PCR screening for common weak D types shows different distributions in three Central European populations

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BACKGROUND: DNA sequencing showed *RHD* mutations for all weak D phenotypes investigated in a study from Southwestern Germany. Molecular classification of weak D offers a more reliable basis than serotyping and is relevant for optimal D transfusion strategies.
STUDY DESIGN AND METHODS: Sequence-specific primers were designed to detect weak D types 1 to 5 and the partial D phenotype HMi in a modular set for conventional PCR analysis. Alternatively, all reactions were multiplexed into a single tube, and the products were identified after automated capillary electrophoresis by their size and fluorescence. Weak D phenotype samples from 436 donors in the Tyrol (Austria) and Northern Germany were investigated by PCR.
RESULTS: More than 90 percent of the weak D types identified by PCR represented type 1, 2, or 3. The distribution among the common types varied between the Tyrol and Northern Germany ($p<0.0001$). Three new *RHD* alleles were identified.
CONCLUSION: A PCR method of detecting the common weak D types was validated. This PCR system introduces a simple and rapid tool for routine DNA typing of weak D samples. The results confirmed that all weak D phenotype samples identified by current serologic criteria carry altered D proteins.

The D antigen (ISBT 004.001, RH1) is the most clinically relevant blood group antigen directly determined by a protein. Both the high immunogenicity of D and the relatively high proportion of D– persons (approx. 20%) among whites contribute to the exceptional significance.

The large number of alleles of *RHD* accounts for the diversity of D variant phenotypes. Those with a weak D phenotype are most common among persons carrying D variants. The weak D phenotype was generally considered to comprise samples with a reduced number of D antigens per RBC without apparent lack of D epitopes. The molecular basis of weak D phenotypes has recently been elucidated.[1]

Sequencing of all 10 *RHD* exons from genomic DNA showed that at least 16 different molecular weak D types exist. Fourteen of the weak D types were caused by a single missense point mutation located within the transmembraneous or intracellular regions of the protein. Two weak D types (types 4 and 14) are each associated with nucleotide

ABBREVIATIONS: HGH = human growth hormone; IAT = indirect antiglobulin test; PCR-SSP = PCR with sequence-specific primers.

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substitutions at three nonadjacent positions. In a population-based investigation of 161 weak D samples from Southwestern Germany,[1] all samples showed an aberrant D sequence. Weak D types 1 to 5 were detected in more than a single sample and thus occurred with a haplotype frequency greater than 1 in 25,000.

These findings prompted us to compare the molecular basis of the weak D samples from Southwestern Germany to weak D blood specimens from 436 persons from the Tyrol region of Austria or from Northern Germany. For this task and for routine *RHD* typing, we developed and validated a new modular system of six PCRs with sequence-specific primers (PCR-SSP) performed under identical thermocycling conditions to detect the mutations specific for weak D types 1 to 5 and the partial D phenotype HMi with a weak D expression. We also mixed all six reactions into a single tube and then performed an automated fragment length analysis. With these strategies, we observed significant differences in the regional distribution of the three most common weak D types. Only a few samples did not provide a positive reaction in the weak D PCR-SSP panel. Sequencing *RHD* exons 1 to 10 of such samples found three new alleles, including one new molecular weak D type.

MATERIALS AND METHODS

Blood samples

EDTA or citrate blood samples were collected from White blood donors in the Tyrol or in Northern Germany. The samples from donors marked as weak D in their files were investigated. If weak D PCR-SSP typing was negative, the serologic weak D assay was repeated by the following method.

Agglutination was tested in both a gel matrix test ([LISS = indirect antiglobulin test [IAT]] 37°C; DiaMed-ID Micro Typing System, DiaMed, Cressier sur Morat, Switzerland) using a monoclonal antiserum (DiaClon Anti-D, IgG + IgM, DiaMed) according to the manufacturer's instructions and in tube tests, performed with a washed RBC suspension using two approved, agglutinating, D typing reagents (Seraclone Anti-D [226], IgM, Biotest AG, Dreieich, Germany; and Gamma-clone Anti-D, monoclonal blend, Gamma Biologicals, Houston, TX). Samples with weak reactions in both the gel matrix and tube test were submitted to further analysis by nucleotide sequencing.

