Presence of *RHD* in serologically D–, C/E+ individuals: a European multicenter study

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BACKGROUND: *RHD* blood group alleles with reduced or absent antigen expression are a clinically significant and heterogeneous group.

STUDY DESIGN AND METHODS: To detail population genetics data on apparently D– individuals in central Europe, a six-center study was performed with participants from Austria, Germany, Slovenia, Switzerland, and Russia. A total of 1700 serologically D– samples, positive for C and/or E, were investigated.

RESULTS: Observed unexpressed *RHD* alleles were 59 *RHD-CE-D+* hybrid alleles, 9 apparently regular *RHD*, 1 new *RHD*(Y401X); DELs were 8 *RHD*(M295I), 6 *RHD*(IVS3+1G>A), and 1 new *RHD*(X418L); and weakly expressed *RHD*s were 2 weak D type 5, 1 weak D type 1, 1 *RHD* category VI type 1, and 1 novel weak D type 26. Although weak D type 26 was shown to have one of the lowest D antigen densities ever observed, it gave rise to anti-D immunization in a transfused D– individual.

CONCLUSION: The relative occurrence of *RHD* among serologically D– samples, positive for C and/or E, differed significantly in the investigated central European regions. Considering the growing use of molecular typing techniques, correct identification of blood group alleles with scarce or missing antigen expression is of utmost clinical importance and requires reliable population-based frequency data.

Negativity for certain blood group antigens is a basic characteristic of practically all human blood group systems—including RhD—and can also be depicted as naturally occurring double-knockout individuals. In Caucasian persons, the main reason for D negativity was found to be linked to the absence of one of the two closely related RH genes; its molecular background was described.[1-3] Approaching RHD in an approximate way, D positivity and negativity could be considered as clearly defined and investigated. Considering antigens of phenotype frequencies with lower than 1 percent, however, further incremental steps are added to this pure positive-negative scheme, as exemplified by weak D and DEL, showing low and minute amount of D antigens, respectively.

ABBREVIATION: PCR-SSP = polymerase chain reaction with sequence-specific priming.

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Weak D were shown to be caused by single-point mutations in most cases, leading to amino acid exchanges in the transmembraneous, or intracellular, parts of the D protein and as a consequence thereof showing a reduced D antigen density.[4] Very weakly expressed weak D, or D only detectable by adsorption-elution techniques—named DELs—were frequently unidentified by routine serologic procedures, and the large group of completely unexpressed *RHD* alleles only became evident by DNA-typing methods. A variety of barely or completely unexpressed *RHD* alleles were already reported in 1994, among D– individuals with positivity for at least C. The identified alleles included *RHD-CE-D* hybrids with large parts of the *RHCE* gene of Caucasian and African origin, the latter found in D– black individuals who exhibited the Cce^s phenotype complex; and apparently complete *RHD* genes, one of which of oriental origin with very low levels of D antigen detectable only by adsorption-elution.[5-10] Further investigations confirmed these findings and pointed to high incidences of unexpressed *RHD* alleles among D– individuals with positivity for at least either C, or E.[11-15] Consequently, more systematic and population-based studies characterizing barely or completely unexpressed *RHD* alleles were carried out and delivered population specific data.

In black African persons, approximately 3 to 5 percent of the population is D–; the vast majority of them are presenting a homozygous or heterozygous combination out of the three predominant D– "alleles": 1) classical *RHD*-deleted RHd haplotype, 2) unexpressed *RHDψ*, and 3) D– *RHD*Ce^S.[7,16-18] What really differentiates the black African population from Caucasian persons in this context is the pronounced difference in proportional "allelic" contribution to D negativity. Whereas (1) contributes up to almost 100 percent to D negativity in Caucasian persons, the above-mentioned alleles seem to contribute 56.45 percent (1), 26.2 percent (2) and 17.3 percent (3) in Mali and apparently 32.2 percent (1), 52.3 percent (2), and 15.5 percent (3) in the black South African population.[17,18] Weak C expression among D– in black African persons is generally encoded by *RHD*Ce^S and therefore correlates directly with the frequency of this allele in the investigated population.[7] Haplotype frequency of *RHD*Ce^S may be as high as 4.3 percent as shown for black African persons from Mali.[18]

In Caucasian persons, approximately 15 percent are D–, and the classical *RHD*-deleted RHd haplotype is practically the only contributor (approximately 100%) to this phenotype. The cumulative population frequencies of 1) unexpressed *RHD* genes, 2) D– *RHD-CE-D* hybrid alleles, and 3) DELs were 1 among 6443, 2018, and 3030, respectively, and hence very low compared to black African and Asian persons.[19] In detail, 754 D– individuals with positivity for at least C or E showed 5 individuals with 4 different unexpressed *RHD* genes, 24 individuals with 9 different *RHD-CE-D* hybrid alleles with *RHD-CE(2-9)-D* representing by far the most frequent and alone having been observed 11 times, and 15 individuals with three different DEL alleles: *RHD*(M295I), *RHD*(K409K), and *RHD*(IVS3+1G>A).[19] These alleles are expected to contribute to D negativity at a frequency of 0.22 percent for (1) and (2) together and 0.11 percent for (3).

In Asian persons—as shown by studies performed in Japan, Taiwan, and China—D negativity as detected by routine serologic methods includes a high percentage of DELs at a frequency in between 12.8 and 16.3 percent, whereas truly D– *RHD-CE-D* hybrid alleles are found at a comparably low frequency of in between 0.44 and 0.98 percent only.[8-10,20,21]

It is noteworthy that apparently different region specific DEL alleles are responsible for their common high prevalence in Asian regions, even located close to each other, as exemplified by the Shenzen DEL lead allele *RHD(K409K)* and the Taiwan DEL lead allele *RHD(delEx9)*.[10,21,22] This regional specificity is further accentuated by the existence of an unexpressed *RHD(G314V)* allele contributing 13.8 percent to D negativity in Japanese (not including DELs), but its complete lack in the other two Asian populations investigated.[8-10,20-22]

Taking into account the above-mentioned regional specificities, we aimed at investigating "the central European" allelism of D negativity among samples with positivity for either at least C or E. Participants were from Innsbruck (Austria), Oldenburg (Germany), Ljubljana (Slovenia), Bern (Switzerland), Braunschweig (Germany), and Kirov (Russia) and contributed between 54 and 738 samples, adding up to a total of 1700 samples investigated. Participating laboratories and their geographic locations are given in Fig. 1.

MATERIALS AND METHODS

Sample origin

The sample origin was defined by the participating laboratories, which are representatives of the regions investigated: Innsbruck for the federal state of Tyrol (Austria); Oldenburg for the federal states of Lower Saxony, Saxony-Anhalt, Thuringia, Oldenburg, and Bremen (Germany); Ljubljana for different towns of Slovenia, which can be considered as a representative Slovenian population; a vast majority (>80%) of the Swiss samples from Bern from the canton of Bern (Switzerland); Kirov for the regional subdivision Kirov Oblast (Russia); and Braunschweig for the city of Braunschweig and eastern parts of Lower Saxony (Germany).

