RHD variants in Flanders, Belgium

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BACKGROUND: D antigen variants may be grouped into partial D, weak D, and DEL types. Cumulative phenotype frequencies of these D variants may approach 1% in certain European regions. Unambiguous and quick identification of D variants is of immediate clinical relevance, with implications for transfusion strategy.

STUDY DESIGN AND METHODS: A total of 628 samples with ambiguous serologic results from different immunohematology laboratories throughout the Flanders region, Belgium, were genotyped using a commercially available weak D typing approach. After exclusion of detectable weak D types, molecular *RHD* exon scanning was performed for the remaining samples, and *RHD* sequencing was performed in two particular cases.

RESULTS: Of all samples investigated, 424 (67.5%) were positive for weak D Type 1, 2, or 3, and 22 cases (3.5%) typed weak D Type 4.0/4.1/4.3, 4.2, 5, 11, 15, or 17. Another 49 (7.8%) samples were partial D variants, with a major proportion being category DVI types (n = 27). One *RHD*(S103P) sample was identified as high-grade partial D, with DIII-like phenotype and anti-D and anti-C immunization. Additionally, a novel DVI Type 3 (A399T) variant was found. Of the remaining 133 samples mainly tested because of ambiguous serologic D typing results due to recent transfusion, 32 (5.1%) were negative for *RHD*, and 101 (16.1%) were indistinguishable from wild-type *RHD* and not investigated further.

CONCLUSION: Despite the enormous diversity of *RHD* alleles, first-line weak D genotyping was remarkably informative, allowing for rapid classification of most samples with conspicuous RhD phenotype in Flanders. The clinical implications are discussed.

The D antigen of the Rh blood group system is of major clinical importance due to its marked immunogenicity. Alloanti-D acquired by transfusion or pregnancy may cause hemolytic transfusion reactions as well as hemolytic disease of the fetus and newborn.[1] Transfusion policy and prenatal investigations depend on reliable D typing that, however, is complicated by more than 200 D variants presently known.[2,3] These include weak D, partial D, and DEL types, with different clinical implications: most weak D individuals are believed to express all D epitopes excluding anti-D alloimmunization, whereas partial D is generally characterized by D epitope loss.[4,5] Only minute D antigen quantities are expressed by DEL types, which may feature complete or partial D epitope composition.[6] All D+ phenotypes including even the weakest D variants may induce anti-D in D– subjects.[7,8] In addition, individuals with partial D antigens may also develop alloanti-D upon exposure to the entire set of D epitopes of normal D+ red blood cells (RBCs); this holds true even with only minimal epitope loss.[9]

It is of clinical importance to identify D variants with potential for anti-D alloimmunization, specifically in women of childbearing age and patients with a need of

ABBREVIATION: SSP = sequence-specific priming.

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Received for publication May 27, 2014; revision received September 22, 2014, and accepted October 10, 2014.

doi: 10.1111/trf.12947

TRANSFUSION 2015;55:1411–1417

chronical transfusions or with anti-D. For this purpose, routine serologic D typing may be efficiently supported by *RHD* genotyping,[10] as serology cannot discriminate variants that safely can be considered as D+ phenotypes from those that need to be treated as D– recipients. Of note, *RHD* genotyping is complicated by a wealth of molecular peculiarities, such as *RHD-RHCE* gene hybrids, *RHD*-null alleles, and Rh mosaicism.[11-13]

In this study, *RHD* genotyping was performed on all patient and blood donor samples with D variants coming to attention by routine serology (weak agglutination in RhD typing or anti-D in a D+ sample). With this approach, for the first time the relative distribution of a D variant in Flanders, Belgium, was determined. Moreover, an individualized and safe transfusion strategy regarding D matching for all studied patients could be attained. In addition, information on two newly characterized partial D variants is provided.

MATERIALS AND METHODS

Studied blood samples

All 628 samples were from the Flanders region, Belgium. About 95% of the tested samples were from patients from different hospitals across Flanders; the rest were donor samples.

Routine serologic RhD typing

Automated D antigen typing was done using a compact analyzer (WADiana, Grifols, Barcelona, Spain) equipment with monoclonal anti-D in gel matrix (Bio-Rad, Cressier, Switzerland), followed by tube testing with blended monoclonal anti-D reagents (DiaClon, Bio-Rad).

Extended serologic analysis of a *RHD*(S103P) sample

Rh antigen (D, C, c, E, e) typing, RBC antibody screening and specification, and the direct anti-human globulin test was performed exactly as described.[6] Anti-G was prepared by a double elution method: polyclonal anti-CD reagent (Biotest, Dreieich, Germany) was adsorbed onto group O C+D– RBCs. After extensive washing and acid elution (DiaCidel, Bio-Rad), the resulting eluate was adsorbed onto group O C–D+ RBCs, followed by repeat washing and elution.[14,15] The eluate and the last RBC washing solutions were examined against a test cell panel in gel matrix indirect antiglobulin test (IAT); the final eluate containing anti-G was employed for typing in low-ionic-strength saline antiglobulin cards (Bio-Rad). Similarly, Sample 1 plasma was tested for anti-G reactivity: plasma was adsorbed onto and eluted from group O C–D+ RBCs; the eluate was adsorbed onto and eluted from group O C+D– RBCs. The final eluate was tested for anti-G reactivity against a cell panel in gel IAT.[14] D epitope[16] mapping was done as described,[17] using 41 human monoclonal anti-D: P3x35, P3x61, P3x290, P3x241, P3x249, HM10, HM16, P3x21223B10, and P3x21211F1 (Diagast; provided by K. Göttfert); MS26, ESD1, LDM1, LHM77/64, LHM70/45, LHM76/55, LHM59/19, and LHM169/80 (Bio-Rad); Brad-2, Brad-3, and Brad-5 (provided by Belinda Kumpel, International Blood Group Reference Laboratory [IBGRL], Bristol, UK); BIRMA D6 and BIRMA D10 (IBGRL); H4111B7, BS221, BS226, BS227, BS228, BS229, BS231, and BS232 (provided by Manfred Ernst, Biotest); BS225 (Sifin, Berlin, Germany); MS-201 and LDM3 (Medion, Düdingen, Switzerland); RUM-1 and D175-2 (Immucor, Rödermark, Germany); HIRO-3, HIRO-5, HIRO-9, and HIRO-94 (provided by M. Uchikawa, Tokyo Metropolitan Blood Center, Tokyo, Japan); B9A4-B2A6A6A1A1 (Bio-Rad); and NaTH109-1G2 (IQ Products, Groningen, The Netherlands).