Genomic DNA was prepared as described previously.[2] RBCs for flow cytometry were glycerolized for storage at –70°C[3] and kept frozen until final analysis.

PCR-SSP for weak D types 1 to 5 and partial D phenotype HMi

DNA from all weak D samples was investigated by PCR-SSP *RHD* exon scanning as described.[2] PCR primers for detection of weak D types and the partial D phenotype HMi were selected with the help of software (MacVector, version 4.5.3, Kodak, New Haven, CT). Oligonucleotides were synthesized and supplied by Microsynth (Balgach, Switzerland).

The concentration of the detection primers (Table 1) was 250 n*M*, with the exception of 700 n*M* for the primers for weak D type 2 and 350 n*M* for HMi. Control primers detecting a fragment from the human growth hormone (HGH) gene (position 5559-5992[4]) were added to each PCR in a final concentration of 60 n*M*. All amplification reactions were performed under previously published conditions.[2]

Single-tube method for all six PCR-SSP

Fluorescence-labeled primers (Table 1; Applied Biosystems, Weiterstadt, Germany) for PCR-SSP for all 5 weak D types and HMi were incorporated into a single tube. The primer pair (4,7,2´,7´-tetrachloro-6-carboxyfluorescein labeling of the sense primer) for exon 2 of the *RHD/C* genes[2]

TABLE 1. Primers for the PCR-SSP for the five weak D types and partial D, Hmi*

Reaction	Specificity	Substitution	SSP pairs (5´- to 3´- end)	Modification	Amplicon size
1	Weak D type 1	T 809 G	acacgctatttctttgcagACTTATGG GGTACTTGGCTCCCCCGAC	FAM	153 bp
2	Weak D type 2	G 1154 C	ctccaaatcttttaacattaaattatgcatttaaacagC gtgaaaaatcttacCTTCCAGAAAACTTGGTCATC	FAM	126 bp
3	Weak D type 3	C 8 G	acagagacggacacaggATGAGATG CTTGATAGGATGCCACGAGCCC	TET	166 bp
4	Weak D type 4	C 602 G	AGACTACCACATGAACATGATGCACA CAGACAAACTGGGTATCGTTGCTC	HEX	138 bp
5	Weak D type 5	C 446 A	GGTGCTGGTGGAGGTGACGGA gagcttttggccctttctccc	HEX	112 bp
6	HMi	C 848 T	AGGAGGCGTGGCTGTGGCTAT GGTACTTGGCTCCCCCGAC	TET	108 bp
7	HGH (control)		TGCCTTCCCAACCATTCCCTTA CCACTCACGGATTTCTGTTGTGTTTC	None	434 bp

* The specificity for the *RHD* alleles, the sequences of the primer pairs (with exception of the HGH control primers, small characters indicate intron positions and capitals indicate exon positions of the nucleotides), the 5´-end label (FAM: 5-carboxyfluorescein, TET: 4, 7, 2´, 7´-tetrachloro-6-carboxyfluorescein, HEX: 4, 7, 2´, 4´, 5, 7´-hexachloro-6-carboxyfluorescein) of the primers modified for the multiplex system and the amplicon size are presented.

was added as internal control instead of the HGH primers with their large product size. Amplification of approximately 50 ng of DNA in a total volume of 20 µL was performed as described above.

After PCR, free primers were removed by using a PCR purification kit (QIAquick, Qiagen, Hilden, Germany). Formamide (10 µL) and size standards (1 µL; Genescan 350, Applied Biosystems) were added to the purified amplicons (5 µL) for automated processing with a genetic analyzer (ABI Prism 310, Applied Biosystems). Each sample was loaded by application of 2 kV for 30 seconds. The amplicons were separated by electrophoresis (15 kV, 20 min) in a 50-cm capillary filled with polymer (POP-6, Applied Biosystems). The data from each run were collected and evaluated batchwise with software (Genotyper, Applied Biosystems) to automatically identify each amplicon by both its size and its fluorescence.