Serologic typing

For all samples, typing for D, C/c, and E/e antigens was performed by gel matrix testing (Micro ID typing system,

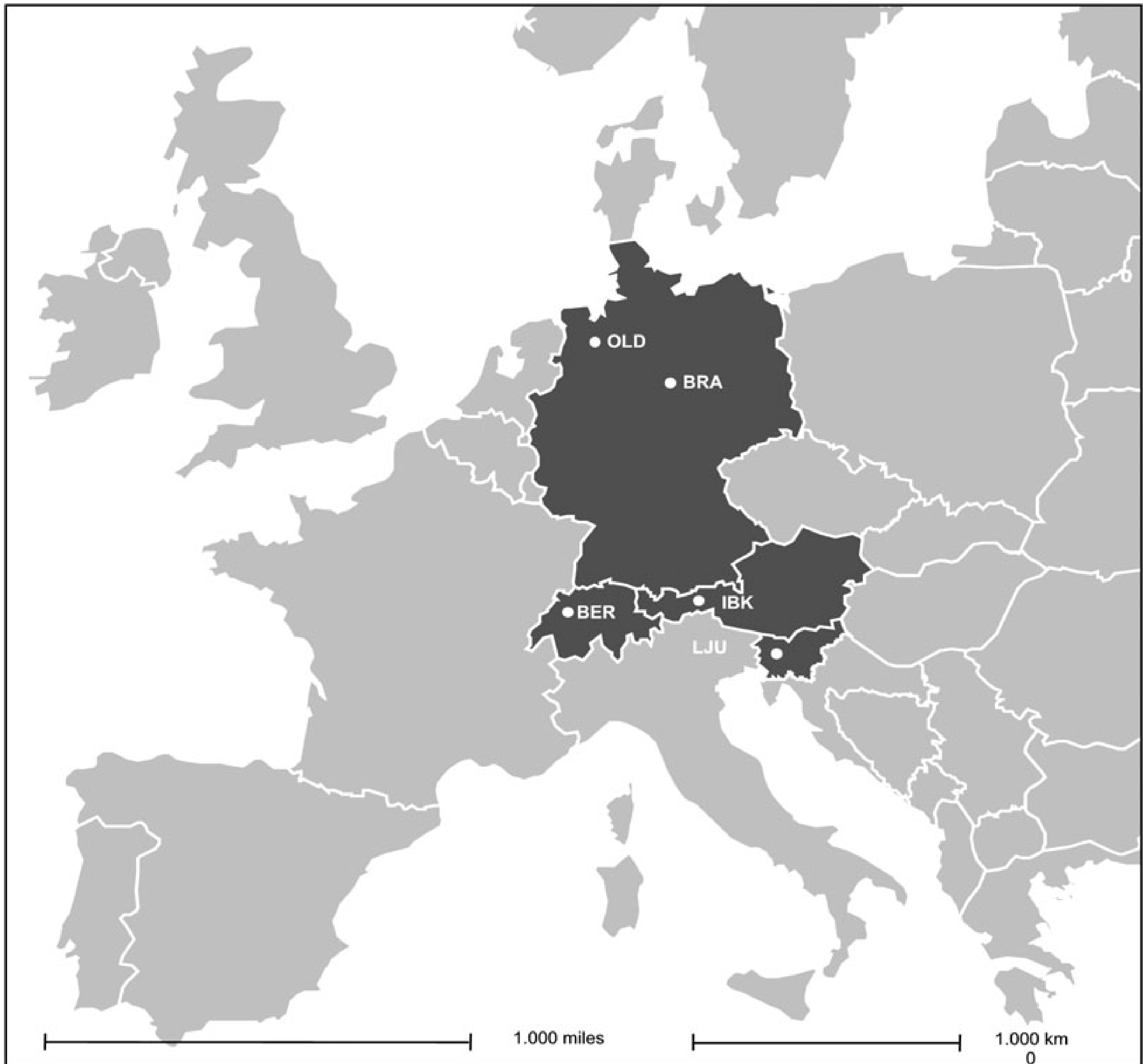

Fig. 1. Participating laboratories and their geographic location in central Europe. Innsbruck, Austria (IBK); Oldenburg, Germany (OLD); Ljubljana, Slovenia (LJU); Bern, Switzerland (BER); Kirov, Russia (KIR); and Braunschweig, Germany (BRA). City of Kirov, Russia, is located outside the shown map.

DiaMed, Cressier sur Morat, Switzerland), including the detection of weak D antigens by indirect antiglobulin test (IAT), despite Oldenburg, which employed an automated blood grouper (PK7200, Olympus, Hamburg, Germany) with a monoclonal IgM (Diagast, Aachen, Germany) and polyclonal IgG (Meridian, Cincinnati, OH), with subsequent IAT for partial and weak D including both tube and gel matrix testing. An estimated 15 percent of the samples from Innsbruck relied on historical data, when testing for weak D was still performed as IAT in tubes. Barely expressed D antigens were confirmed by adsorption of human polyclonal anti-D from an anti-D-immunized individual with test and control red blood cells (RBC) and subsequent acid elution (Elu-*Kit*II, Gamma, Houston, TX) for further antibody specification.

DNA screening

Ethylenediaminetetraacetate-anticoagulated blood samples were collected for DNA preparation. DNA preparation was performed with a DNA preparation kit (Nucleon BACC2, Amersham Biosciences, Freiburg, Germany) for

all samples except Oldenburg, which used another kit (Puregene DNA blood kit, Gentra Systems, Minneapolis, MN).

All samples were screened for *RHD*-specific DNA sequences in the 5′-untranslated region, exon 3 and exon 10 by polymerase chain reaction with sequence-specific priming (PCR-SSP). Screening for exon 3 and exon 10 was performed as described earlier, screening for the 5′-untranslated region was performed exactly as described with primers given in Table 1A (primer 1).[14]

The exon scanning method and testing for weak D types 1 to 5 was performed as described earlier, supplemented by the eight PCR-SSPs as described in the next paragraph for additional information on the *RHD* 5′-untranslated region, exons 2 and 8.[14,23] *RHD* zygosity was determined by checking for the presence of hybrid, upstream, and downstream Rhesus box to predict the presence of a *D*– Rhesus haplotype, by *Pst*I RFLP as described elsewhere.[3]

