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REVIEW

Molecular Biology of partial D and weak D: Implications for Blood Bank Practice

WILLY A. FLEGEL, FRANZ F. WAGNER

Abteilung Transfusionsmedizin, Universitätsklinikum Ulm and DRK Blutspendedienst Baden-Württemberg, Institut Ulm, Ulm, Germany

SUMMARY

Two genes, *RHD* and *RHCE*, encode the antigens of the RH blood group system. The clinically most important antigen D is determined by the presence of a functional and grossly normal *RHD* gene. About 18% of Europeans do not express an antigen D, most often but not always caused by the *RHD* gene deletion. Rhesus negative phenotypes in Africans are caused by the *RHD* gene deletion, the *RHD* pseudogene *RHD*ψ, and the *Cde*^{s} allele. About 1% of Europeans carry *RHD* alleles with aberrant structures encoding for diminished Dimmunoreactivity. In Africans the frequency of aberrant *RHD* alleles is much higher. Aberrant *RHD* alleles encode partial D, some of which were dubbed D categories, and weak D. Since we defined the molecular basis of the *RHD* deletion, a specific detection of heterozygous carriers became feasible. (Clin. Lab. 2002;48:53-59)

KEY WORDS

blood group; Rhesus; Rh; partial D; weak D; *RHD*; *RHCE*; immunohematology; genotyping; red blood cell;

Structure of *RH* gene locus and Rh proteins

The *RH* locus consist of two genes, *RHD* and *RHCE*, that are highly homologous, comprise about 60 kilobases (kb) each, face each other by their tail ends, and are separated by a DNA segment of 30,000 kb only (Figure. 1, panel A). The RhD and RhCE proteins consist of 417 amino acids each, form transmembranous proteins with 6 extracellular loops and function as ammonia transporter (1,2). Depending on the alleles considered, RhD and RhCE differ by 32 to 35 amino acids that are dispersed throughout the protein (Figure 1, panel B); however, the extracellular differences between RhD and the C allele of RhCE are restricted to protein loops 3, 4, and 6.

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§ see references

Molecular basis of partial D

Partial D have altered RhD proteins that differ sufficiently from normal RhD to allow allo-anti-D production or to be non-reactive with some monoclonal anti-D. Today, D categories represent a subgroup of partial D. Three types of molecular bases have been found: *RHD/CE* hybrid alleles, missense mutations in the extracellular protein segments, and dispersed missense mutations.

Hybrid Alleles

The structure of the *RH* gene locus strongly favors the generation of hybrid alleles of a *RHD-CE-D* or *RHCE-D-CE* type. More than 20 partial D alleles of this type have been described (3) and the substituted gene segments range from a few to more than 10,000 base pairs (bp). A regularly updated listing of partial D, weak D and D negative alleles can be viewed at the RhesusBase website[§]. However, the phenotype is mostly determined by the extracellular loops involved, resulting in only 8 different phenotype clusters (Table 1). Alleles of the

