or antigen E or both (Table 1).

In a subsequent survey, we checked 7,374 ccddee samples, which were tested in pools of twenty samples for *RHD* promoter, intron 4 and exon 10. This survey aimed to increase the power of our study for ccddee donors, which represent 92% of all antigen D negative [28]. Two *RHD* positive donors were detected (Table 1). In summary, 50 *RHD* positive donors were found in the two population surveys. They were further characterized by a detailed molecular work-up including *RHD* exon specific PCR-SSP, PCR of intron polymorphism or nucleotide sequencing.

Table 1: Population surveys of D negative blood donors documented D negative and screened by *RHD* PCR-SSP

	Samples (n)		
Documented phenotype	screened	PCR-SSP positive	D positive*
Testing as single samples †			
Ccddee	433	34	0
ccddEe	271	5	2
CCddee	24	4	0
CcddEe	19	4	2
ccddEE	6	1	0
CcddEE	1	0	0
ccddee	314	0	0
Testing as pools of 20 samples ‡			
ccddee	7,374	2	1
Total		50	5

*Samples uncovered on further analysis as weak D, partial D or $D^{+/-}$ chimera. † Positive for at least one of four *RHD* specific polymorphism tested (promoter, intron 4, exon 7 and 3' UTR). ‡Positive for at least one of three *RHD* specific polymorphism tested (promoter, intron 4, and 3' UTR).

Exclusion of five antigen D positive donors

The molecular and serologic work-up revealed that 5 donors, previously documented as antigen D negative, were weakly antigen D positive (Table 1). Two donors of phenotype CcDEe carried *D category VI type I* and *weak D type 2,* respectively. Two donors of phenotype ccDEe carried *D category VI type* I and the new partial D *DIM* [29], respectively. One donor of phenotype ccDee was a $D^{+/-}$ chimera.

Molecular analysis of 45 antigen D negative RHD gene positive samples

The remaining 45 samples were investigated by *RHD* exon specific PCR for exons 3, 4, 5, 6, 7, and 9. Samples with discrepant results for *RHD* promoter and exon 3 were investigated for intron 1 and intron 2, those with discrepant results for exon 7 and 9 were investigated for intron 7 and intron 8, and those with discrepant results for exon 9 and exon 10 were investigated for intron 9. 24 samples could be assigned to one of nine distinct *RHD* PCR patterns and 21 samples were positive for all *RHD* specific polymorphism investigated (Fig. 1). **(i) Hybrid alleles.** The *RHD* PCR patterns could be explained by nine *RHD-CE-D* hybrid alleles (Fig. 1A). Only two of these alleles could be definitively related to prior descriptions of *RHD* positive antigen D negative alleles: One of the three carriers of the *RHD-CE(8-9)-D* allele was a donor previously communicated by us as "CCD^{nex} ee", who was negative in an *RHD* exon 9 PCR [27]. Another pattern was identified as *Cde* ^{s} (Fig. 2 to Fig. 4). We cannot exclude the possibility that some of the seven remaining alleles have been observed previously [18,19,20,21,22, 24,25,26]. Because of the limited published data for those observations, we found more than one "compatible" allele in our study for each previous observation. It should be noted that the hybrid structure was predicted from the PCR pattern and alternative explanations like combinations of two hybrid genes or partial deletions of the *RHD* gene were not formally excluded, **(ii) Other alleles.** The twenty-one samples positive for the nine *RHD* specific polymorphism tested were assigned to one of eight different *RHD* alleles (Fig. 1B). One allele was identical with *RHDψ* [14], the other seven alleles were novel. Each allele was characterized by nucleotide sequencing of the ten *RHD* exons in at least one sample. Once a new allele was characterized, the remaining samples were assigned by nucleotide sequencing of the informative exons (Fig. 1B).

D_{el} phenotype

D_{el} is defined by expressing trace amounts of antigen D that can be detected by an adsorption/elution study only [30]. Because current routine serology cannot discriminate D negative from the D_{el} phenotype, at least one RBC sample of each allele (Fig. 1) was tested by adsorption and elution. Three alleles represented the D_{el} phenotype (Fig. 1B) and were characterized by one missense and two splice site mutations, respectively. For each allele, only a single sample was sequenced, and the influence of the mutations on mRNA splicing was not verified by cDNA analysis. Because lack of material, we could not formally exclude the D_{el} phenotype for the two *RHD-CE(2-7)-D* $_{1}$ samples (Fig. 1A). However, a D_{el} expressed by this allele was very unlikely, as several other hybrid alleles carrying smaller gene conversions, like *RHD-CE(2-7)-D* $_{2}$, were unequivocally D negative.

Population frequencies

The population frequencies of the alleles were calculated (Table 2). The cumulative frequency of all antigen D negative *RHD* gene positive haplotypes was estimated to be 1:1,537. The most frequent allele was *RHD-CE(2-9)-D* $_{2}$ with a frequency of 1:5,682, representing about 27% of antigen D negative *RHD* gene positive alleles. Hybrid alleles lacking *RHD* exon 4 to exon 7 accounted for 68% of antigen D negative *RHD* gene positive alleles. 84% of antigen D negative *RHD* positive haplotypes carried the antigen C, compared to less than 3% of all D negative haplotypes [28]. The cumulative allele frequency of D_{el} was 1:3,030.

Analysis of SCARF samples

We obtained 2 ccddEE, 1 CcddEe and 1 ccddee (G+) DNA samples through the SCARF Exchange and tested them for promoter, intron 4, exon 7 and the 3' untranslated region by *RHD* PCR-SSP. Positive reactions were obtained with the CcddEe sample only, which was assigned to the *RHD-CE(8-9)-D* allele by *RHD* exon-specific PCR-SSP.

Optimized RHD PCR

Based on the population frequencies, we calculated the expected positive predictive values of a positive result for different RHD PCR strategies (Table 3). RHD PCR based on intron 4 and exon 7 had a considerably higher positive predictive value than testing exon 10 alone. Testing for RHD*ψ* [14] improved specificity. Even greater improvements were effected by testing for other allele like RHD-CE(8-9)-D or RHD(W16X). An optimized PCR strategy would comprise checking for RHD intron 4, exon 7 and intron 7 complemented by the specific detection of RHD(W16X) and RHD*ψ* Antigen prediction in the rare samples positive for either of these alleles necessitates complementary methods, since the allele in trans may be D positive. The five polymorphism tested can be multiplexed in two PCR tubes (Fig. 5). This assay was about twice as reliable as current exon scanning approaches [6,26] requiring, if not multiplexed [26], up to 8 separate PCR tubes [6].

Table 2: Estimated population frequencies for antigen D negative *RHD* positive and D_{el} haplotypes in Europeans

	Frequencies			
	In population		Within haplotype	
Allele	Estimate	95% confidence interval	Estimate	Haplotype
D negative alleles				
RHD-CE(2-9)-D $_2$	1:5,682	1:3,046 - 1:13,837	1:62	*Cde*
RHD-CE(2-9)-D $_1$	1:15,152	1:5,610 - 1:55,568	1:167	*Cde*
RHD-CE(8-9)-D	1:15,152	1:5,610 - 1:55,568	1:167	*Cde*
RHD-CE(4-7)-D $_1$	1:18,036	1:6,678 - 1:66,145	1:101	*cdE*
RHD-CE(2-7)-D $_1$	1:22,727	1:6,798 - 1:128,041	1:250	*Cde*
RHD-CE(2-7)-D $_2$	1:22,727	1:6,798 - 1:128,041	1:250	*Cde*
RHD(W16X)	1:22,727	1:6,798 - 1:128,041	1:250	*Cde*
RHDψ	1:37,431	1:7,032 - 1:733,950	1:14,748	*cde*
RHD-CE(4-7)-D $_2$	1:45,455*	1:8,539 - 1:891,266	1:500*	*Cde* or *cdE*
Cde ^{s}	1:45,455	1:8,539 - 1:891,266	1:500	*Cede*^{s}
RHD(G212V)	1:45,455	1:8,539 - 1:891,266	1:500	*Cde*
RHD(Y330X)	1:45,455	1:8,539 - 1:891,266	1:500	*Cde*
RHD(IVS8+1G>A)	1:45,455	1:8,539 - 1:891,266	1:500	*Cde*
RHCE(1-9)-D	1:54,107	1:10,164 - 1:1,060,924	1:303	*cdE*
associated with *Cde*	1:1,818	1:1,262 -1:2,711	1:20	*Cde*
associated with *cdE*	1:13,527	1:5,638 - 1:39,610	1:75	*cdE*
associated with *cde*	1:37,431	1:7,032 - 1:733,950	1:14,748	*cde*
Total	1:1,537	not applicable	not applicable	
D $_{el}$ *alleles*				
RHD(M295I)	1:6,493	1:3,302 - 1:13,837	1:71	*Cde*
RHD(K409K)†	1:9,091	1:4,067 - 1:23,073	1:100	*Cde*
RHD(IVS3+1G>A)	1:15,152	1:5,610 - 1:55,568	1:167	*Cde*
Total	1:3,030	1:1,913 - 1:5,610	1:33	*Cde*