Sequencing of the 10 *RHD* exons from genomic DNA

Nucleotide sequencing was performed as described previously.[1,5]

Flow cytometric measurement of D density

Epitope densities were determined with two MoAbs, H41 and BS221 (Biotest AG), by indirect immunofluorescence using a standard cell of known antigen density as described previously.[5,6]

Serologic characterization

Agglutination was tested in a gel matrix test (LISS-IAT 37°C, DiaMed-ID Micro Typing System) as reported.[5]

Statistical analysis

Frequencies were compared by using the χ^2 test for 3-×-4 or 2-×-4 contingency tables. The Bonferroni-Holm adjustment was applied to correct for multiple testing. Analyses were performed with software (SPSS 5.1 for Macintosh, SPSS, Chicago, IL).

RESULTS

PCR-SSP systems for weak D types 1 to 5 and partial D phenotype HMi

SSP systems for the weak D and HMi panel were designed (Table 1) to generate specific amplicons at identical thermocycling conditions. Results of this modular PCR system are presented in Fig. 1.

As an alternative screening method, fluorescence-labeled primers were added (Table 1) to combine all six PCR-SSP in one reaction tube. After capillary electrophoresis, the reaction products were identified by both their size and the type of fluorescence. The instrument for fragment length analysis (ABI Prism 310 genetic analyzer) automatically processes up to 96 samples in a single run. The method reliably detected samples from persons with weak D types 1 to 5 and HMi. It did not generate any false-positive signal in a series of DNA samples from 100 consecutive donors with a normal D+ phenotype.

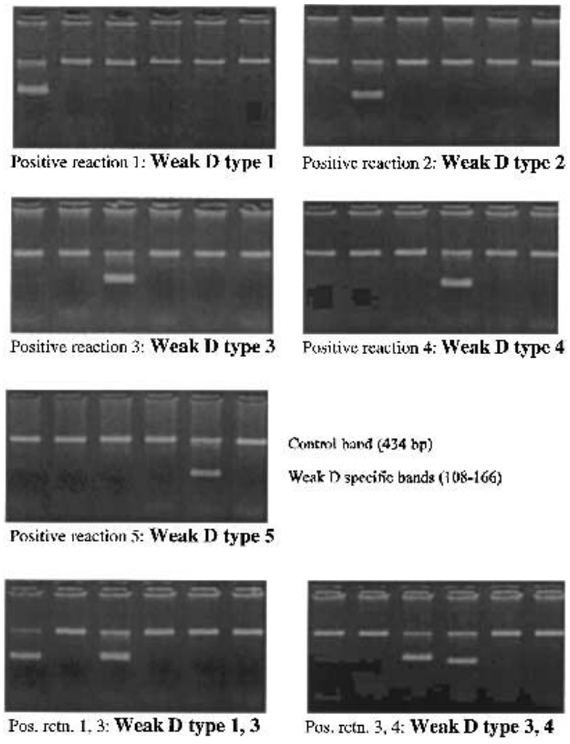

Fig. 1. Results of the modular weak D PCR-SSP system. The upper five panels show specific products of weak D PCR-SSP identified by agarose gel electrophoresis of samples with weak D types 1 to 5. The lowest two panels show typing result of two persons who combined type 3 with either type 1 or type 4. The specificity, the nucleotide substitution detected, the primers for each reaction, and the amplicon lengths are summarized in Table 1.

Weak D collection and evaluation

Blood samples from 436 persons with a history of a weak D phenotype were collected in the Tyrol and Northern Germany. All samples were tested with PCR-SSP for exon D screening as described previously[2] and with weak D PCR-SSP. A total of 21 samples without positive reactions in the weak D PCR-SSP or in the *RHD* exon D screening PCR-SSP were reevaluated by applying the current serologic methods and criteria for the detection of weak D. For only one sample was the weak D phenotype confirmed. Genomic DNA sequencing demonstrated an abnormal *RHD* for this sample. For the other 20 samples, weak D phenotype could

not be confirmed: their Rh phenotypes were CcDee in 13 cases and CCDee in 7 cases. Of these samples, the single CcDee sample from one collection site with a history of weak D not confirmed by the reevaluation was investigated by sequencing; it also revealed an abnormal *RHD* allele.