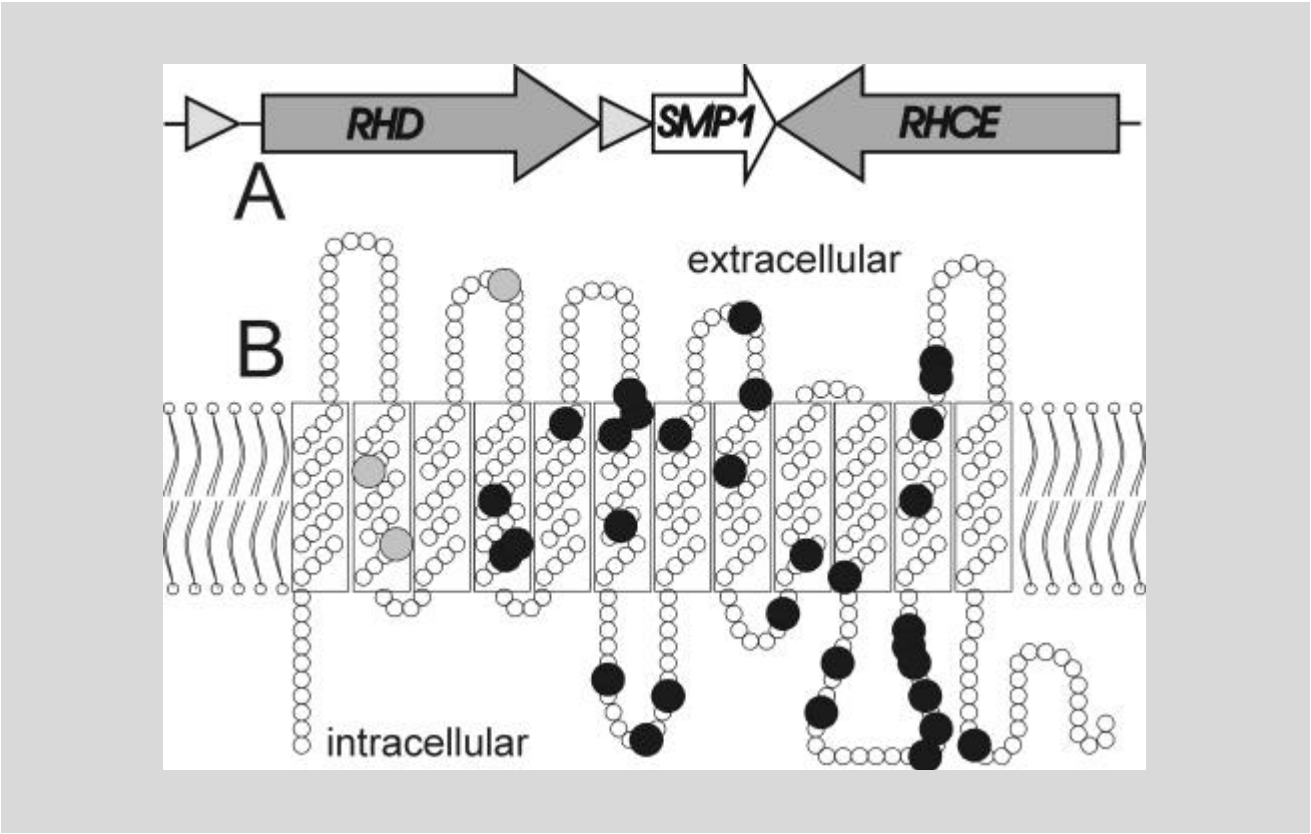

Figure 1: Structure of the *RH* gene locus and Rh protein.
(A) *RH* gene locus on the short arm of chromosome 1. The two *RH* genes, *RHD* and *RHCE*, have opposite orientation and face each other with their 3' ends. A third gene, *SMP1*, is located between *RHD* and *RHCE*. The *RHD* gene is flanked by two highly homologous *Rhesus boxes* (gray triangles) of identical orientation. (B) Rh protein in the red cell membrane. Amino acid positions differing between RhD and the ce allele of RhCE are dispersed throughout the protein (black and gray circles). Three amino acid positions do not differ between the C allele of RhCE and RhD (gray circles). The Rh proteins form twelve transmembrane helices and six extracellular loops. Only extracellular loops 3, 4, and 6 contain amino acids that differ between C and D.

Table 1: Phenotype cluster and extracellular loops involved.

Phenotype cluster	Extracellular			No. of known alleles	Examples
	3	4	6		
D^{III}	+	+	+	2	D^{IIIb}, D^{IIIc}
DFR	-	+	+	2	DFR type I
D^{V} *	+	-	+	>5	D^{Va} type I
D^{IVb}-like	+	+	-	4	D^{IV} type III
D^{VI}	-	-	+	4	D^{VI} type III
DBT	+	-	-	2	DBT-1
R_0^{Har}	-	+	-	1	R_0^{Har}
D negative	-	-	-	>5	Cde^{s}

* Additional major heterogeneity has been observed and is based on the an Ala/Pro polymorphism at codon 226 (4).