* Assuming a *Cde* haplotype; a *cdE* haplotype would result in a frequency of 1: 54,107 (95% confidence interval: 1:10,164 - 1:1,060,924; frequency within haplotype 1:303). † Silent mutation adjacent to an intron/exon boundary, probably affecting splicing.

Gene conversions in cis

The gene conversion of five hybrid alleles observed in this study involved intron 2 (Fig. 1) which was utilized to delineate the allele origin of the *RHCE* gene segment found in the *RHD* gene. In all five haplotypes, a gene conversion *in cis* was likely (Fig. 6A). We proposed that gene conversions *in cis* occur during hairpin formation, which is favored by the clustered gene arrangement (Fig. 6B).

Anti-D immunizations

A flow cytometry study of the RBC from the $D^{+/-}$ chimera revealed 94% D negative RBC and an admixture of 6 % D positive RBC (Fig. 7). This chimera was confirmed in a 3 month follow-up. The 24 year old donor was healthy and had no twin. A look-back of this donor revealed 13 units that had been issued as D negative. Two D negative recipients were traced and available for an antibody screen, both of whom were anti-D immunized.

Table 3: Expected rates of false positive results and expected positive predictive values for different *RHD* PCR strategies*

PCR strategy	Rate of false positives	Positive predictive value of positive result	Number of polymorphism tested
Exon 10 only [1,2]	1:1,276	0.999216	1
Intron 4/Exon 7 [13]	1:4,081	0.999755	2
Intron 4/Exon 7/*RHDψ* [14]	1:4,700	0.999787	3
Intron 4/Exon 7/W16X	1:5,212	0.999808	3
Intron 4/Exon 7/Intron 7	1:6,051	0.999835	3
Exons 3, 4, 5, *6, 7, 9* [26]	1:6,051	0.999835	6
Exons 2, 3, 4, 5, 6, 7, 9, 10 [27]	1:6,051	0.999835	8
Intron 4/Exon 7/W16X/*RHDψ*	1:6,267	0.999840	4
All *Exons*/*RHDψ*	1:7,520	0.999867	9
Intron 4/Exon 7/Intron 7/W16X	1:8,921	0.999888	4
Intron 4/Exon 7/Intron 7/W16X/*RHDψ*	1:12,533	0.999920	5

* Rates were calculated based on the population frequencies of different alleles determined in the Table 2. The exact rates are population dependent and may vary according to the prevalence of alleles in the population tested.

Discussion

In a systematic population survey including more than 8,000 antigen D negative blood donations, we identified 14 different *RHD* positive antigen D negative and 3 different D_{el} haplotypes, the majority of which were novel. The molecular bases were alleles comprising *RHD/RHCE* hybrids, stop codons, missense mutations and splice site mutations. The cumulative frequency of *RHD* gene positive antigen D negative haplotypes was about 1:1,500; that of the D_{el} alleles was about 1:3,000. We determined the specificity of antigen D prediction by PCR and devised an optimized *RHD* PCR strategy with a calculated positive predictive value greater than 0.9999. Five antigen D positive samples missed by routine D typing were uncovered and two anti-D immunizations traced.

For practical purposes, two groups of *RHD* alleles that do not express antigen D can be distinguished. *RHD* alleles of the first group lack some or many *RHD* specific polymorphism and usually represent *RHD/CE* hybrids. For alleles of this group, a correct antigen D prediction may be accomplished by a prudent selection of the *RHD* specific polymorphism utilized for *RHD* genotyping. *RHD* alleles of the second group carry all *RHD* specific polymorphism and most often harbor point mutations. For alleles of this group, a correct antigen D prediction necessitates the specific detection of an aberration that is usually unique to the allele. The identification of four new alleles in this group increased the number of known alleles from 3 to 7 and was critical for improving *RHD* genotyping.

The data of this study allowed for the first time to calculate population frequencies of *RHD* positive antigen D negative and D_{el} alleles. This information was indispensable to derive rational *RHD* typing strategies and will be essential for establishing cost-efficient approaches. The majority of samples belonging to the first group of D negative alleles (probable *RHD/CE* hybrids) was compatible with *RHD-CE-D* hybrid alleles, in which the DNA segment derived from the *RHCE* gene encompassed at least exon 4 to exon 7. These samples would be correctly typed, if exon 4/intron 4 and exon 7 were used for *RHD* genotyping, as proposed previously [13]. With the exception of *RHD* exon 9, testing additional *RHD* exons would not have improved the specificity of antigen D prediction. Improving this specificity, however, became possible by the specific detection of frequent alleles of the second group, like *RHDψ* and *RHD*(W16X). We demonstrated that testing 5 carefully selected polymorphism would have resulted in an assay yielding false positive results at a rate less than 1:12,000, and hence would have doubled the specificity compared to contemporary approaches testing all informative *RHD* exons [6,26]. Further improvements may be achieved by the specific detection of additional alleles, that might become practical in massively parallel molecular assays.

The detailed analysis including intron polymorphism revealed that the first group of alleles (probable *RHD/CE* hybrids) represented at least 9 different molecular events. We proposed that the proximity and inverse orientation of both *RH* genes favored gene conversions occurring *in cis* (Fig. 6), which have also been noted in partial D [31]. An exact definition of the molecular bases of the *RHD/CE* hybrids would allow their specific detec-

tion, even if they were positioned *in trans* to the regular *RHD* allele. Such a detection would be necessary, if molecular *RH* zygosity testing is expected to achieve the same specificity as antigen D prediction.

A considerable proportion of seemingly D negative samples carrying the *RHD* gene presented a D_{el} phenotype. Interestingly, *RHD*(M295I) coded for weak D, if associated with a *ce* haplotype [32], but for D_{el}, if associated with a *Ce* haplotype; this observation may be explained by the suppressive effect of C *in cis* [33].

The nature and frequency of *RHD* gene positive antigen D negative alleles differ among populations. Apart from a probably lower absolute frequency, we detected in Europeans many parallels to oriental populations: Both populations shared the diverse nature of *RHD* haplo

types of the first group (probable *RHD/CE* hybrids) [18,19,20], the preferential occurrence of *RHD* positive antigen D negative alleles in *Cde* haplotypes [18], and the comparatively frequent observation of D_{el} phenotypes [19]. In contrast, *RHDψ* and Cde^s are predominant in African populations [14]. Still another situation may be present in the middle-west USA, where 6 of 26 *RHD* gene positive antigen D negative samples had aberrations limited to a single exon yet detectable by PCR [34].