RHD DNA sequencing

Genomic DNA of the respective samples was used for specific amplification of *RHD* exons 1 to 10 and their flanking intronic sequences. All eight reactions worked at the same cycling conditions, which were an initial denaturation step at 94°C for 2 minutes; 10 cycles of denaturation at 95°C for 15 seconds, annealing at 65°C for 90 seconds, and synthesis at 72°C for 90 s; and 25 cycles of denaturation at 95°C for 15 seconds, annealing at 61°C for 1 minutes, and synthesis at 72°C for 2 minutes. Amplification was carried out in a final volume of 75 µL containing 50 mmol per L KCl, 10 mmol per L Tris-HCl (pH 8.3), 0.01 percent gelatin (vol/wt), 5.0 percent glycerol (vol/wt), 100 µg per mL cresol red, 200 µmol per L of each dNTP, and 250 ng of genomic DNA. Primers are given in Table 1 (Table 1A, primers 1-8 for amplification; Table 1B for sequencing). PCR products were purified with a PCR purification kit (QIAquick, Qiagen, Hilden, Germany). DNA sequencing was performed at the Microsynth DNA service facility (Microsynth, Balgach, Switzerland) with dye terminator technology from Applied Biosystems (Vienna, Austria). The minimal length of the exon-flanking intronic sequences analyzed was 35 bp. New *RHD* alleles were deposited at the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/). Alternatively, samples of Oldenburg were sequenced with the dye terminator cycler sequencing method (ABI310, Applied Biosystems, Darmstadt, Germany) as published previously.[4,19,24]

Enhanced *RHD-CE(2-9)-D* molecular analysis

The analysis of *RHD-CE(2-9)-D* hybrid alleles was carried out by one PCR-SSP procedure, which detected an *RHD*-specific polymorphism located 1038 bp 5′ of the intron 1–exon 2 boundary (Table 1C, primer 1); the control primers used and cycling conditions were exactly as described previously.[14] PCR fragments for sequencing 5′ and 3′ breakpoint of *RHD-CE(2-9)-D* were amplified with reactions D1 (Table 1D, primer 1; exactly as described for C1, without control primers) and long-range PCRs D2 and D3 (Table 1D, primers 2 and 3). Long-range PCR reagents were those from a PCR system (Expand Long Template PCR system, Roche, Vienna, Austria) and exactly used as described by the manufacturer. Cycling conditions were an initial denaturation step at 92° for 2 minutes; 10 cycles of denaturation at 92°C for 10 seconds, annealing at 65°C for 30 seconds, and synthesis at 68°C for 8 minutes; and 25 cycles of denaturation at 92°C for 10 seconds, annealing at 65°C for 30 seconds, and synthesis at 68°C for 8 minutes plus an incremental 20 seconds per cycle; and final elongation for 7 minutes at 68°C. Amplification was carried out in a final volume of 50 µL; PCR fragments were purified and sequenced with primers given in Table 1E as described above.

Detection of D antigen density of weak D type 26

D antigen density of weak D type 26 and control RBCs was determined by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany) as described.[25] Fifty microliters of 2 percent suspensions of washed RBCs in phosphate-buffered saline were incubated at 37°C for 30 minutes with 50 µL of human anti-D. Nine human IgG anti-D monoclonal antibodies (MoAbs) were used: P3x290, P3x35, P3x241, P3x249, and HM16 (Diagast); MS26 and ESD1 (DiaMed); and BRAD-3 and BRAD-5 (provided by G.L. Daniels, Bristol Institute for Transfusion Sciences, Bristol, UK). The human clone AEVZ5.3 served as a negative control. After incubation with the primary antibody, the cells were washed and the bound antibody was detected by the addition of fluorescein isothiocyanate-conjugated $F(ab')_2$ fragment rabbit anti-human IgG (Dako, Glostrup, Denmark). After incubation with the secondary antibody (30 min at 4°C) and washing, 30,000 events per sample were acquired. Absolute D antigen densities (D antigens per RBC) were assessed by use of a standard RBC sample with 18,332 D antigens per cell (provided by V. Curin Serbec and M. Urbajs, Blood Transfusion Center of Slovenia, Ljubljana, Slovenia) deduced from the standard of the “Fourth International Workshop on Monoclonal Antibodies against Human Red Blood Cells.” For antigen density calculations, the recommended algorithm was applied.[26]

Flow cytometric assessment of RhAG expression of weak D type 26 samples and control RBCs was performed by use of murine anti-human MoAb 2D10 (provided by L. de Jong, Sanquin, Amsterdam, the Netherlands). Mouse IgG-negative controls and phycoerythrin-conjugated rabbit anti-mouse IgG $F(ab')_2$ were from Dako.

TABLE 1. Primers used for PCR and DNA sequencing

A	Primer Name	Exon sequencing PCR primer	Gene location	Primer conc. (nmol/L)	Product length (bp)
1	RHD_i1+578R	ATGGTGGTGCCCTCCTGTGGTC	Exon 1	350	902
1	RHDpro-132F	GTTAACTCCATAGAGAGGCCAGCAGAA	Exon 1	350	
2	nRHD_i1-1405F	CATTTCCCCTATTTAACAGACAAGAACAAG	Exon 2	350	1710
2	RHD_i2+61R	GGCAATATCCCAGATCTTCTGGAACC	Exon 2	350	
3	RHD_i2-182F	AGGCCACCTTAACGGGAGAAGAG	Exon 3	400	677
3	RHD_i3+301R	GCTATGTTGCCCAGCTCGGTCC	Exon 3	400	
3	nRHD_i3-45F	AAGGACTATCAGGGCTTGCCCCGTGC	Exons 4 and 5	300	983
3	RHD_i5+149R	CCACTGTGACCACCCAGCATCCTA	Exons 4 and 5	300	
4	nRHD_i5+1463F	AGGCAGTAGCGAGCTGGCCCCTCA	Exon 6	350	554
4	nRHD_i6+57R	GCACTGCACAGTGGCCCATCAGGTCC	Exon 6	350	
5	RHD_i6-160F	CTCTTCATTTCAACAAACTCCCCGA	Exon 7	350	665
5	RHD_i7+326R	TGGGAGCACGTCCACAGCAAAG	Exon 7	350	
6	RHD_i7-327F	TGGAGGCTCTGAGAGGTTGCGG	Exon 8	350	603
6	nRHD_i8+151R	GCCTCACAGTCCACATTAGCAGCAG	Exon 8	350	
7	RHD_i8-67F	TGAGATACTGTCGTTTTGACACACAATACTTC	Exon 9	350	268
7	RHD_i9+62R	GTTTTACTCATAAACAGCAAGTCAACATATATCCT	Exon 9	350	
8	DEX10-SP-1358-as	CAGTGCCTGCGCGAACATTG	Exon 10	350	567
8	RHi9-417F	CACTCCAGCCTGAGACAAGAGCGAAAC	Exon 10	350	