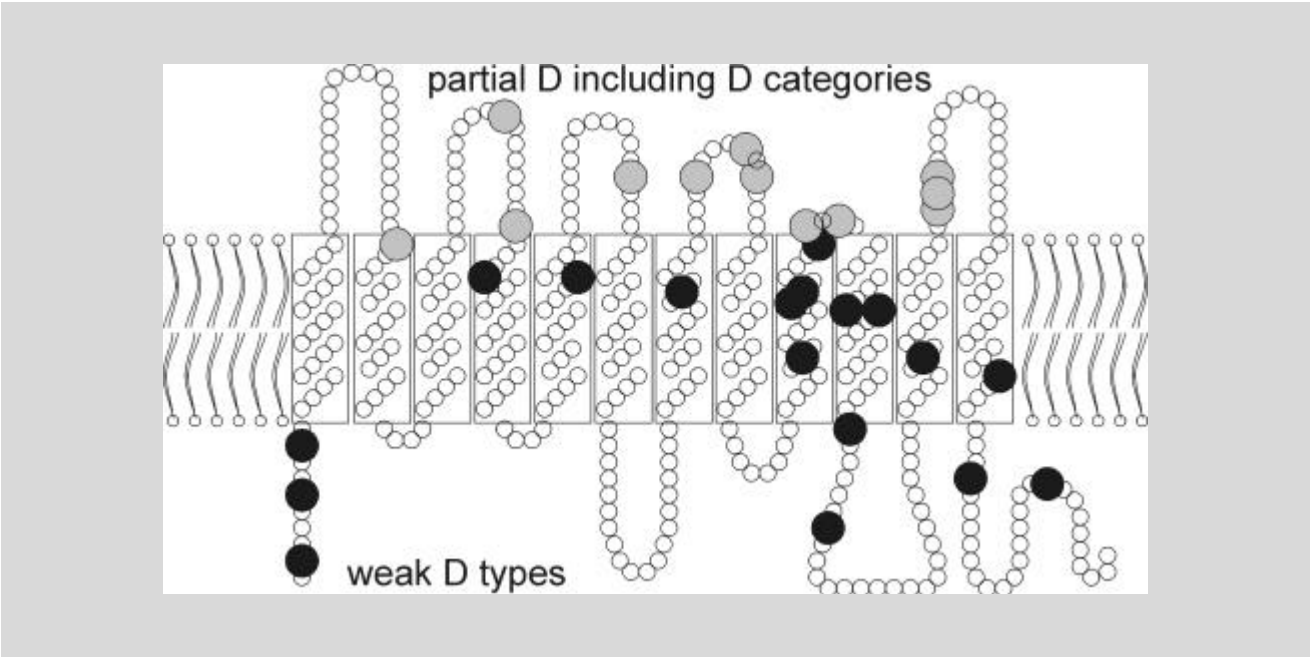

Figure 2. Aberrant *RHD* alleles with single missense mutations.
Amino acid positions affected in weak D types are shown in black; those affected in partial D, including D categories, are shown in gray. Amino acid substitutions in weak D are located in the intracellular or transmembraneous protein segments, those found in partial D phenotypes in the extracellular protein segments.

same phenotype cluster usually differ in antigen density or binding of a few anti-D only and it may be hard or impossible to distinguish them by serology methods. Although there are exceptions, D category samples are typically caused by hybrid alleles.

Missense mutations

More than 10 partial D are caused by single missense mutations located in the extracellular loops (Figure 2). The phenotypes of these partial D are much more diverse than the phenotypes caused by gene conversion and determined by the localization and type of substitution. Generally, less epitopes are affected than in partial D that were caused by gene conversions. Although there are exceptions, partial D samples other than D categories are typically caused by missense mutations.

Dispersed amino acid substitutions

Several partial D comprise multiple amino acid substitutions that are dispersed in the RhD protein. Examples are D category IIIa, D category III type IV, D category IVa and DAR. These partial D are almost never found in Europeans but appear to be frequent in Africans. They are often difficult to recognize by serologic methods including monoclonal anti-D and may hence pose considerable diagnostic problems in Africans. Dispersed substitutions may either be associated with D category samples or partial D samples other than D categories.