Blood group serologists might note the observation of 5 D positive samples in our study with disturbance. In many centers, donors are checked for antigen D by sensitive methods at first and second donations only. On subsequent donations, carriers of partial D, like D^{vI} or DIM, some weak D and $D^{+/-}$ chimerism may pass unnoticed in tests based on direct agglutination, even with the most avid IgM anti-D. Immunizations caused by units of such donors will generally be missed, because the occurrence of an anti-D in a patient is usually not further investigated [35]. For example, the two anti-D immunizations induced by units of the chimerical donor of this study were found only in a look-back triggered by our molecular screen. Chimeras in the Rh system have repeatedly been observed [36,37] and chimeras may be a more widespread phenomenon than anticipated [38]. A lower antigen density threshold for anti-D immunization has not been established yet, and future studies might indicate a need to exclude even D_{el} donors from transfusion to D negative recipients. A routine investigation of all samples by adsorption and elution is not feasible. However, checking D-negative samples, especially those occurring with a C or E or both, for *RHD* specific sequences by nucleic amplification techniques may become practical in the near future. The knowledge of the detected alleles is also important for fetal genotyping assays using fetal DNA in maternal plasma, because false positive results will be obtained in mothers harboring *RHD* positive D negative alleles.

Subjects and Methods

Blood samples

EDTA- or citrate-anticoagulated blood samples were collected from blood donors characterized as D negative in routine typing including an antiglobulin test with anti-D. The D antigen determination in antiglobulin technique was performed as part of routine blood donor typing over a period of more than 15 years with varying commercial anti-D in tube or column agglutination. For each donor, this antiglobulin test was done only once, if the donor lacked the antigens C or E, and two times from independent samples, if the donor was either C or E positive. Subsequent donations were checked by direct agglutination using an Olympus PK7200 autoanalyzer only. Samples were collected at random for specific CcEe phenotypes. DNA was isolated by a modified salting-out procedure as described [27,39].

Screening by PCR with sequence specific priming (PCR-SSP)

For the first population survey, 314 ccddee, 433 Ccddee, 271 ccddEe, 19 CcddEe, 24 CCddee, 1 CcddEE and 6 ccddEE samples were tested individually for the presence of *RHD* specific polymorphism located in the *RHD* promoter, intron 4, exon 7 and the 3' untranslated region of exon 10 by PCR-SSP. The donor previously reported as "CCD-^{nex}ee" [27] returned by chance and his allele was further characterized as *RHD-CE(8-9)-D*.

For the second population survey, 7,374 ccddee samples were analyzed in pools. Equal volumes of 20 samples were mixed. To confirm the sensitivity of the pool testing, 1% of D positive blood was added to an aliquot as positive control. DNA was extracted and checked for *RHD* promoter, intron 4, and exon 10 using modified PCR-SSP to enhance sensitivity. Repeated testing of donors was minimal, because the mandatory donation interval exceeded the collection period.

The donors were representative for the population currently living in the county of *(Land)* Baden-Württemberg. The ethnic origin of individual donors, in particular of those carrying Cde^s or *RHDψ* was not identified. Independent of the population surveys, 2 ccddEE, 1 CcddEe and 1 ccddee DNA samples of unknown ethnic backgrounds were obtained from the SCARF Exchange (Hahnemann University, Philadelphia, USA).

Further molecular characterization

All samples positive for any of the above mentioned PCR-SSP assays were further investigated for the presence of *RHD* specific polymorphism in exon 3, exon 4,

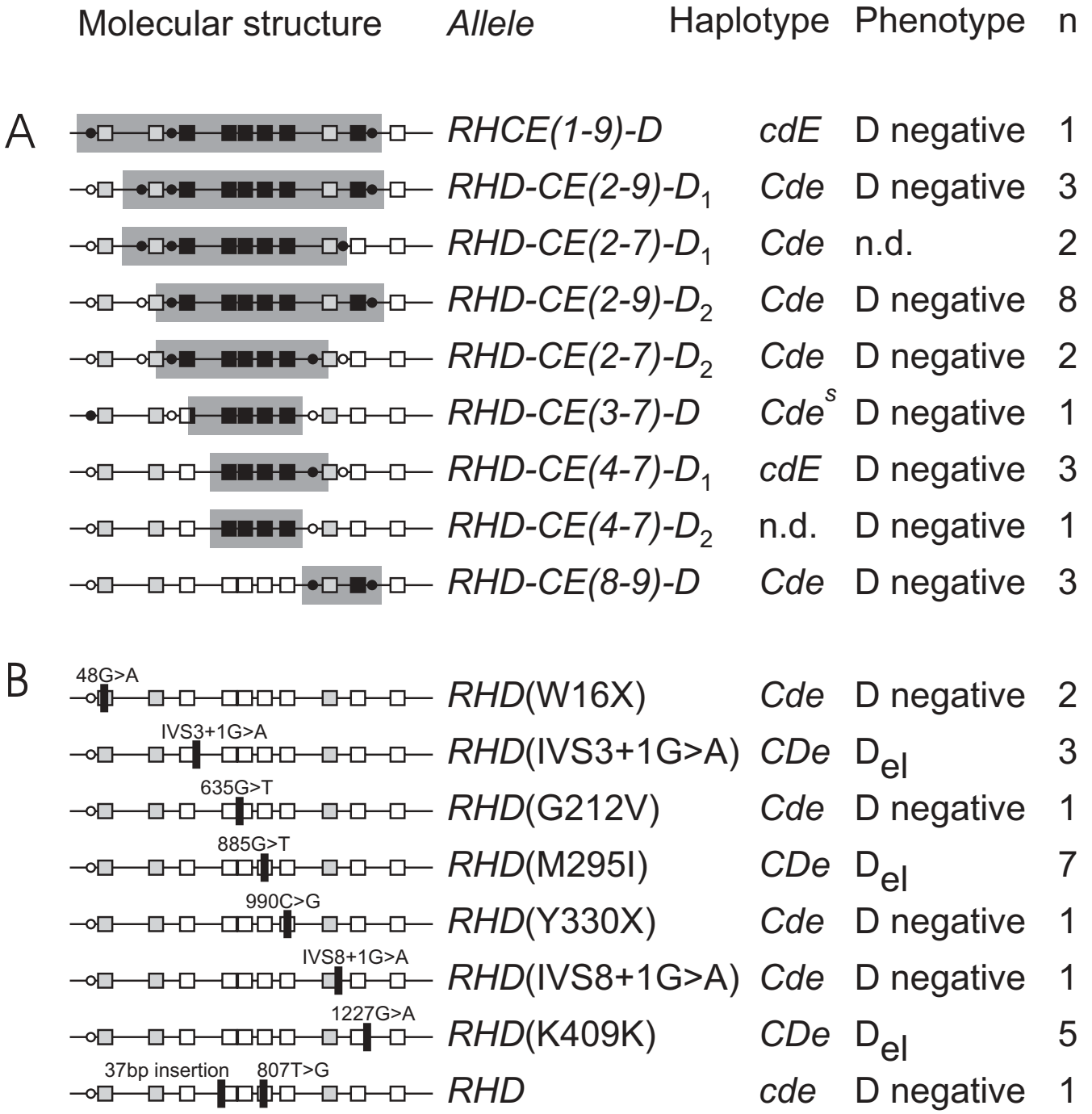


Figure 1
Predicted molecular structure of the 17 *RHD* positive, D negative or D_{el} alleles detected. For each haplotype, a schematic representation of the molecular structure is shown along with a designation, haplotype association, phenotype, and numbers of samples observed. Each *RHD* exon is indicated by a box, intron and promoter polymorphism investigated are shown as circles. White symbols indicate the presence of *RHD* specific sequences, black symbols their lack as predicted form the *RHD* exon-specific PCR-SSP results. Exons 1, 2, and 8 are shown in gray, because they are identical in *RHD* and some *RHCE* alleles. Panel A: Hybrid alleles. The molecular structures are represented as single hybrid alleles; it should be noted that the PCR patterns could also be caused by combinations of hybrid alleles or by partial *RHD* deletions. Panel B: Other alleles. The nature of the aberration is indicated, and its position visualized by a vertical bar. The *RHD(M295I)* allele is similar to weak D type 11 [32] but represents a different haplotype and phenotype.