After the selection, 416 samples were finally included in the molecular investigation of weak D samples from the Tyrol and Northern Germany (Table 2). Approximately 10 percent of the samples from the Tyrol represented D category VI type 1 and about 1 percent of the samples from Northern Germany represented D category VI type 2. Three additional samples with a negative reaction in the *RHD* exon D scanning were sequenced, and they represented two weak D samples (type 13, type 14) and one partial D (D category V type VII). A total of four samples represented D category VII with a similar frequency for Tyrolean and German samples. The partial D phenotype HMi was detected in only two samples. One new partial D type (D HO) was identified.

Molecular structure and phenotype of the new *RHD* alleles identified

Sequencing of the weak D phenotype samples without a positive weak D PCR-SSP identified two rare weak D types described previously (types 13 and 14) and three new *RHD* alleles (Table 3).

TABLE 2. Distribution of individual partial D types and the totality of all molecular weak D types investigated

Aberrant RhD	Tyrol	Northern Germany
D^{VI} type I	15	0
D^{VI} type II	0	3
D^{VII}	1	3
D HMi	0	2
D HO*	0	1
D^{V} type VII	0	1
Weak D, all types†	130	260
Total	146	270

* See Table 3.
† See Table 6.

TABLE 3. Molecular basis of new *RHD* alleles identified in this study

Trivial name	Allele	Nucleotide substitution	Localization*	Haplotype
Weak D type 21	*RHD*(P313L)	C 938 T	IC	CDe
D HO	*RHD*(K235T)	A 704 C	EC (loop 4)	CDe
D^{V} type VII	*RHD- CE*(5: F223 - G263R)-*D*	Multiple:		CDe
	F 223 V	T 667 G	TM	
	E 233 Q	G 697 C	EC (loop 4)	
	V 238 M	G 712 A	EC (loop 4)	
	V 245 L	G 733 C	TM	
	—	C 744 T		
	G 263 R	G 787 A	IC	

* The predicted localization of the amino acid substitutions in the cell membrane is intracellular (IC), exofacial (EC), or transmembraneous (TM). The *RHD* alleles have been deposited in GenBank/EMBL nucleotide database/DDBJ under accession numbers AJ276015 (weak D type 21), AJ276016 (D HO), and AJ276017 (D category V type VII).

Weak D type 21 had a missense mutation in its intracellular segment. On serologic testing, it possessed all D epitopes evaluated (Table 4) and had an antigen density of about 5200 per cell. Despite its weak D-like molecular structure and reduced antigen density, it may be typed as normal D by current serologic criteria.

A second allele, dubbed D HO (Table 3), had a single missense mutation located in the fourth extracellular loop), similar to that of D HR. It was not agglutinated by antibodies directed to epitopes epD4, epD11, and epD31; however, its antigen density of 1300 per cell was rather low, and it is not clear whether the negative reactions are due to qualitative or quantitative changes in D expression.

A third allele, dubbed D category V type VII, had previously been observed by us in three samples from Switzerland and in a sample from Southwestern Germany (unpublished data). It represented an *RHD-CE(5)-D* hybrid allele (Table 3) with a subtotal substitution of *RHD* exon 5 by RHCE exon 5. Its serologic profile was similar to that of D category Va (Table 4) without the differentiation between type I and II. However, it had a much lower antigen density (estimate based on the two DMoAbs: 1180-1280 antigens/cell), and it failed to react with several examples of anti-D probably due to the low antigen density.

Distribution of weak D types in three Central European regions

The results of the genomic *RHD* typing are compared to those in a previous population-based molecular analysis in Southwestern Germany.[1] In all three regions with German-speaking populations, persons with a weak D DNA type 1, 2, or 3 consistently contributed more than 90 percent of all molecular weak D types (Table 5). The distribution of these three weak D types differed among the three regions ($p<0.0001$: χ^2 test for 3-×-4 contingency tables, $\chi^2 = 123$; Northern Germany vs. Southwestern Germany, $\chi^2 = 35$; Tyrol vs. Southwestern Germany, $\chi^2 = 85$; Tyrol vs. Northern Germany, $\chi^2 = 72$). In Northern and Southwestern Germany, weak D type 1 represented almost two-thirds of the total number. Weak D type 3 was present in about half of the Tyrolean persons. Weak D type 2 and type 3 were observed with similar frequencies in Northern Germany, but not in Southwestern Germany. In the Tyrol, only 10 samples were type 2, but 4 type 5 samples and 1 type 14 sample were observed. Two persons in Austria combined type 3 with either type 1 or type 4. The phenotype (CCD.ee in weak D type 1/type 3; CcD.ee in weak D type 3/type 4) was compatible with a weak D heterozygosity in both samples.