Molecular basis of weak D

All published weak D samples have been demonstrated to carry missense mutations in their *RHD* alleles. The most frequent weak D type in Europeans is weak D type 1 (Figure 3). Currently, more than 20 weak D types can be distinguished (see RhesusBase in the Internet). In contrast to partial D, missense mutations in weak D are exclusively located in the transmembranous or intracellular parts of the RhD protein (see Figure 2). Each molecularly defined weak D type has a distinct phenotype, with a circumscribed antigen density and often minor alterations of the D antigen (5,6). The extreme form of weak D is the D_{el} phenotype, in which the D antigen is so weakly expressed that it may be demonstrated only by adsorption and elution. Similar to weak D, a host of *D_{el}* alleles may be distinguished by molecular methods (7,8).

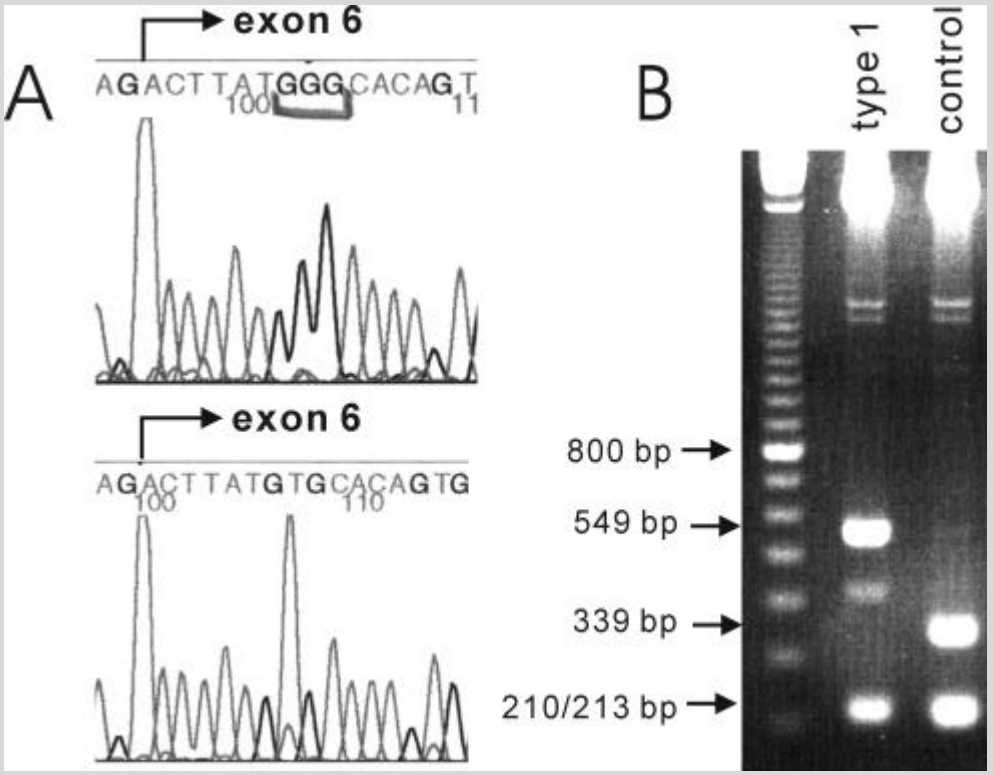

Figure 3. Molecular structure of *weak D type 1* as determined by *RHD* exon-specific sequencing from genomic DNA and PCR-RFLP.
(A) Electrophoretograms of exon 6 of a weak D type 1 sample (upper insert) and a control D-positive sample (lower insert) are shown. (B) The G to T mutation destroys an *Alw44*I site (GTGCAC). Hence, in PCR-RFLP using *Alw44*I, a *weak D type 1*-specific 549 bp fragment is amplified instead of two 339 and 210 bp fragments. Additional fragments (210 bp and >2,000 bp) are invariably present.

Does knowledge of partial D and weak D status serve a clinically useful purpose?