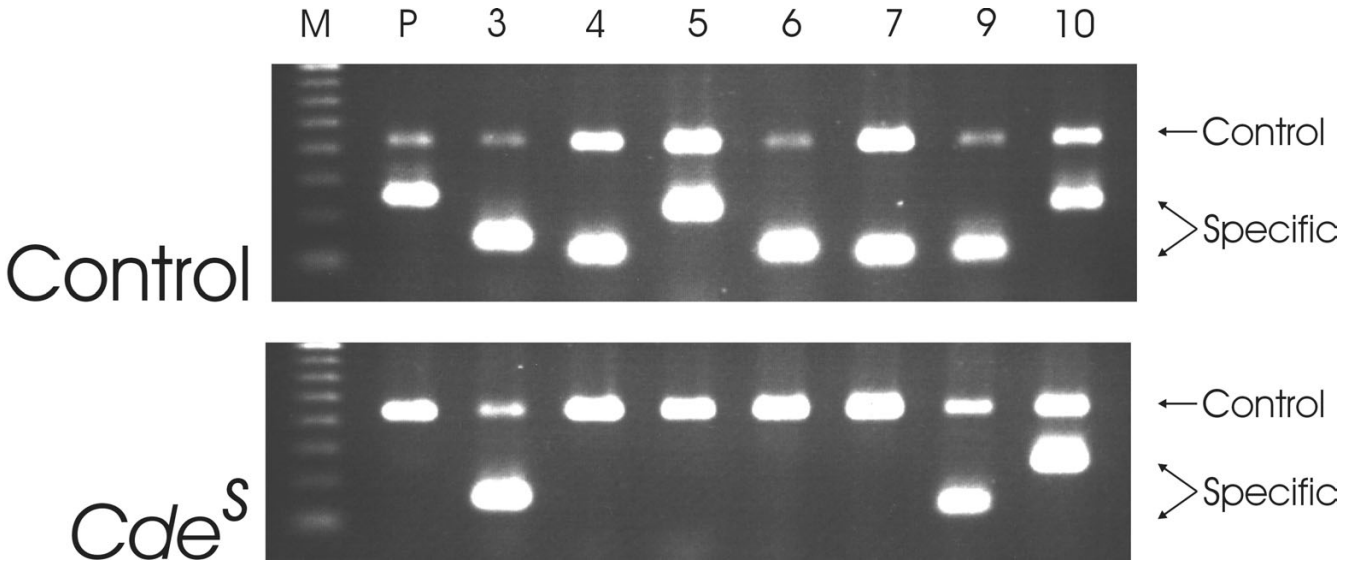


Figure 2

RHD exon specific PCR-SSP of *Cde*^{s}. In an *RHD* positive control, *RHD* specific PCR products are obtained for the *RHD* promoter (lane marked P, 255 bp), exon 3 (154 bp), exon 4 (123 bp), exon 5 (228 bp), exon 6 (133 bp), exon 7 (123 bp), exon 9 (119 bp) and exon 10 (232 bp). The 434 bp control product derives from the *HGH* gene. In the *Cde*^{s}sample, *RHD* specific amplicons are obtained for exon 3, exon 9, and exon 10, only.

exon 5, exon 6, exon 7, and exon 9 by PCR-SSP. Samples positive for all PCR-SSP were sequenced, until they could be assigned to a distinct *RHD* allele. Samples negative for some PCR-SSP were checked for informative polymorphism in intron 1, intron 2, intron 7, intron 8, and intron 9.

Analysis for Cde^s and RHDψ

RHD(N152T) and *RHCE*(L245V) present in *Cde*^{s} [16] were checked by PCR-SSP. The 37 bp insertion present in *RHDψ* was detected by PCR-SSP. The 37 bp insertion, the M218I, F223V and S225F missense mutations and the Y269X nonsense mutation previously described for *RHDψ* [14] were confirmed by sequencing of all 10 exons; no additional aberrations were detected.

Nucleotide sequencing

The ten *RHD* exons were sequenced as described [29,32]. The promoter was amplified with primers rend31k (for *RHD* alleles) or re04 (for *RHCE)* and rb45 and a DNA stretch encompassing primer re012 was sequenced using primers re08 and re09.

RHD PCR

Most *RHD* PCR-SSP were similar to the *RHD* exon specific PCR-SSP previously described [27]. Cycling conditions consisted of an initial denaturation of 2 min at 94°C, followed by ten cycles of 10 s denaturation at 94°C and 1 min annealing/extension at 65°C; and finally 25 cycles of 30 s denaturation at 94°C, 1 min annealing at 61°C and 30 s extension at 72°C. 0.4 U Taq polymerase (Qiagen, Hilden, Germany) were used in a final volume of 10 μl. Primers (Table 4) were re012 and re011d for the promoter; re41 and rb12 for intron 4; ga71 and ga72 for exon 7 in the PCR-SSP screening; rea7 and rr4 for exon 10; ga31 and rb21 for exon 3; ga41 and ga42 for exon 4; rb24 and ga51 for exon 5; ga62 and ga61 for exon 6; rb26 and re71 for exon 7 in the molecular work-up; re83 and re94 for exon 9; rb51 and rb52 for intron 7; RhPsiF and RhPsiB for *RHDψ;* Rh152Tb and ga31 for *RHD*(N152T); and Rh223Vf and Rh245Vb for *RHCE*(L245V). Primer concentrations were 0.2 μM except for exon 6 (0.1 μM), *RHD*(N152T)(0.3 μM), and intron 7 and exon 9 (both 0.4 μM). For most samples intron 4/exon 7 was tested as multiplex reaction containing 0.2 μM of exon 7 (ga71/ga72) and 0.1 μM of intron 4 primers. As internal control, two primers amplifying an *HGH* gene fragment were added in concentrations of 0.05 μM for promoter, intron 4, and exon 7 (ga71/ga72); 0.075 μM for exon 10; 0.1 μM for intron 7, RHDψ, *RHD*(N152T) and RHCE*(L245V);* 0.15 μM for exon 3, exon 4, exon 7 (rb26/re71), and exon 9; 0.2 μM for exon 5 and exon 6. Mg^{2+} concentration was 0.15 μM, except 0.4 μM for intron 7. For exon 6, 20 % solution Q (Qiagen) was added. To enhance sensitivity, the pools were tested with *RHD* primers in a concentration of 0.3 μM and *HGH* primers at 0.1 μM.

Intron 1 was tested 1173 and 1174 bp 5' of the intron 1/exon 2 boundary by *RHD* specific amplification of exon 2 as described [32]. Intron 2 was evaluated by PCR with