B	Primer Name	Exon sequencing, sequencing primer	Gene location
	SYpro-118F	CACAGCCAGCCTTGCAGCC	Exon 1
	SYi1-147F	ATTCAGTTGAGAACATTGAGGC	Exon 2
	SYi2-151F	GAGATGGTCACTCCACTCTGTAG	Exon 3
	SYi4+103R	TGATGGAAGGGCTTCAGACACC	Exon 4
	SYi5+127R	CCTAGAGCTCCACTGTAGAGGC	Exon 5
	SYi5-149F	TCCACTGATGAAGGACACGTAG	Exon 6
	SYi6-130F	GTGCACATTCAAGTCTGAGAAG	Exon 7
	SYi7-121F	ATGTACCAGCCAGGGAGAGGAC	Exon 8
	SYi9-58R	CAAGTCAACATATATACCCAGG	Exon 9
	SYi9-119F	TCCAAGATCTCTTCCAATTCAG	Exon 10

C	Primer Name	Intron 1 RHD-SSP	Gene location	Primer conc. (nmol/L)	Product length (bp)
	RHD-i1-1042F	GGGTGACGAGTGAAACTCTATCTCGAT	Intron 1	400	306
	RHD-i1-783R	GACAAACTTGGGTTCAAATCAGGAGTC	Intron 1	400	

D	Primer Name	Breakpoint long-range PCR primer	Gene location	Primer conc. (nmol/L)	Product length (bp)
1	nRHD_i1-1405F	CATTTCCCCTATTTAACAGACAAGAACAAG	Intron 1	400	670
1	RHD-i1-783R	GACAAACTTGGGTTCAAATCAGGAGTC	Intron 1	400	
2	RHD-i1-1042F	GGGTGACGAGTGAAACTCTATCTCGAT	Intron 1–exon 3	300	7382
2	EX3-US-451-as	ACTGATGACCATCCTCAGGTTGCC	Intron 1–exon 3	300	
3	RHD-i8-88F	GAGAAAAAGGATTTCTGTTGAGATACTGTCG	Intron 1–exon 10	300	7275
3	DEX10-SP-1358-as	CAGTGCCTGCGCGAACATTG	Intron 1–exon 10	300	

E	Primer Name	Intron sequencing, sequencing primer	Gene location
	RD-i1-1398F	AACAGACAAGAACAAGAAGAGG	Intron 1
	RD-i1-788F	CACACAGTTAGAGGGTGCCA	Intron 1
	RD-i1-173F	CGGCATGTTATGTTATCCCC	Intron 1
	RD-i2+945R	AACGTGTATAGCTTGATGTG	Intron 2
	RD-i2-866F	GTTACCTGGGCAGGAGGTGG	Intron 2
	RD-i2+1480F	GCTGGGCATGGTGGTTCA	Intron 2
	RD-i2+2403F	GAGTTAATTCCCACCGGGATTC	Intron 2
	RD-i2+3083F	AGTGCAGTGGTACAATCATAGC	Intron 2
	RDi9-5537F	CACTTCCTCCTGCTCCTTTG	Intron 9
	RDi9-4795F	CCAGCTCAGTCACTTGAGTTGC	Intron 9
	RDi9-4226F	CATATTGCAAGCCTACAGTTTC	Intron 9
	RDi9+2983R	AATTAGCCGTGGTGGCAGG	Intron 9
	RDi9+2710R	GAAGTGCATGTCAGTGAGCCC	Intron 9
	RDi9+2007R	GCCAATGGATGATCTTCAGCAC	Intron 9
	RDi9-2216F	AGGTGGGGAGCATCTGCAAG	Intron 9
	RDi9-1989F	GAAGATCATCCATTGGCTTCTG	Intron 9
	RDi9-1495F	GGAGTGGTTGCAGTGAGCCGAG	Intron 9
	RDi9-1162F	GCATTTAAACAGGTTTGCTCC	Intron 9
	RDi9-981F	GGGTTCCACCACTGCCAGCTC	Intron 9
	RDi9-785R	GCTGAGTTCTGAAGTGGTGCC	Intron 9
	RDi9-482F	GAGGCAGAATTGCTTGAACCCG	Intron 9

The presence for anti-LW[a] in the serum sample of the weak D type 26–immunized mother was tested by comparing adsorption of the antibody in question to three samples each of D+ and D– cord and adult blood cells.[27]

Population genetics and statistical analysis

Haplotype frequencies of cde, Cde, and cdE were calculated from an appropriate number of donors from the respective transfusion centers in Innsbruck, Oldenburg, and Ljubljana. The haplotype associations of the respective *RHD* genes with either the haplotype Cde or the haplotype cdE were apparent. *RHD* allele frequencies were calculated from their frequencies in the respective haplotype and the calculated frequency of these haplotypes in the local population. Confidence intervals (CIs) were calculated according to the Poisson distribution.[28] Donors were not checked for kinship.

Contributions

Sample collection, serologic typing, and DNA preparation was performed in each participating laboratory. Serologic retesting of all Kirov samples was performed in Göttingen, Germany. PCR screening with three PCR-SSP reactions and RHD exon scanning was performed in Innsbruck for samples from Innsbruck, Bern, Kirov, and Braunschweig and in Oldenburg (with capillary electrophoresis for exon scanning) and Ljubljana. DNA sequencing for all but the Oldenburg samples was performed in Innsbruck, including two samples from Oldenburg, which were sequenced in Oldenburg and Innsbruck, but did not show any mutation(s). Flow cytometry analysis of weak D type 26 was performed in Vienna.

RESULTS

Blood transfusion centers from Innsbruck, Oldenburg, Ljubljana, Bern, Kirov, and Braunschweig contributed 738, 400, 333, 104, 71, and 54 samples, respectively. All 1700 samples were from blood donors typed D– by serology but C+ and/or E+ (Table 2A). They were collected for laboratory-dependent time frames in 2002 and 2003 by chance, with an exception from Bern, which overweighted its collection of samples with E.

DNA samples were screened individually by three PCR-SSP procedures specific for the *RHD* 5′-untranslated region and exons 3 and 10. All 89 partially and complete *RHD+* samples among D– individuals were then analyzed further with an *RHD* exon scanning PCR-SSP, specific for *RHD* exons 3, 4, 5, 6, 7, 9, and 10 and an additional PCR-SSP system with additional specificities for the *RHD* 5′-untranslated region and exons 2 and 8. The absence of *RHD*-specific amplification products led to the recognition of 1 *RHD* category VI type 1 sample and 59 *RHD-CE-D* hybrid alleles. With respect to these hybrid alleles, partial deletions of the *RHD* gene, or heterozygous combinations of different *RHD-CE-D* hybrid alleles, could not be excluded formally at this stage of investigation. The remaining 30 samples with the presence of all *RHD*-specific amplification products were further analyzed with five PCR-SSP procedures, capable of properly identifying weak D types 1 to 5. This approach yielded 1 weak D type 1 and 2 weak D type 5. The remaining 25 samples with the *RHD* signal were DNA sequenced for all 10 *RHD* exons including parts of the adjacent introns: among these, 1 novel weak D type 26 allele, 1 novel DEL allele *RHD*(X418L), 8 DEL *RHD*(M295I), 6 DEL *RHD*(IVS3+1G>A), and 1 novel unexpressed *RHD*(Y401X) allele, together with 9 unexpressed *RHD* alleles, of which no molecular cause could be recognized. Summarized results are shown in Table 2C.