(i) Carriers of most partial D and of a few weak D types are prone to anti-D immunizations. Hence, it would be advantageous to have them typed in a way that avoids their exposure to Rhesus positive blood.
(ii) Carriers of most weak D alleles cannot be anti-D immunized and should be transfused with Rhesus positive blood to avoid the common practice of wasting Rhesus negative red cell units.
(iii) Molecular typing is going to uncover many weak D donors in the "Rhesus negative" donor pools of most blood centers - a conclusion which unfortunately also applies to our center (8).
(iv) As the discrimination of the underlying molecular types is becoming routine, considerable knowledge will be gained regarding the clinical implications of known and new *RHD* alleles alike.

Molecular basis of Rhesus negative

Antigen D negative haplotypes are caused by deletions of the whole *RHD* gene (9), *RHD/CE* hybrid alleles involving exofacial loops 3 to 6, nonsense or splice site mutations and a multitude of other changes, like partial deletions or insertions. The most frequent D negative allele in whites is caused by an *RHD* deletion, which was triggered by the *Rhesus boxes*, two highly homologous 9,000 bp DNA segments flanking the *RHD* gene. This deletion may today be detected specifically by PCR (10). In Africans, two other D negative alleles are also frequent, the *RHD-CE-D* hybrid allele Cde^{s} and the *RHD* "pseudogene", *RHD?* (11).

Implications for Rhesus typing strategies: serology

The risk of anti-D immunization depends on the qualitative changes of the D antigen and may not sufficiently be predicted by the overall antigen density as determined with polyclonal anti-D.

Clinical problems in transfusion recipients and pregnant women most often occur with a few aberrant *RHD* alleles only. Those alleles are rather frequent in the population and their carriers may be readily anti-D immunized. D^{VI} was considered most important. Since 1996 antigen D routine typing in Germany (12) is performed with monoclonal anti-D that do not react with D^{VI}. Hence, carriers of D^{VI} are deliberately "mistyped" as D negative to avoid exposure to Rhesus positive blood and to prevent anti-D immunization. Based on an ongoing international survey (13), most anti-D immunizations currently occurring in D positive individuals are associated with the various D^{IV} types and with DNB (14). Future improvements in routine typing strategy may take account of those partial D.

In contrast, no allo-anti-D immunization had been observed in the weak D types prevalent in whites (weak D type 1, type 2 and type 3). Our surveillance strongly suggests that patients with these alleles may safely be transfused Rhesus positive, as this is the recommended transfusion strategy according to the guidelines in Germany since 1996 (12). These alleles should be typed as D positive by using monoclonal anti-D with high affinity. Little is known about the anti-D immunization risk of rare *RHD* alleles with lower antigen density than weak D type 2, therefore we recommend Rhesus negative transfusion of their carriers.

The immunohematologic relevance of aberrant *RHD* allele must be evaluated empirically. However, the loss of many D epitopes in combination with a decreased Rhesus D similarity index (6,15) may be a hint to the risk of anti-D immunization.

In individuals of African descent, the *RHD* allele frequency differs considerably from that of whites. Several *RHD* alleles, such as DAR (also known as weak D type 4.2) or $D^{III a}$ are frequent and prone to anti-D immunization but not recognized by any current serologic typing strategy.

Antigen density variation among samples of one weak D type is much less than among samples of different weak D types. As it became possible to distinguish molecularly defined weak D types, these special red cell samples may be utilized for reagent and method standardization. We recommend the use of weak D type 2 for quality assurance in routine laboratories (16).

Implications for Rhesus typing strategies: molecular methods

Currently the following indications for *RH* genotyping should be considered (17):

(i) in the fetus from amniotic fluid or trophoblastic cells ("chorionic villi");

(ii) in poly-transfused patients if standard serology has failed;

(iii) in case of auto- and allo-immunohemolytic anemia if standard serology has failed;

(iv) to check the presence of *weak D types* and other forms of aberrant *RH* alleles if serology is inconclusive for deciding the transfusion therapy or anti D prophylaxis.