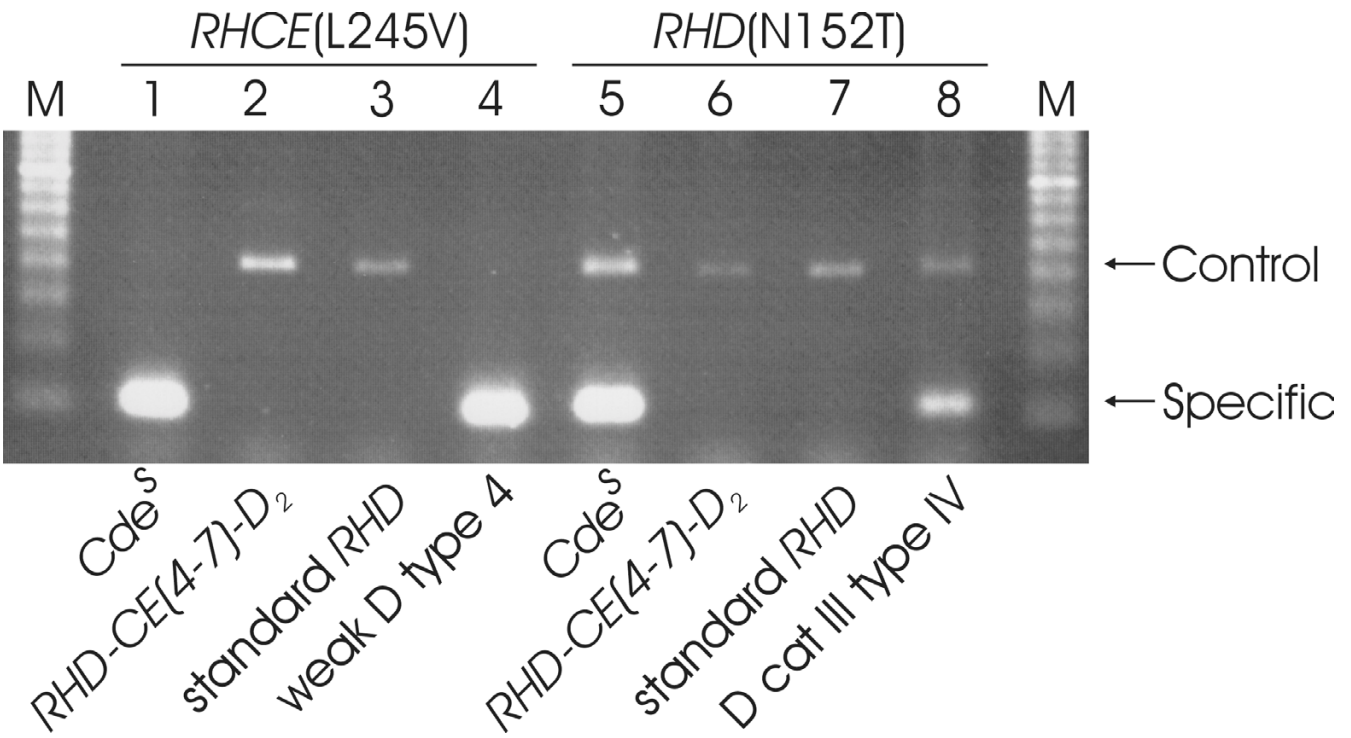


Figure 3

Demonstration of the *RHCE*(L245V) and *RHD*(N152T) substitutions characteristic of *Cde*^{s}. PCR-SSP were performed to detect single nucleotide polymorphism characteristic for *Cde*^{s} [16] and indicative of *RHCE*(L245V) (lanes 1 to 4, 110 bp specific product) and *RHD*(N152T) (lanes 5 to 8, 120 bp specific product). Both polymorphism were present in the *Cde*^{s} sample as expected (lanes 1 and 5). The *RHD-CE(4-7)-D* $_2$ sample was compatible with *Cde*^{s} according to the *RHD* exon specific PCR (Fig. 1) but lacked both polymorphism (lanes 2 and 6). Negative controls were standard *RHD* (lanes 3 and 7), positive controls weak D type 4 (lane 4) and D^{III} type IV (lane 8), respectively.

length polymorphism as described [31]. The *BamH*I restriction site introduced by the 9 bp deletion in *RHD* intron 8 position 1114 to 1122 (Genbank accession number AL139426) was checked after amplification with primers re74 and re93 and digestion with *BamH*I. The 980 bp deletion starting at position 633 in *RHD* intron 9 (Genbank accession number AL139426) was evaluated using primers re93k and re916.

Optimized RHD PCR-SSP for routine DNA typing

Reaction A contained primers ga71 and ga72 at 0.3 μM, rb12 and re41 at 0.1 μM, and *HGH* primers at 0.1 μM. Mg^{2+} was at 0.175 μM. Reaction B contained primers RhPsiF and RhPsiB at 0.5 μM, re11d and RhX1f1 at 0.3 μM, re721 and rb9 at 0.2 μM and as control primers rend9b1 and rend9b2 at 0.2 μM. Mg^{2+} was at 0.15 μM.

Immunohematology

One sample of each *RHD* positive allele was evaluated by direct agglutination with two monoclonal anti-D (Seraclone anti-D, clone BS226; Biotest, Dreieich, Germany; and Frekaklon anti-D, clone MS201; Gull, Bad Homburg, Germany). Indirect antiglobulin test was done in a gel matrix test (LISS-Coombs 37°C, DiaMed-ID Micro Typing System, DiaMed, Cressier sur Morat, Switzerland) using an oligoclonal anti-D (Seraclone anti-D blend, clones H41 11B7, BS221 and BS232; Biotest). Samples reactive in gel matrix technique were further investigated using the monoclonal anti-D HM10, HM16, P3x61, P3x35, P3x212 11F1, P3x212 23B10, P3x241, P3x249, P3x290 (Diagast, Loos, France) and H41 11B7 (Biotest). The presence of a D_{el} phenotype was determined by adsorption of 500 μl of a polyclonal anti-D (human incomplete anti-D; Lorne Laboratories, Reading, UK) to 500 μl packed red blood cells (RBC) for 1 h at 37°C and elution using a chloroform technique [35]. A detailed serologic report of the *RHD*(C285Y) sample, dubbed DIM [29], has been published separately.

Flow cytometry

Flow cytometry was performed as described [40,41] using a polyclonal anti-D (anti-D Molter; Ortho Clinical Diagnostics, Neckarsgmünd, Germany) as primary and goat anti-human IgG, F_{ab}-fragment, FITC-conjugated