The highest D density among the three common types of weak D is ob-

TABLE 4. Epitope profile of new *RHD* alleles*

Epitope (1-37)	Antibody tested	Weak D type 21	D HO	D^{V} type VII	D^{Va}
1	LHM169/81	+	+	–	–
2	LHM70/45	+	+	–	–
3	LOR12-E2, P3X249	+	+	+	+
4	LOR28-7E6	+	–	–	+
5	LHM76/55, P3X290	+	+	+	+
6	LOR17-6C7	+	+	+	+
7	HIRO-6	+	+	–	–
10	C205-29, P3X35, P3X241	+	+	–	–
11	819	+	–	–	–
12	P3AF6	+	+	+	+
12	RUM1, P3X61	+	+	–	+
13	D89/47	+	+	+	+
15	BTSN4	+	+	+	+
17	BS232, HM16	+	+	w/–	+
18	HM10	+	+	–	+
21	D90/12	+	+	–	+
22	LHM59/19	+	+	+	+
22	P3x212, 11F1	+	+	–	+
23	P3x212, 23B10	+	+	–	+
31	NOU	w	–	–	–
32	ZIG-1B9	+	+	–	–
33	NaTH87-4A5	+	+	–	+
34	LORA	+	+	–	+
35	SALSA-12	+	+	–	+
36	BTSN6	+	+	+	+
37	822	+	+	+	+

* The results are repesented by + for a normal positive reaction, w for a weak positive reaction, w/– for weak or negative reactions and – for a negative reaction. Epitope profile established for D category V in the Workshop on Monoclonal Antibodies against Human Red Blood Cells and Related Antigens.[7]

TABLE 5. Distribution of the molecular weak D types in the three regions

		Germany	
Weak D	Tyrol	Northern	Southwestern
Type 1	43 (33%)	169 (65%)	95 (60%)
Type 2	10 (8%)	44 (17%)	43 (27%)
Type 3	65 (50%)	45 (17%)	7 (4%)
All other types	12 (9%)	2 (1%)	14 (9%)
Total	130	260	159

served for type 3. We measured the antigen densities of type 3 RBCs from the three regions by flow cytometry. A similar distribution of the values for the individual samples was observed in all three regions (Fig. 2). The median value of 1505 epitopes per cell in the Tyrol did not differ from the medians of 1732 for Northern Germany and 1768 for Southwestern Germany.

The occurrence of weak D types other than 1, 2, or 3 is shown in Table 6. Statistical analysis of the frequencies of the rare weak D types was not planned because of the low numbers expected. However, retrospective comparison of the data revealed a north–south gradient of the weak D types underlying ccD.Ee samples. In Northern Germany, all 44 such samples were weak D type 2. In Southwestern Germany, the vast majority (43) were type 2, and yet two type 5 and several rarer alleles were also observed.

DISCUSSION

A method for PCR-SSP detection of the five most common molecular weak D types[1] was established. This set and the partial D HMi PCR extend the modular system of seven reactions for *RHD* exon screening and of six reactions for C, C^{w}, c, E, and e DNA typing by PCR-SSP.[2,8,9] All these reactions are designed to reliably operate under identical thermocycling conditions. This strategy simplifies the set-up for DNA typing of diverse alleles and of larger numbers of samples.

Depending on the specific resources available to a laboratory, the typing of hundreds of DNA samples and thus the need for analyzing thousands of separate reactions are laborious challenges. Therefore, we also combined the six primer pairs plus a control reaction into a single reaction tube. Combination methods with three or more primer pairs have also been developed for blood group typing.[10,11] These methods enhance the accuracy and the speed of typing and minimize the DNA amount. Their successful applications underscore the fact that many different PCRs can reliably be combined in a single tube.