Indication (i) is considered the method of first choice for Rhesus determination in the fetus because less invasive and less hazardous sampling procedures are required. If applied prudently, indications (ii) to (iv) can be very cost-efficient procedures.

Implications for Rhesus typing strategies: detection of *RHD* heterozygous status in fathers

For decades, the discrimination of *RHD* heterozygosity from homozygosity was not practical, because serologic methods lacked sensitivity. One of the latest developments is the specific detection of the *RHD* deletion in Rhesus positive individuals (10). If a woman is anti-D immunized, the chance of conceiving an Rhesus negative fetus can be unambiguously determined by testing the Rhesus positive father for the *RHD* deletion. Suitable techniques have been published elsewhere (10), improved versions of which may become available in the future.

RHD genotyping: Recommended procedure

RHD genotyping is performed in different populations and for different purposes. The equipment for and experience with particular genotyping systems, which may be available in a given laboratory, will often guide the selection for a *RH* genotyping system (17). Generally, testing of two or more "diagnostic sites" is required to limit the rate of false results. Checking one of the *RHD*-specific nucleotides in *RHD* exon 7 and in at least one second region, preferably in exon 4 or intron 4 was advocated (17). *RHD* genotyping based solely on *RHD*-specific sequences in the 3' UTR of *RHD* exon 10 was not considered a safe procedure and should be abandoned (18).

Recently we devised an *RHD* PCR with sequence specific priming (PCR-SSP) that was optimized for specificity in the German population (8) and which we would currently recommend for *RHD* genotyping (Figure 4). The expected rate of false positive results in less than 1:12,000 yielding a positive predictive value for posi-