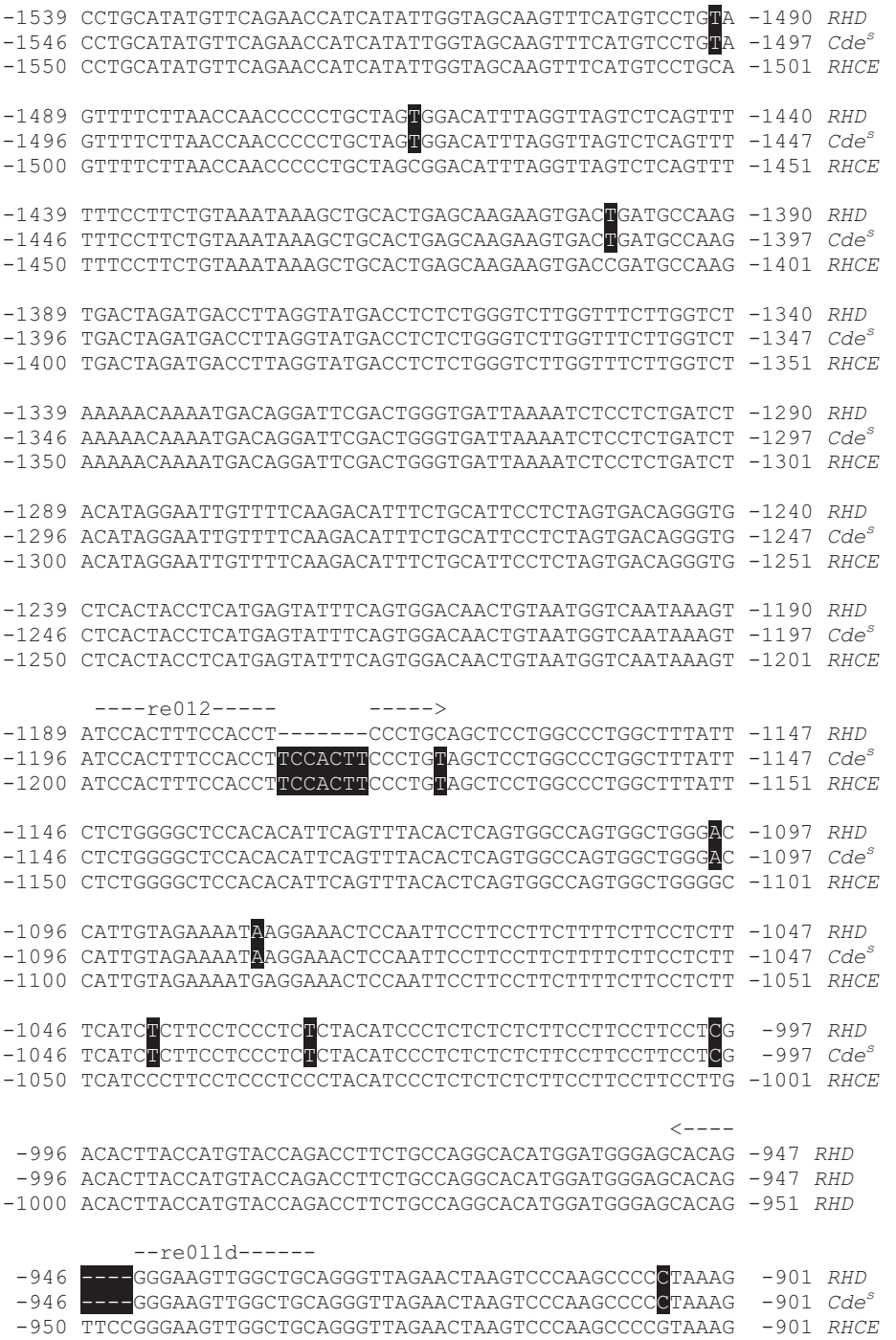


Figure 4

Molecular cause of the negative *RHD* promoter PCR in *Cde*^{s}. The nucleotide sequence of the *Cde*^{s} , *RHD* and *RHCE* promoter reaching from about 1550 to 901 bp 5' of the A of the start codon is shown. The positions of the *RHD* specific primers re011d and re012 used for the *RHD* promoter PCR are given. Nucleotides indicating *RHD* or *RHCE* origin of the *Cde*^{s} sequence are highlighted. The *Cde*^{s} promoter sequence represents *RHD*. A small DNA stretch of at least 13 bp in the region of re012 is replaced by the corresponding sequence of *RHCE*. This gene conversion caused the negative result obtained in the *RHD* promoter PCR-SSP (Fig. 2). *RHCE* nucleotide sequence is according to GenBank accession number AL031284.

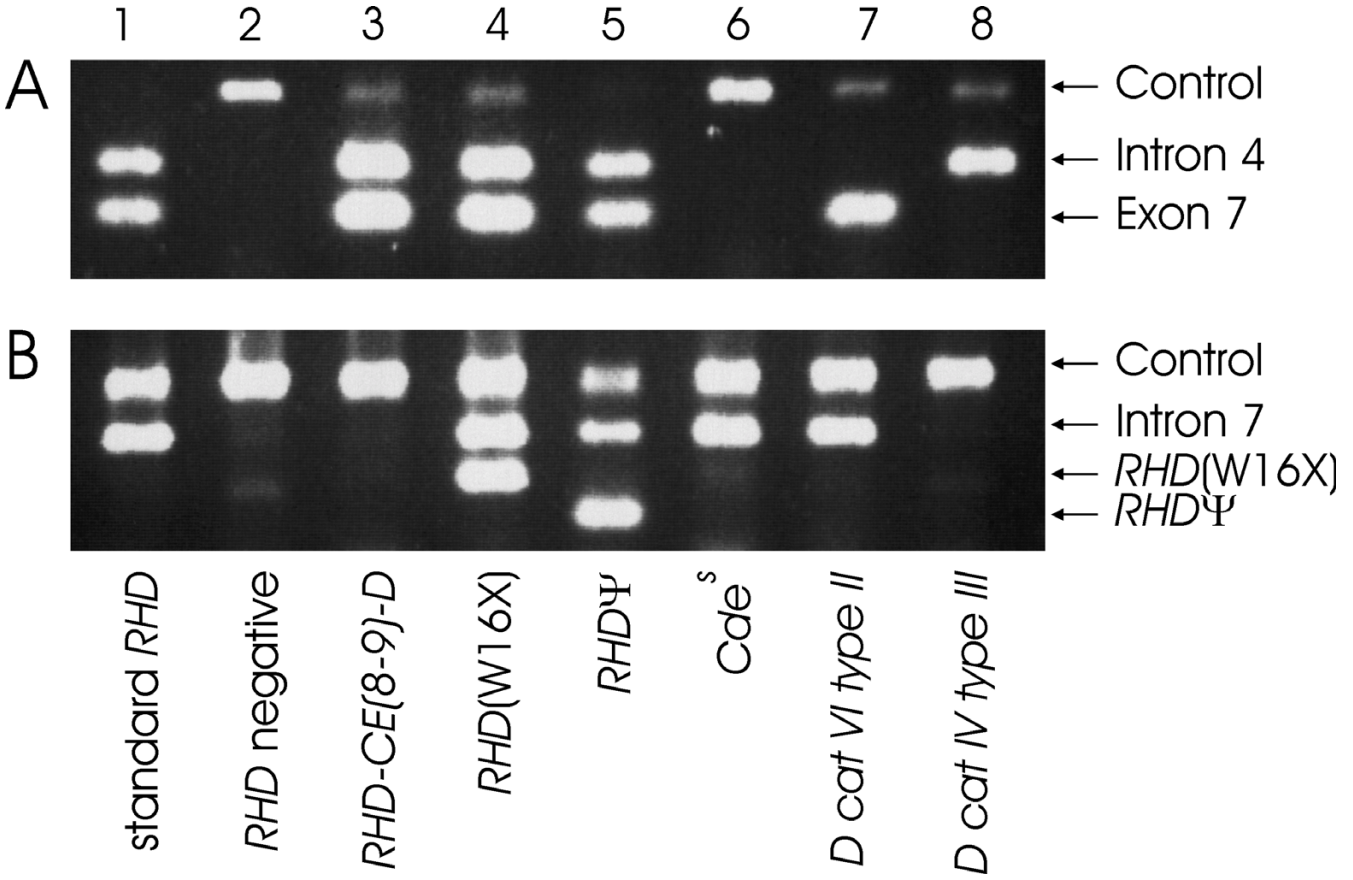


Figure 5
RHD PCR-SSP optimized for specificity. The PCR is performed as a modular system consisting of two multiplex reactions. An *RHD* intron 4/exon 7 multiplex PCR-SSP (Panel A) is combined with an *RHD* intron 7 PCR that is multiplexed with reactions for the specific detection of *RHD*(W16X) and *RHDψ* (Panel B). Results are shown for a normal D positive sample (lane 1), a normal D negative sample (lane 2), several rare D negative samples (lanes 3 to 6) and major D positive *RHD* variants (lanes 7 and 8). Standard D positive and D negative samples and D categories VI and IV are recognized in panel A. *RHD-CE(8-9)-D* is detected in panel B by the absence of the intron 7 band (lane 3). The presence of *RHD*(W16X) and *RHDψ* is detected in panel B because of their specific amplicons (lanes 4 and 5). Amplicon size is Panel A, control, 434 bp (*HGH* gene); intron 4, 226 bp; exon 7, 123 bp; Panel B, control, 659 bp (chromosome 1 genomic sequence about 90,000 bp 5' of *Rhesus box);* intron 7, 390 bp; *RHD*(W16X), 248 bp; *RHDψ,* 154 bp. The internal control amplicons, which were devised to be larger than the specific amplicons, may be suppressed because of competition, if a specific product is amplified.

(Dianova, Hamburg, Germany) as secondary antibody. Markers were set to encompass >99.5% of a D positive control and less than 0.5% of a D negative control. The percentage of cells in the marker area was evaluated.