We coupled the combined PCR system to automated capillary electrophoresis and fluorescence detection of the amplicons. This semi-automated method efficiently analyzed large sample numbers. Capillary electrophoresis reliably separates amplicons differing in size by only a single base pair, and the inclusion of internal size standards into each separation ensures a very high reproducibility of the absolute size measurement of the PCR products. Generating amplicons with different fluorescence labels further reduces a potential risk of misidentifying PCR products of similar size, and both the single-tube reaction and the automated processing should help to minimize potential sample mix-ups. A disadvantage of this approach is the need for the special instrumentation that also serves nucleotide sequencing.

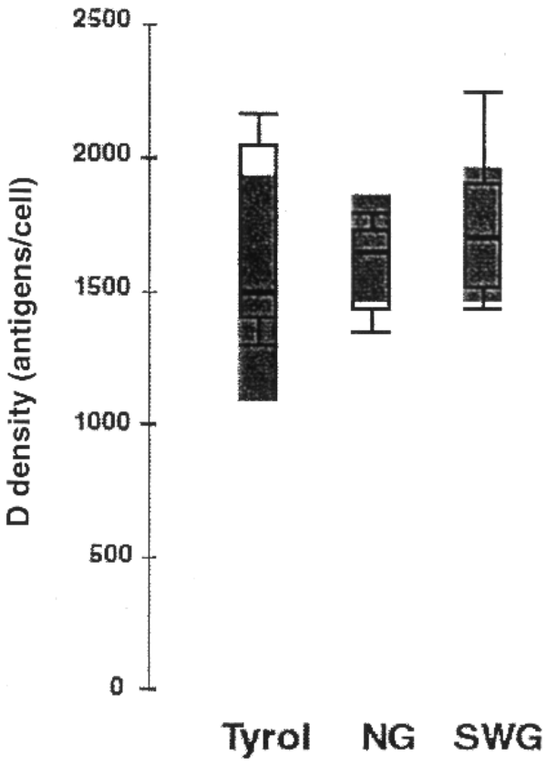

Fig. 2. Antigen densities of weak D type 3 samples collected in the three regions. The distribution of the antigen densities measured by flow cytometry in weak D type 3 samples collected in the Tyrol (n = 6), Northern Germany (NG; n = 13), and Southwestern Germany (SWG; n = 10) is represented by the boxplots. The shaded area indicates the 95% CIs for the median (thick center line).

The PCR-SSP for molecular weak D typing may offer a valuable tool for the evaluation of weak D phenotypes independently of its application as a semi-automated combined or a conventional method. We evaluated the new method for PCR-SSP weak D typing by our screening of samples with a history of a weak D phenotype from the Tyrol and Northern Germany. The study was not designed as a prospective investigation applying prespecified criteria for the phenotypic selection of samples. The cutoff between "normal" and "weak" D is not internationally standardized and may have varied between laboratories and time periods. Therefore, the results may be affected by selection bias. Despite these limitations, DNA typing of these samples provided several important new insights.

The molecular weak D types 1, 2, and 3 contributed more than 90 percent of all molecular weak D samples in both Austria and Northern Germany (Table 5). Among the less frequent molecular weak D types, only types 4 and 5 were identified in more than one sample (Table 6). All other weak D types were so rare that their frequency cannot be reliably estimated from the available data. This finding corroborates the results from the previous study[1] that the molecular weak D types 1 to 3 represent the vast majority of samples of all 21 types reported so far.

TABLE 6. The number of rare molecular weak D types in the three regions compared to the sum of the common weak D types 1, 2, and 3

		Germany	
Weak D type	Tyrol	Northern	Southwestern
1, 2, or 3	118	258	145
4	4	1	6
5	4	0	2
6 to 11	0	0	6*
13	1	0	0
14	1	0	0
21	0	1	0
1 & 3	1	0	0
3 & 4	1	0	0
Total	130	260	159

* Each type was found in a single sample.[1]

Beyond this general pattern in the molecular causes of weak D, a more detailed analysis revealed substantial differences between the populations (Table 5): In Northern Germany, type 1 was most frequent, with 60 percent and more. In the Tyrol, type 3 represented about half of all samples. Type 2 and 3 were observed with similar frequencies in Northern Germany. In Southwestern Germany, type 3 samples represented less than 20 percent of the type 2 samples. Because weak D type 3 has a rather high antigen density, different cutoff values of the weak D serotyping could explain these differences. However, we observed no obvious difference in the antigen densities of weak D type 3 samples collected at different sites (Fig. 2). This finding suggests that samples identified by molecular typing may be of value for the standardization of serologic methods.