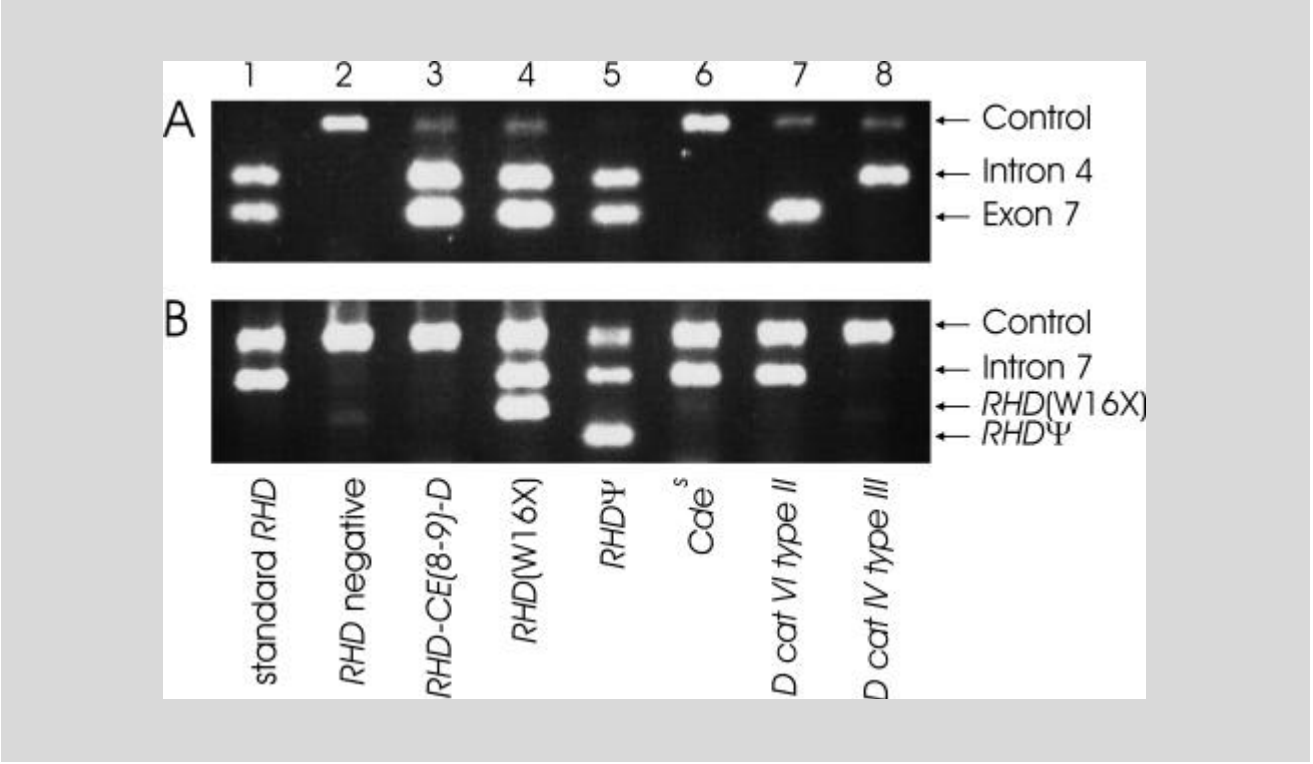

Figure 4. *RHD* PCR-SSP optimized for specificity.
The PCR is performed as a modular system consisting of two multiplex reactions. An *RHD* intron 4/exon 7 multiplex PCR-SSP (Panel A) is combined with an *RHD* intron 7 PCR that is multiplexed with reactions for the specific detection of *RHD*(W16X) and *RHD*? (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 *RHD*? 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; *RHD*? , 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. The technical details of this recommended *RHD* genotyping method have been published recently and are available online (8). ? **2001 Wagner et al, licensee BioMed Central Ltd. Reprinted with permission.**

tive results of 0.999920. These values compare favorably with almost all genotyping approaches in man. Only two multiplexed PCR reactions are required, in which five polymorphisms are tested. All other published or commercially available *RHD* genotyping systems are associated with a higher rate of false positive results (Table 3 in reference 8), albeit they may require eight or more separate PCR reactions.

ZUSAMMENFASSUNG

Die beiden Gene *RHD* und *RHCE* kodieren die Antigene des Blutgruppensystems RH. Antigen D ist das klinisch wichtigste Antigen dieses Systems und das Produkt eines normalen funktionalen *RHD* Gens. Zirka 18% aller Europäer tragen kein Antigen D, was meist - aber nicht immer - durch die *RHD* Deletion bedingt ist. Der Rhesus negative Phänotyp in Afrikanern ist verursacht durch die *RHD* Deletion, das *RHD* "Pseudogen" *RHD?* oder das *Cde*^{s} Allel. Ungefähr 1% der Europäer weisen aberrante (strukturell veränderte) *RHD* Allele auf, die ein verändertes Antigen D bedingen. In afrikanischer Bevölkerung ist die Frequenz aberranter *RHD* Allele wesentlich höher. Aberrante *RHD* Allele kodieren partial D-Phänotypen, von denen ein Teil als D Kategorien bezeichnet werden, oder Phänotypen mit abgeschwächtem Antigen D, deren molekulare Ursache von uns nachgewiesen wurde und die man seitdem in unterschiedliche "weak D" Typen differenzieren kann. Nachdem wir die molekulare Basis der *RHD* Deletion geklärt haben, ist der spezifische Nachweis heterozygoter Träger möglich geworden.

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§ see page XXXX:
RhesusBase: http://www.uni-ulm.de/~fwagner/RH/RB/
Related internet resources:
The Rhesus Site: http://www.uni-ulm.de/~wflegel/RH/
OMIM Rhesus blood group: http://www.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?111680
4th International Workshop on Monoclonal Antibodies to Human Red Blood Cells and Related Antigens: http://www.ints.fr/4thworkshop/
Blood Group Antigen Gene Mutation Database: http://www.bioc.aecom.yu.edu/bgmut/rh.htm
SCARF Rh blood group system: http://jove.prohosting.com/~scarfex/blood/4.html

Correspondence: Priv.-Doz. Dr. med. Willy A. Flegel
Universitätsklinikum Ulm
Abteilung Transfusionsmedizin
Helmholtzstraße 10
D-89081 Ulm, Germany
willy.flegel@medizin.uni-ulm.de
http://www.uni-ulm.de/~wflegel/DRK/indxengl.htm