Haplotype frequencies

For alleles observed more than once, the haplotype association with Cde and cdE was obvious, because of their repeated observations in association with the rare phenotypes Ccddee or ccddEe, respectively. Based on the paucity of RHD positive samples among the ccddee samples, alleles that were observed only once were assumed to be associated with the Cde or cdE haplotype rather than the cde haplotype. An allele occurring in an unique CcddEe sample was counted as Cde. The RHDψ allele was assumed to be associated with the ce(W16C) allele, because RHCE specific sequencing of exon 1 revealed a C/G heterozygosity at position 64 and ce(W16C) is almost absent from the cde haplotypes in our population [27]. The frequency of a given aberrant RHD allele in its haplotype was calculated as the number of observed samples divided by the number of the corresponding haplotypes under observation (500 Cde, 303 cdE). For cde, the haplotype frequency was calculated from the 14,748 haplotypes checked in the second survey. The population frequency of an RHD allele was calculated from the frequency of this allele in its haplotype and the known frequency of the haplotype in the local population [28]. Confidence intervals were calculated according to the Poisson distribution [42]. Donors were not generally

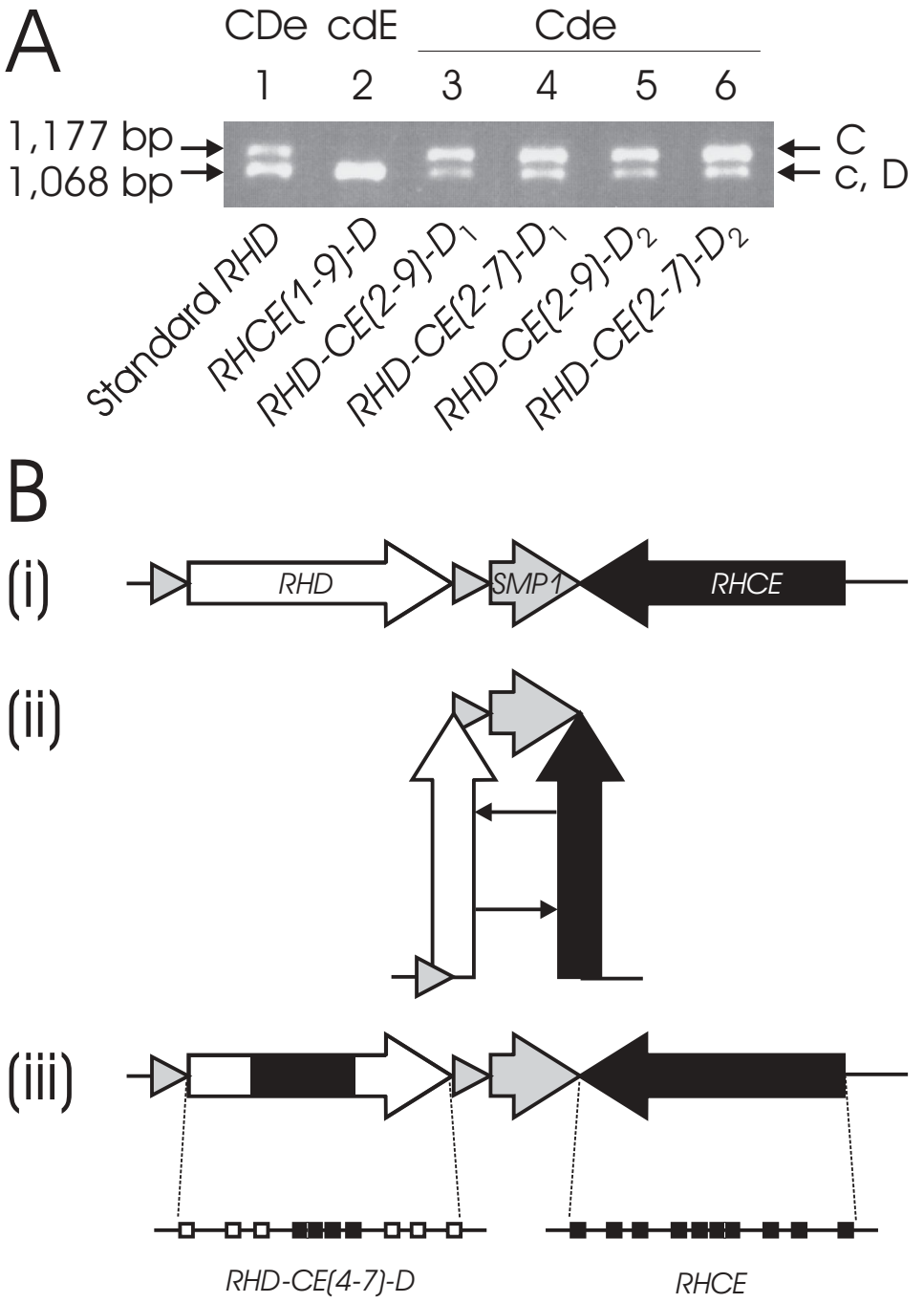


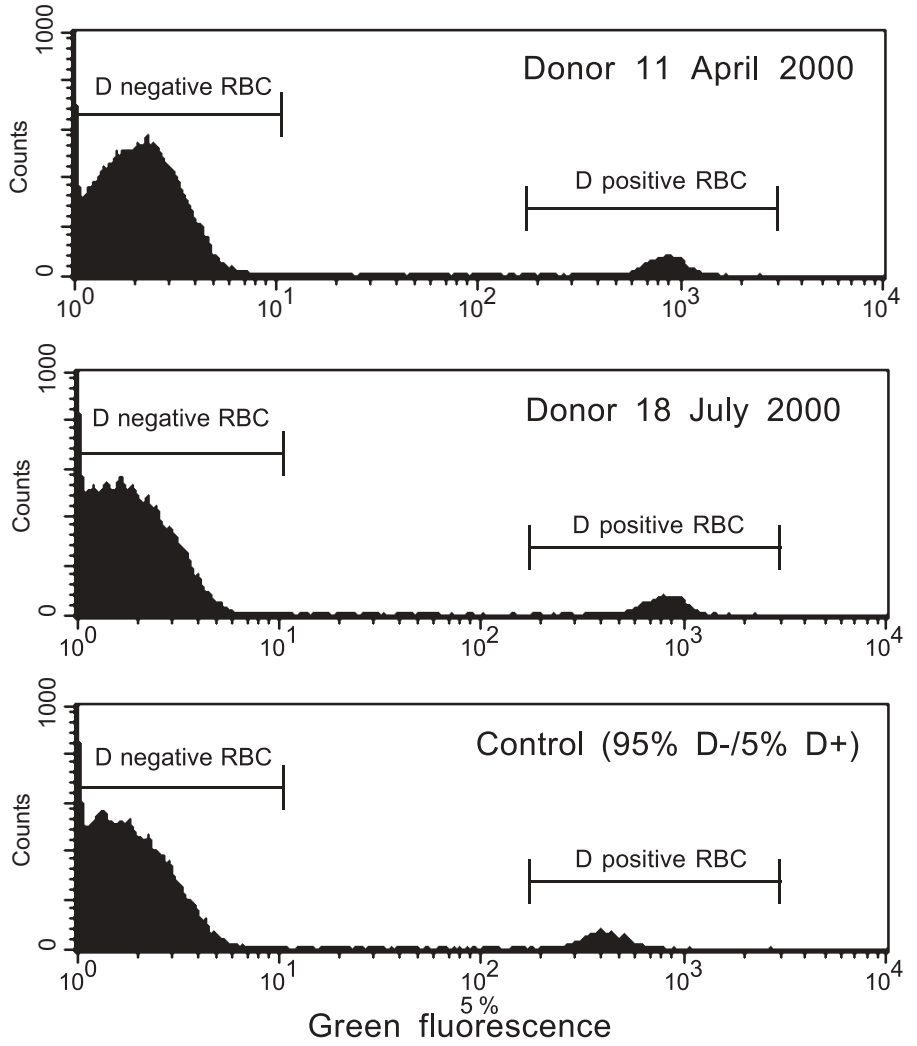
Figure 6
Gene conversion *in cis.* Panel A: Origin of the *RHCE* gene segments. The allele origin of the *RHCE* segments in the *RHD* gene was analyzed by a PCR length polymorphism in intron 2 [31,43]. The 1,177 bp product is specific for the *C* allele of *RHCE,* the 1,068 bp product for the *c* allele of *RHCE* and for *RHD*. The CcDee control shows a strong band at the c/D position and a weaker band at the C position (lane 1). The cE associated *RHCE(1-9)-D* hybrid allele lacks the C band (lane 2), indicating that the intron 2 of the hybrid allele derives from c. In contrast, all Ce associated hybrid alleles involving intron 2 show a strong C band and a weaker c/D band (lanes 3 to 6), indicating that the introns 2 of those hybrids derive from C. Panel B: Proposed mechanism of gene conversion *in cis.* (i) The *RHD* and *RHCE* genes are inversely orientated [7] as typical for clustered genes. (ii) A putative hairpin formation of the chromosome allows the close proximity of homologous segments in identical orientation. This structural feature is instrumental for gene conversion events *in cis.* (iii) Resolving the hairpin yields an *RHD-CE-D* hybrid gene structure, many of which have been observed to date at the *RH gene* locus. As an example, the *RHD-CE(4-7)-D* hybrid exon structure is shown. Symbols are according to Fig. 1.