RHD-CE-(D) hybrid alleles

A total of 59 *RHD-CE-(D)* hybrid alleles were identified. The majority of samples with any positive *RHD*-specific PCR-SSP procedures were *RHD-CE-D* hybrid alleles with large parts of the *RHCE* gene, for example, 39 *RHD-CE(2-9)-D*, 13 *RHD-CE(2-10)*, 4 *RHD-CE(2-8)-D*, 2 *RHD-CE(4-8)-D,* and 1 *RHD-CE(4-7)-D*. The alleles *RHD-CE(2-10)*, *RHD-CE(2-8)-D,* and *RHD-CE(4-8)-D* were new alleles and had not been reported previously.

With respect to *RHD-CE(2-10)*, the claimed specificity for the *RHD* 5′-untranslated region—defined only by one PCR-SSP procedure specific for adenine, positioned 132 nucleotides in front of the start codon ATG (please refer to reaction 1 of Table 1A)—was further investigated by the analysis of *RHD* zygosity, for example, testing the presence of an *RHD* hybrid box in these cases. All above-mentioned samples showed *RHD* hybrid box homozygosity, indicating that there was no *RHD* gene at all, but rather an *RHCE* variant allele with A-132 in its genomic DNA sequence.[3]

For all these alleles, PCR-SSP characterization was definitive, beside the most common, which were *RHD-CE(2-9)-D* and *RHD-CE(2-8)-D*. Because of their DNA sequence similarity, unambiguous PCR-SSP typing for *RHD* exon 2 in the presence of *RHC* is blocked. Analysis of 34 of all 39 *RHD-CE(2-9)-D* hybrid alleles identified in this study (only lacking 3 samples from Oldenburg and 2 samples from Ljubljana) with a PCR-SSP detecting *RHD*-specific adenine, however, 1038 bp 5′ of the intron 1–exon 2 boundary, showed positivity in all cases (Fig. 2). No evidence for allelic subgroups, for example, “D1 and D2”—as described earlier—could be observed.[19]

DNA sequencing of the 3′ part of intron 1 and exon 2, the 5′ part of intron 2, and the 3′ part of intron 9 was carried out of a *RHD-CE(2-9)-D* sample to define possible breakpoints. Sequences were deposited under AJ633649 for intron 1 and exon 2, AJ633650 for intron 2, and

TABLE 2. Investigated sample material of each transfusion center, their Rh haplotype composition, and their respective *RHD+* fraction*

	IBK	OLD	LJU	BER	KIR	BRA	SUM
A. Number of phenotypes investigated							
C – dd E –	0	0	0	1	0	0	1
C c dd E –	0	0	0	0	0	0	0
– c dd E –	3	3	0	0	0	0	6
C – dd E e	0	0	0	1	0	0	1
C c dd E e	7	0	1	3	0	0	11
– c dd E e	189	125	36	50	5	17	422
C – dd – e	9	5	12	3	2	1	32
C c dd – e	530	267	284	46	64	36	1227
Total	738	400	333	104	71	54	1700
B. Rh haplotypes included							
C – d – e	555	277	309	56	68	38	1303
– c d E -	202	131	37	53	5	17	445
C – d E – (assumption: no CdE in D– C+ c– E+ e+ sample)	0	0	0	3	0	0	3
c d – e	719	392	320	96	69	53	1649
Total	1476	800	666	208	142	108	3400
C. All samples with *RHD* gene							
C c dd – e, *RHD-CE(2-10)-D*	0	0	13	0	0	0	13
C c dd – e, *RHD-CE(2-9)-D*	29	3	4	1	0	2	39
C c dd – e, new allele *RHD-CE(2-8)-D*	0	4	0	0	0	0	4
C c dd – e, new allele *RHD-CE(4-8)-D*	0	1	1	0	0	0	2
– c dd E e, *RHD-CE(4-7)-D*	1	0	0	0	0	0	1
C c dd – e, DEL *RHD*(M295I)	5	1	1	0	0	1	8
C c dd – e, DEL *RHD*(IVS3+1G > A)	1	0	3	2	0	0	6
C c dd – e, new allele DEL 1252T(Tins)1253 A(X418L)†	1	0	0	0	0	0	1
– c dd E e, new allele D– T1203A(Y400X)‡	0	0	0	0	1	0	1
– c dd E e, *RHD* category VI type 1	1	0	0	0	0	0	1
C c dd – e, weak D type 1	0	0	1	0	0	0	1
– c dd E e, weak D type 5	2	0	0	0	0	0	2
C – dd – e, new allele weak D type 26 T26A (V9D)§	1	0	0	0	0	0	1
C c dd – e, regular *RHD* ?	0	8	1	0	0	0	9
Total	41	17	24	3	1	3	89

* A shows the number of different Rhesus phenotypes investigated from each participating transfusion center: Innsbruck, Austria (IBK); Oldenburg, Germany (OLD); Ljubljana, Slovenia (LJU); Bern, Switzerland (BER); Kirov, Russia (KIR); and Braunschweig, Germany (BRA). B gives the numbers of respective Rh haplotypes investigated. For all D– C+ c+ E+ e+ samples, heterozygosity of Rh haplotypes Cde/cdE was assumed. C shows all single observations of *RHD+* alleles identified in the various participating transfusion centers. For all panels, on the left-hand side, Rh phenotypes (haplotypes) are indicated, and the column on the far right gives the total sum for each observation of all participating transfusion centers. Total sums for each transfusion center are given at the bottom of each panel.

† Insertion of T at coding nucleotide 1253 (exon 10).

‡ Substitution of T1203A (Y401X) premature STOP (exon 9).

§ Substitution of T26A (V9D) (exon 1).

AJ633651 for intron 9. *RHD* and *RHCE* genomic sequences were compared to respective DNA sequences from genomic contigs AL928711 and BX640519. The 5′ breakpoint area showed an *RHD*/*RHC*-specific sequence until an *RHC*-specific 109-bp insertion in intron 2 of *RHD-CE(2-9)-D*. Interestingly, *RHD-CE(2-9)-D* shows one specific nucleotide exchange at coding nucleotide 203, which is changed from G in *RHD*/*RHC* to A, giving rise to a predicted amino acid exchange from serine to threonine at amino acid position 68. The deduction of the 3′ breakpoint in *RHD-CE(2-9)-D* was ambiguous, because nucleotide position –4359 suggests the most 5′-located *RHD*-specific nucleotide in intron 9 *of RHD-CE(2-9)-D,* whereas the further 3′-located nucleotide position –4159 shows *RHCE* specificity again, before exon 10 cDNA nucleotide position 1359 (and following nucleotides) finally exhibited *RHD* specificity. Nucleotide positions –4757 and –1862 were specific for *RHD-CE(2-9)-D* (Fig. 3).