The relative frequency of weak D type 2 among samples with weak D expression diminished from north to south. Both type 2 and type 5 have a very low antigen density and certainly would be considered weak D in any laboratory, so that this comparison cannot be due to a selection bias. The diminishing role of weak D type 2 in the Tyrol becomes even more impressive if D category VI with its low D antigen density is also considered. We observed no D category VI type I sample in Northern Germany, but 15 such samples in the Tyrol. Hence, weak D type 2 makes up 100 percent of all ccD.Ee samples with low D antigen in Northern Germany, but only 35 percent of those in the Tyrol.

All samples with a weak D phenotype according to the current standard that were not D category VI or VII represented either previously reported rare weak D types (1 sample each of types 13 and 14 in the Tyrol) or new alleles (Table 3).

Weak D type 21 harboring a substitution in its intracellular segment fits the structural scheme previously ob-

served in most weak D types. It had a comparably high antigen density, and it is now likely that such samples would often not be recognized as weak D. Its high antigen density further supports the hypothesis[5] that alleles with substitutions in the intracellular part tend to have only moderately reduced antigen density. The identification of this allele indicates that further aberrant *RHD* alleles may be present in samples with only moderately reduced antigen densities. These alleles were, however, not the focus of this study.

D HO, named after its observation in Oldenburg, shares with D HR an amino acid change in extracellular loop 4. Its epitope distribution lacking epitopes 4, 11, and 31 did not resemble D HR, which lacks epitopes 1, 2, 12, and 20. The low antigen density of both D HO (1300 D antigens/cell) and D HR (3800 D antigens/cell[12]) might have confounded epitope determination. However, it should be noted that the nonconservative amino acid substitution in D HO (lysine eliminated) differs from the conservative one in D HR. Furthermore, the distance of six amino acids is likely to correspond to considerable different parts of the extracellular loop 4.

D category V type VII is a very interesting allele. It represents an *RHD-CE-D* hybrid allele involving exon 5, and it is similar to all observed D category V alleles[13-16] in that it possesses the E233Q substitution. The only difference in D category V types II and VII is the maintenance of the ultimate, D-typic lysine in exon 5 at codon 267, and the only difference from D category V type VI is the arginine at codon 263. However, D category V type VII has a considerably reduced antigen density that is probably unique among D category V, which results in negative reactions with many antibodies directed at epitopes present in D category V. This observation underscores the possibility that minor molecular differences may lead to considerable phenotypic variation. It is interesting that, before this study, we observed this type in several samples from Switzerland that were investigated because of the combination of a low antigen density and absent reactivity with many D MoAbs. These observations probably indicate a local accumulation of this otherwise rare *RHD* allele in Switzerland.

If we exclude those samples that do not fit the current weak D serotyping criteria, our results support the original observation of missense mutations in all 161 weak D samples collected in Southern Germany for a total of 577 weak D samples collected from a larger region of Central Europe. It also corroborates the finding that the molecular weak D types 1 to 3 represent the vast majority of all weak D samples. The only other genotype occurring with considerable frequency among samples with a weak D expression in whites is D category VI.[17] Because this genotype is also detected by our combination of exon-specific PCR-SSP and weak D PCR, we are able to identify the underlying genotype for more than 90 percent of all samples with weak D expression. During the course of our study, the presence of an *RHD* pseudogene (RH–) was reported in a substantial portion of D– Africans.[18] RH– is not expected to affect the detection of the common weak D alleles by our PCR system. Our combination of serotyping and DNA analysis for the investigation of weak D samples is essential to a reliable assessment of samples with an African background. A pure DNA typing strategy, however, should include the detection of the RH– gene[18] or the *RHD* deletion.[19]

Our observations found substantial differences in the regional distribution of genotypes underlying samples classified as weak D by the regional laboratory. The PCR method of rapidly identifying the common weak D types will ensure that, in the future, different laboratories will be able to share observations on defined molecular entities rather than a "weak D" phenotype defined by serologic testing, which may represent quite different entities.