Table 4: Primers used

Name	Nucleotide sequence	Genomic region	Position*	Strandedness	specificity
ga31	ttgtcggtgctgatctcagtgga	exon 3	362 to 383	sense	*RHD*
ga41	acatgatgcacatctacgtgttcgc	exon 4	503 to 527	sense	*RHD*/*RHCE*
ga42	cagacaaactgggtatcgttgctg	exon 4	625 to 602	antisense	*RHD*/*RHCE*
ga51	ctgctcaccttgctgatcttccc	intron 5/exon.	5 8 to 787	antisense	*RHD*
ga61	caggtacttggctcccccgac	exon 6	936 to 916	antisense	*RHD*
ga62	ttatgtgcacagtgcggtgttgg	exon 6	804 to 826	sense	*RHD*/*RHCE*
ga71	gttgtaaccgagtgctggggattc	exon 7	944 to 967	sense	*RHD*/*RHCE*
ga72	tgccggctccgacggtatc	exon 7	1066 to 1048	antisense	*RHD*
rb12	tcctgaacctgctctgtgaagtgc	intron 4	198 to 175	antisense	*RHD*
rb21	aggtccctcctccagcac	intron 3	28 to 11	antisense	*RHD*/*RHCE*
rb24	agacctttggagcaggagtg	intron 4	-53 to -34	sense	*RHD*/*RHCE*
rb26	aggggtgggtagggaatatg	intron 6	-62 to -43	sense	*RHD*/*RHCE*
rb45	acactgttgrctgaatttcggtgc	intron 1	164 to 139	antisense	*RHD*/*RHCE*
rb51	gcatgacgtgttctgcctcttg	intron 7	-3365 to - 3386	antisense	*RHD*
rb52	ccaggttgttaagcattgctgtacc	intron 7	-3433 to -3409	sense	*RHD*
re04	aggtcacatccatttatcccactg	promoter	-2498 to -2474	sense	*RHD*/*RHCE*
re08	gggcttgggacttagttctaac	promoter	-858 to -879	antisense	*RHD*/*RHCE*
re09	cgactgggtgattaaaatctcc	promoter	-1280to-1259	sense	*RHD*/*RHCE*
re011d	gcagccaacttcccctgtg	promoter	-883 to -905	antisense	*RHD*
re012	tccactttccacctccctgc	promoter	-1148to-1122	sense	*RHD*
relld	agaagatgggggaatctttttcct	intron 1	129 to 106	antisense	*RHD*/*RHCE*
re41	cgatacccagtttgtctgccatgc	exon 4	608 to 631	sense	*RHD*/*RHCE*
re71	acccagcaagctgaagttgtagcc	exon 7	1,008 to 985	antisense	*RHD*
re74	tatccatgaggtgctgggaac	intron 7	-244 to -224	sense	*RHD*/*RHCE*
re721	ctggaggctctgagaggttgag	intron 7	-348 to -326	sense	*RHD*
re83	gagattaaaaatcctgtgctcca	intron 8	-54 to - 34	sense	*RHD*/*RHCE*
re93	cacccgcatgtcagactatttggc	intron 9	320 to 297	antisense	*RHD*/*RHCE*
re93k	gccaaatagtttgacatgcgggtg	intron 9	297 to 320	sense	*RHD*/*RHCE*
re94	cttggtcatcaaaatatttagcct	exon 9	1216 to 1193	antisense	*RHD*
re916	gtttttgaggcaaagtctcgctc	intron 9	1689 to 1666	antisense	*RHD*/*RHCE*
rea7	tgttgcctgcatttgtacgtgag	3' UTR†	1311 to 1333	sense	*RHD*/*RHCE*
rend31k	cctccccaacccagacagaattag	AJ252311‡	8506 to 8529	sense	not applicable
rend9b1	cactgcacttggcaccattgag	AL031432	29489 to 29468	antisense	not applicable
rend9b2	ttccgaaggctgcttttccc	AL031432	28840 to 28859	sense	not applicable
Rh152Tb	gatattactgatgaccatcctcatgg	exon3	480 to 455	antisense	*RHCE*
Rh223Vf	ttgtggatgttctggccaagtg	exon 5	646 to 667	sense	*RHCE*
Rh245Vb	gctgtcaccactctgactgctac	exon5	755 to 733	antisense	*RHD*
RhPsiB	tctgatctttatcctccgttccctc	exon 4	601 to 577	antisense	*RHD*
RhPsiF	agacagactaccacatgaacttac	intron 3	-38 to-15	sense	*RHDψ*
RhX1f1	cgctgcctgcccctctga	exon 1	31 to 48	sense	*RHD*(W16X)
rr4	agcttactggatgaccacca	3'UTR	1,541 to 1,522	antisense	*RHD*

*The positions of the synthetic oligonucleotides are indicated relative to their distances from the first nucleotide position of the start codon ATG for all primers in the promoter and in the exons including the 3' untranslated part of exon 10, relative to their adjacent exon/intron boundaries of *RHCE* for primers in introns; and according to the numbering in the genomic sequences indicated. Primers rh1 [44], ga31 (previously dubbed D-3-383), ga41 (D-4-527), ga42 (D-4-602), ga51 (D-5-787), ga61 (D-6-916), ga62 (D-6-826), ga71 (D-7-967), ga72 (D-7-1048) [27], rb5, rb12, rb24, rh5, rh7 [31], rb21, rb26, re11d, re71, re74, re83, re93, rr4 [32], re012 [29], re011d and rea7 [7] have been published previously. † 5' UTR: 5' untranslated region of exon 1; 3' UTR: 3' untranslated region of exon 10. ‡ Accession number of nucleic acid sequence in EMBL/GenBank/DDBJ; AJ252311 represents upstream *Rhesus box;* AL031431 Chromosome 1 genomic clone dJ465N24.

checked for kinship. However, the three RHD-CE(8-9)-D donors were siblings; a fourth sample was independently observed in the single RHD positive DNA from the SCARF Exchange.



A

Marker	Events	% Total
All	20000	100.00
D negative RBC	18618	93.09
D positive RBC	1162	5.81

B

Marker	Events	% Total
All	20000	100.00
D negative RBC	18726	93.63
D positive RBC	1190	5.95

C

Marker	Events	% Total
All	20000	100.00
D negative RBC	18857	94.28
D positive RBC	1042	5.21

Figure 7

Flow cytometric analysis of a $D^{+/-}$ chimera. The fluorescence histograms obtained by indirect immunofluorescence with a polyclonal anti-D are shown for the index donation of the chimerical donor (Panel A), a second donation three month later (Panel B) and a control mixture containing 5% D positive RBC (Panel C). There are two peaks separated by a large gap indicating that two different RBC populations are present. The left peak represents D negative RBC, the right peak D positive RBC carrying a normal strength antigen D. The positive RBC population of the donor was about 6%.

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