Weak D type 26

During the fourth pregnancy of a D– C+ c– E– e+ woman (with documented anti-D– throughout her previous three pregnancies), anti-D alloantibody formation was observed after having received 3 units of RBCs of D– C+ c– E– e+ phenotype. The presence of anti-LW[a] was excluded by demonstrating complete adsorption of the antibody in question to D+ but not to D– cord blood cells. The husband of the mother typed D– C– c+ E– e+ in serology and DNA; all children were of D– C+ c+ E– e+ phenotype as shown by serologic methods (including IAT). Further investigation of the donations revealed two *RHD* DNA–

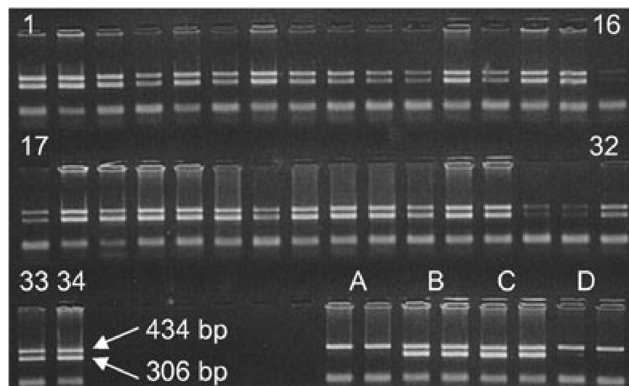

Fig. 2. PCR-SSP analysis of *RHD-CE(2-9)-D* hybrid alleles PCR-SSP analysis of 34 of 39 *RHD-CE(2-9)-D* hybrid alleles is shown. The presence of *RHD*-specific adenine located 1038 bp 5′ of the intron 1–exon 2 boundary gives rise to a 306-bp *RHD*-specific amplification product, which can be identified in all *RHD-CE(2-9)-D* samples investigated. RH DNA types of the following samples served as controls: 2 D– C– c+ E– e+ (A), 2 D+ C+ c+ E– e+ (B), 2 D+ C– c+ E– e+ (C), and 2 D– C+ c+ E– e+ (D). In each reaction, a 434-bp control band was coamplified as a positive amplification control.

positive RBC units among the three given. One of them—IBK376—had already been included in the presented study (historical record). Both *RHD+* donor samples represented siblings; their weak D were originally overseen by routine IAT performed in tubes, but could clearly be shown to be positive when retested with a gel matrix IAT system. DNA sequencing of the entire *RHD* gene of both samples revealed a changed nucleotide position 26 from T in *RHD* to A, leading to an amino acid exchange at an amino acid position 9 from valine to aspartate in the observed allele. The amino acid exchange was predicted to be located intracellular on the D peptide and is therefore unlikely to behave as a partial *RHD*. Consequently the new allele was named weak D type 26; its exon 1 DNA sequence was deposited under EMBL Accession Number AJ534720.

D serology from both the related donors showed the presence of a weak D, as shown by weak positive reactions in IAT. D antigen density was determined and differed in the female and the male (IBK376) sample, which showed 29 and 70, compared to 10.215 and 19.770, D antigens per cell in D+ C+ c+ E– e+ and D+ C+ c– E– e+ RBCs, respectively. The obvious difference in D antigen numbers between the two weak D type 26 individuals was also evident when using anti-D typing reagents in IAT in gel matrix. Here, one weak D type 26 sample was reactive with all anti-D reagents, whereas the other sample displayed consistently weaker or even negative reactions. These circumstances corroborated the flow cytometric results. Testing for *RHD* zygosity demonstrated the presence of hybrid and upstream Rhesus box in both siblings and consequently excluded homozygosity for weak D type 26 in both samples. Thus, the more than twofold increase of D antigens per cell in the male sample (IBK376) could not be explained by an existing *RHD* gene dosage effect. The influence of *RHAG* could also be excluded, because both samples demonstrated regular RhAG expression.

DELs

With respect to the 15 DEL alleles observed in total, 6 were *RHD*(IVS3+1G>A), 8 were *RHD*(M295I), and 1 was a new DEL allele: *RHD*(X418L). The respective sample—IBK475—showed a negative IAT and positivity in the adsorption-elution test. The allele itself is caused by an insertion of a single T in between coding nucleotide 1252 and 1253 of exon 10 of the *RHD* gene (“1252T(Tins)1253A”), hereby causing a frameshift, which changed the previous *RHD* translation stop codon TAA(X) to TTA(L) leading to a predicted extension of the RhD protein from 417 to 488 amino acid. The DNA sequence of all 10 *RHD*(X418L) exons was deposited under EMBL Accession Numbers AJ630375 to AJ630384.

One sample with an unexpressed *RHD* gene with an identified mutation

Kirov sample KIR39—presenting an apparently regular RHD gene, but not showing any antigen D expression at all (negative IAT, negative adsorption-elution)—showed a transversion T1203A. This T1203 mutation changed coding sequence of codon 401 from TAT to TAA, and the respective amino acid sequence of the D peptide from a tyrosine to a stop codon, leading to a predicted premature stop of translation (Y401X). The DNA sequence of all 10 *RHD*(Y401X) exons was deposited under EMBL Accession Numbers AJ630385 to AJ630394.

Nine samples with an unexpressed *RHD* gene, but without identifiable mutation(s)

An additional nine samples could be identified without D expression, but an apparently intact *RHD* gene. DNA sequencing of all coding exons and adjacent intron sequences did not reveal any specific point mutation(s). A vast majority of these alleles—eight of nine—were observed in Oldenburg; only 1 of them was observed in Ljubljana. No additional DNA sequencing of intronic sequences was performed in these cases. The molecular reason for the lack of expression in these alleles could not be identified.

Transfusion reactions

Analysis of transfusion reactions to transfused RBC units was controlled for the 5 DEL *RHD*(M295I) individuals of

A: intron 1 exon 2	-1060	-1041	-1039	-1024	-994	-936	-804	-783	-778	-763	-658	-610	-482	-416	-335	-322	-201	-17	150	178	201	203	307
RHc	AA	A	A	A	A	T	C	C	T	A	A	T	A	T	A	A	C	A	C	C	A	A	C
RHC	AA	A	A	A	A	T	T	G	C	G	G	C	G	C	G	G	T	G	T	A	G	G	T
RHD	–	G	T	-	G	C	T	G	C	G	G	C	G	C	G	G	T	G	T	A	G	G	T
RHD-CE(2-9)-D	–	G	T	-	G	C	T	G	C	G	G	C	G	C	G	G	T	G	T	A	G	C	T

B: intron 2	3021	3026	3087	3091	3205	3262	3265	3283	3296
RHc	A	TTT	C	——	C	T	G	G	G
RHC	T	—	T	109 bp	C	C	G	G	G
RHD	T	—	T	——	G	C	A	T	A
RHD-CE(2-9)-D	T	—	T	109 bp	C	C	G	G	G