The predominance of weak D types 1, 2, and 3 is an important finding consistently observed for all involved regions and laboratories. No alloanti-D has been found in persons with these most common weak D types.[5] Therefore, our results qualify the PCR-SSP detection of these three types as a simple and valuable tool for identifying the molecular basis of the majority of weak D phenotypes. The transfusion of D RBCs or becoming pregnant may be associated with a minimal risk of alloimmunization for these persons.[5]

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REFERENCES

1. Wagner FF, Gassner C, Müller TH, et al. Molecular basis of weak D phenotypes. Blood 1999;93:385-93.
2. Gassner C, Schmarda A, Kilga-Nogler S, et al. RHD/CE typing by polymerase chain reaction using sequence-specific primers. Transfusion 1997;37:1020-6.
3. Hogman CF, Hornblower ML, Flodin M, et al. A simple method for high-quality frozen red cells in blood group serology. Transfusion 1986;26:434-6.
4. Chen EY, Liao YC, Smith DH, et al. The human growth hormone locus: nucleotide sequence, biology, and evolution. Genomics 1989;4:479-97.
5. Wagner FF, Frohmajer A, Ladewig B, et al. Weak D alleles express distinct phenotypes. Blood 2000;95:2699-708.
6. Wagner FF, Gassner C, Müller TH, et al. Three molecular structures cause rhesus D category VI phenotypes with distinct immunohematologic features. Blood 1998;91:2157-68.

7. Scott M. Rh serology–coordinator's report. Transfus Clin Biol 1996;3:333-7.
8. Flegel WA, Wagner FF, Müller TH, Gassner C. Rh phenotype prediction by DNA typing and its application to practice. Transfus Med 1998;8:281-302.
9. Rozman P, Dovc T, Gassner C. Determination of autologous *ABO, RHD, RHCE, KEL, JK* and *FY* blood group genotypes by analysis of peripheral blood samples of patients who have recently received multiple transfusions. Transfusion 2000;40:936-42.
10. Maaskant-van Wijk PA, Faas BH, de Ruijter JA, et al. Genotyping of RHD by multiplex polymerase chain reaction analysis of six RHD-specific exons. Transfusion 1998;38:1015-21.
11. Yip SP. Single-tube multiplex PCR-SSCP analysis distinguishes 7 common ABO alleles and readily identifies new alleles. Blood 2000;95:1487-92.
12. Jones JW, Lloyd-Evans P, Kumpel BM. Quantitation of Rh D antigen sites on weak D and D variant red cells by flow cytometry. Vox Sang 1996;71:176-83.
13. Rouillac C, Colin Y, Hughes-Jones NC, et al. Transcript analysis of D category phenotypes predicts hybrid Rh D-CE-D proteins associated with alteratin of D epitopes. Blood 1995;85:2937-44.
14. Omi T, Takahashi J, Tsudo N, et al. The genomic organization of the partial D category DVa: the presence of a new partial D associated with the DVa phenotype. Biochem Biophys Res Common 1999;254:786-94.
15. Avent ND, Reid ME. The Rh blood group system: a review. Blood 2000;95:375-87.
16. Omi T, Okuda H, Iwamoto S, et al. Detection of Rh23 in the partial D phenotype associated with the D(Va) category. Transfusion 2000;40:256-8.
17. Wagner FF, Kasulke D, Kerowgan M, Flegel WA. Frequencies of the blood groups ABO, Rhesus, D category VI, Kell, and of clinically relevant high-frequency antigens in southwestern Germany. Infusionsther Transfusionsmed 1995;22:285-90.
18. Singleton BK, Green CA, Avent ND, et al. The presence of an RHD pseudogene containing a 37 base pair duplication and a nonsense mutation in Africans with the Rh D-negative blood group phenotype. Blood 2000;95:12-8.
19. Wagner FF, Flegel WA. RHD gene deletion occured in the Rhesus box. Blood 2000;95:3662-8.