C: intron 9 exon 10	-5443	-5369	-5083	-4757	-4359	-4159	-1862	1359-
RHCE	G	A	C	G	C	T	G	TTATGT
RHD	A	G	T	G	T	C	G	AATGTT
RHD-CE(2-9)-D	G	A	C	C	T	T	A	AATGTT

Fig. 3. Breakpoints in between genomic DNA sequences for *RHD* and *RHCE* for *RHD-CE(2-9)-D*. Intronic nucleotide positions are given relative to the respective exon–intron boundaries; exonic nucleotide positions are given according to *RHD* cDNA sequence. *RHD*-specific sequences are underlayed in gray, *RHD-CE(2-9)-D*-specific sequences are underlayed in black with white letters. (A) Informative polymorphisms for the *RHD-CE(2-9)-D* 5′ breakpoint. It compares *RHc*, *RHC*, *RHD*, and *RHD-CE(2-9)-D* beginning 1060 bp 5′ of intron 1–exon 2 boundary, ending with exon 2. *RHD-CE(2-9)-D* shows one specific nucleotide exchange at coding nucleotide 203, which is changed from G in *RHD* and *RHC* to A, giving rise to a predicted amino acid exchange from serine to threonine at amino acid position 68. (B) Informative polymorphisms for the *RHD-CE(2–9)-D* 5′ breakpoint. It compares *RHc*, *RHC*, *RHD* and *RHD-CE(2-9)-D* beginning 3021 bp 3′ of exon 2–intron 2 boundary, ending with nucleotide 3296. An *RHC*-specific 109-bp insertion is present in *RHD-CE(2-9)-D* at nucleotide position 3091. (C) Informative polymorphisms for the *RHD-CE(2-9)-D* 3′ breakpoint. It compares *RHCE*, *RHD*, and *RHD-CE(2-9)-D* beginning 5443 bp 5′ of intron 9–exon 10 boundary and ends with exon 10 cDNA position 1359. Deduction of the *RHCE-RHD* breakpoint is ambiguous, because nucleotide position -4359 suggests the most 5′-located *RHD*-specific nucleotide in intron 9 of *RHD-CE(2-9)-D*, whereas the further 3′-located nucleotide position -4159 shows *RHCE* specificity again, before exon 10 cDNA nucleotide position 1359 (and following nucleotides) finally exhibit *RHD* specificity. Nucleotide positions -4757 and -1862 are specific for *RHD-CE(2-9)-D*.

the sample group of 738 from Innsbruck (Austria). A total of 7 units of RBCs of the 5 donors were transfused to D– patients without previous knowledge, and their antibody status was checked by chance between 17 and 415 days after transfusion (mean, 141 days). No anti-D could be detected in these recipients by IAT. The other D+ RBC units from Innsbruck of individuals with *RHD* category VI type 1 and *RHD*(IVS3+1G>A) were not; weak D type 5 and *RHD*(X418L) was only transfused once to D– patients. Again, no D-specific antibodies could be detected in these recipients.

Population genetics of Innsbruck, Oldenburg, and Ljubljana

The Rhesus haplotype frequencies of cde, Cde, and cdE were calculated for Innsbruck, Oldenburg, and Ljubljana, which together contributed more than 86 percent (Table 2B). The frequency of Rhesus haplotype cde was almost identical in all three centers investigated (0.41999, 0.42228, and 0.41691), but differed considerably for Cde (0.01496, 0.00959, and 0.02022) and cdE (0.00686, 0.00382, and 0.00250), respectively.

Additional differences among the three transfusion centers were evident with respect to the regional-specific alleles of multiple occurrence: Regional exclusivity but no respective significance ($p < 0.05$) was given for 13 *RHD-CE(2-10)* in Ljubljana and 4 *RHD-CE(2-8)-D* in Oldenburg. The *RHD*(IVS3+1G>A) was only identified in the southern parts of the investigated area, for example, in the centers of Innsbruck and Ljubljana (also in Bern), but was completely lacking in the northern center of Oldenburg (also in Braunschweig and Kirov), again with no significance ($p < 0.05$). With respect to *RHD-CE(2-9)-D*, its frequency differed significantly in between Innsbruck and Oldenburg ($p < 0.01$), but did not for *RHD*(M295I). Estimated frequencies and respective 95 percent CIs for some alleles encountered in this study are given in Table 3.

DISCUSSION

This study was predominantly carried out to quantify the proportion of *RHD* positivity among serologically D– individuals, positive for antigens C and/or E. The study should also help to assess the risk of alloimmunization against D after transfusing weakly expressed D+ RBC units to D– recipients. These weakly expressed D+ donations are usually missed by routine serologic methods and most commonly found among donors of the above-mentioned phenotype. Six central European blood transfusion centers participated in the collection and analysis of a total of 1700 samples, also providing important population genetics data.

New *RHD* alleles could be discovered in the course of this study, DEL *RHD*(X418L) with a predicted polypeptide

TABLE 3. Estimated population frequencies and 95 percent CIs of selected *RHD* alleles found in Innsbruck (IBK), Oldenburg (OLD), and Ljublana (LJU)*

Alleles observed	Number	Estimate	95% CI Lower	95% CI Upper
IBK				
RHD-CE(2-9)-D	29	1:640	1:453	1:974
RHD-CE(2-8)	0	NO	1:5646	Infinite
DEL *RHD*(M295I)	5	1:3710	1:1660	1:9415
DEL *RHD*(IVS3+1G>A)	1	1:18550	1:3485	1:363667
DEL *RHD*(K409K)	0	NO	1:5646	Infinite
Weak D type 26	1	1:18550	1:3485	1:363667
RHD unexpressed	0	NO	1:5646	Infinite
OLD				
RHD-CE(2-9)-D	3	1:4817	1:1784	1:17663
RHD-CE(2-8)	4	1:3613	1:1506	1:10577
DEL *RHD*(M295I)	1	1:14449	1:2715	1:283297
DEL *RHD*(IVS3+1G>A)	0	NO	1:4399	Infinite
DEL *RHD*(K409K)	0	NO	1:4399	Infinite
Weak D type 26	0	NO	1:4399	Infinite
RHD unexpressed	8	1:1807	1:969	1:4399
LJU				
RHD-CE(2-9)-D	4	1:1911	1:796	1:5594
RHD-CE(2-8)	0	NO	1:2326	Infinite
DEL *RHD*(M295I)	1	1:7641	1:1436	1:149823
DEL *RHD*(IVS3+1G>A)	3	1:2547	1:943	1:9341
DEL *RHD*(K409K)	0	NO	1:2326	Infinite
Weak D type 26	0	NO	1:2326	Infinite
RHD unexpressed	1	1:7641	1:1436	1:149823

* Estimated population frequencies and lower and upper limits of the 95 percent CIs of selected *RHD* alleles of three of the participating transfusion centers are given. NO = not observed.

length of 488 amino acid and unexpressed NEX *RHD*(Y401X) with a predicted length of 400 amino acids being two of them.

For NEX *RHD*(Y401X), its complete lack of expression could be explained by the lack of D404 in the truncated protein. D404 of D corresponds to D399 of the AG protein as demonstrated by DNA and protein alignment analysis (data not shown). Lack of D399 in AG abolishes the association of AG to the D2 domain of ankyrin R, which further causes the lack of the Rh complex in the RBC membrane.[29] Therefore, the absence of a NEX *RHD*(Y401X) protein on the erythrocytes of the investigated individual could be explained by a comparable mechanism.

With respect to *RHD-CE(2-9)-D*, PCR-SSP analysis for *RHD* in intron 1 and genomic DNA sequencing of long-range PCR fragments did not show any evidence for two suballelic versions (D1 and D2), as proposed earlier.[19] Interestingly, the sequenced example (and one other, of totally two sequenced in exon 2, data not shown) showed a nucleotide substitution at coding nucleotide 203, leading to a predicted S68T amino acid exchange. This location of the substitution would be predicted to be in the second intracellular domain of the respective protein and, therefore, would probably not exhibit any immunologic features. Owing to the conformational nature of the Rh epitopes, however, amino acid substitutions in a membrane-spanning domain may well contribute to phenotypes as exemplified by the VS antigen and its associated abnormal e expression.

The rationale of the intense study of the *RHD-CE(2-9)-D* samples was to improve *RHD* zygosity testing, because *RHD/RHD-CE(2-9)-D* heterozygous individuals would be wrongly interpreted as *RHD/RHD* homozygotes by currently used methods.[3,31] In fact, *RHD-CE(2-9)-D* hybrid alleles do show an upstream box indicative for *RHD* (data not shown), but are actually D–. This becomes even more important when considering the relatively high frequency of *RHD-CE(2-9)-D* in the Caucasian population. The presented data can now be used to devise a *RHD-CE(2-9)-D* specific PCR-SSP to circumvent these problems.

Recently, a new *RHD-CE-D* hybrid allele—*RHD(1)-CE(2-10)*—has been described.[32] But whereas "our" Caucasian *RHD(1)-CE(2-10)* is upstream box negative, the Shenzen *RHD(1)-CE(2-10)* shows this additional *RHD* specificity, clearly indicating a different molecular background for these two alleles.

Another novel *RHD* allele was weak D type 26, which proved to be clinically important. Respective RBC units had been transfused to a 24-years-old D– C+ c– E– e+ mother who had received two respective RBC units and subsequently developed a positive direct antiglobulin test, and alloanti-D. The two *RHD+* donor samples represented siblings. In the course of this analysis, antigen density was determined to be in between 29 and 70 D antigens per RBC. Usually, transfusion of a relatively large amount of highly immunogenic D+ RBCs (200 mL or more) causes alloanti-D induction in more than 80 percent of the recipients within 2 to 5 months.[33] More recent data hint toward an inverse correlation between the number of transfused units and the probability of antibody formation, moving transfusion of minor amounts of D or weak D into the focus of interest.[34] The described finding of weak D type 26 is of particular interest, because the least amount of D antigens required for anti-D immunization is not known. Proven immunizations with low amounts of D antigens are those reported for weak D type 2 (antigen density of 450), for a D+ chimera (revealing 94% D– RBCs and an admixture of only 6 percent D+ RBCs) and examples of deliberate immunization of, for example, 5 mL of D+ RBCs.[19,35,36] In the late 1940s and early 1950s, it was reported that blood of weak D phenotypes could cause

anti-D alloimmunization in D– individuals, but the first detailed description with the defined causing molecular type involved was only published in 2000.[27,35] The weak D type 26 described here, with its extremely low antigen density, is the weakest D known to cause anti-D immunization so far.

Our study uncovered a total of 15 DEL carriers, 1 of them showing a new DEL allele, *RHD*(X418L). *RHD*(M295I) was encountered eight times and *RHD*(IVS3+1G>A) six times. With these identifications of DEL carriers, we focused on the effect of DEL RBCs when transfused to D– individuals. All transfusions of DEL *RHD*(M295I) RBC units in the Innsbruck center were traced and analyzed. No anti-D immunization could be observed in the seven transfusion events investigated in total. This is of interest, because information about the general potential of DEL RBCs leading to anti-D immunization is scarce. Considering these data, we assumed, that anti-D immunization against DEL RBC is rare, if at all—at least for DEL *RHD*(M295I).

This study also includes facts about the economic aspect of the proposed diagnostic screening procedure. Because DNA preparation contributes significantly to the total costs, the preparation strategy has a big influence on final total costs. Methodically, blood samples of interest can be prepared individually or be pooled following various strategies, but with the disadvantageous delay of individual results. Taking 100 blood samples as the calculative number, however, approximately 50 DNA preparations (considering the "worst-case scenario" of a high frequency of RHD positives among D– and employing specific pooling regimens), screening of each individual, further PCR-SSP analysis of about 6, and finally *RHD* DNA sequencing of about 1 sample seem to be necessary and bring the total costs for 1 sample to approximately $25 USD. With respect to the 738 samples investigated in the Innsbruck center, 1 anti-D immunization could have been avoided definitely by a timely screening procedure. An additional 3 samples—1 *RHD* category VI type 1 and 2 weak D type 5—were identified, which are thought to cause anti-D when transfused to D– recipients, because of their known D antigen density of 1050 and 296, respectively.[24] The category VI protein, however, lacks most of the D epitopes, which could severely reduce its immungenicity.[30] In any case, the respective samples should have been identified earlier by routine serologic methods. Therefore, to decide on the establishment of the proposed screening procedure for routine purposes, one influential variable is given by the quality of routine serologic methods, especially to identify weakly expressed D. Other variables can be national health policies and the level of priority in avoiding anti-D immunization. Nevertheless, the authors—at least—are strongly recommending the individual molecular diagnostic analysis of transfusion events, involving C or E homozygous D– donors.

Numerous studies have already been addressing *RHD* positivity among D– individuals. It is intriguing that regional differences in the relative composition of these groups in even nearby regions is generally encountered. This poses the important question as to which extent locally confined findings can be extrapolated to a complete ethnic group; for example, it seems precarious to claim Caucasian ("European") significance for a regional area. Comparing our findings to a previous study, significant differences in allele frequencies and expected "phenotype" frequencies could be observed, as exemplified by the D– alleles *RHD-CE(2-9)-D*, *RHD-CE(2-8)-D,* and a DEL *RHD*(K409K). Especially, *RHD(K409K)* was estimated to be encountered at a frequency of about once among 100 Cde haplotypes earlier, but could not be observed once among all 1303 Cde haplotypes investigated in this study.[19] Further support for the need of a careful interpretation of population specific data comes from earlier reports on pronounced regional differences found among three Central European populations when investigating weak D.[23] Reliable and detailed population genetic data will have to be taken into consideration and used as a basis to devise generally acceptable molecular typing strategies for *RHD* in the near future.

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