Flow cytometry

The D antigen density of variant D and control RBCs was determined by flow cytometry exactly as described,[9] using the following five primary anti-D: P3x35, P3x290, P3x241, P3x249, ESD1, and Brad-3.

RHD genotyping and sequencing

Standard *RHD* genotyping was performed employing polymerase chain reaction with sequence-specific priming (PCR-SSP). Each studied sample was first analyzed with the weak D-type SSP kit (BAG Health Care, Lich, Germany) to discriminate weak D Types 1, 2, and 3 from other prevalent weak D types in European populations (4.0, 4.1, 4.2, 4.3, 5, 11, 15, and 17). As this genotyping approach positively detects the presence of specific weak D mutations, *RHD* genotypes of other rare weak D types, partial and hybrid alleles, as well as different D+ and D– alleles, cannot be discriminated with this test. The most common partial D variants were therefore tested in a second step using the partial D-type SSP kit (BAG). Genotyping for *RHD-CE-D* hybrid genes, causative of some partial D types, for example, Category III, IV, V, and VI, takes advantage of "*RHD* exon scanning," a principle described for the first time in 1997.[10] Although modified, key elements of the partial D-type SSP kit and comparable products still take advantage of the described *RHD* exon scanning principle.[18]

RHD sequencing of DNA from the sample with *RHD*(S103P) was done exactly as described previously.[8] *RHD* sequencing of DNA from a sample with *RHD**VI Type 3 (A399T) was done as detailed previously.[19]

RESULTS

Sample analysis for *RHD* using PCR-SSP

Over a period of 2 years, a total of 628 D+ samples with either weak agglutination in serologic D typing (n = 627)

or alloanti-D formation (n = 1; in this case combined with anti-C, with apparently normal D strength by routine serology) were *RHD* genotyped by PCR-SSP technique. Starting with a PCR-SSP kit for the identification of weak D types, 424 samples (67.5%) were identified as weak D Type 1, 2, or 3. A further 22 samples were genotyped weak D type 4.0/4.1/4.3, 4.2, 5, 11, 15, or 17. Of note, 446 of 628 (71.0%) of all serologically conspicuous samples could thus be identified using one typing kit with only eight PCR-SSP reactions.

An additional 47 samples were identified as partial D variants, including category DVI Types 1, 2, and 3 with 10, 14, and three cases, respectively. Eight samples showed the *RHCE-D*(5)-*CE* hybrid allele (*DHAR*). Hence, partial D phenotypes with proven D epitope loss including weak D Type 4.2, 11, and 15[2,5] represented 11.5% of all D variants identified in this study. The complete list of all *RHD* variants observed in this study is given in Table 1. Of all samples tested, 101 (16.1%) yielded a normal *RHD*+ typing result without evidence for a *RHD* variant, whereas 32 (5.1%) were genotyped *RHD*-negative. Two samples (Sample 1 and Sample 2) gave inconclusive *RHD* genotyping results using the PCR-SSP tests and were therefore further analyzed by *RHD* sequencing.

Sample 1 analysis by *RHD* sequencing

This sample was genotyped because of the presence of anti-D (and anti-C) in the patient's serum. This patient was of Caucasoid origin and had previously been typed as normal D+ by routine serology. The DNA of the sample showed unexpected negativity in one single diagnostic PCR-SSP of the kit used. Positivity of the respective PCR-SSP is indicative of the simultaneous presence of coding nucleotides 201G and 307T on one allele, normally found in Exons 2 of the regular *RHD* and *RHC* alleles, respectively. Sequencing revealed a *RHD* variant with a 307T>C nucleotide substitution in Exon 2. This missense mutation predicts a serine-to-proline exchange in the second extracellular loop of the RhD polypeptide. This *RHD* variant had already been found in two Dutch samples in close geographical vicinity to Belgium.[21] Both the two Dutch and this sample exhibited a C–c+E+e+ phenotype; therefore, *RHD*(S103P) is most likely linked to a *RHCE*cE* haplotype. So far, it is listed as "incompletely characterized weak or partial D."[2] Our sequencing results were deposited at EMBL database under Accession Number FR748227. *RHD*(S103P) was assigned the name *RHD**39 by the International Society of Blood Transfusion (ISBT), Working Party for Blood Group Allele Terminology.[3]

An apparently normal D-positive phenotype with anti-D and anti-C

This *RHD*(S103P) sample was strongly reactive with all routine monoclonal anti-D reagents in plate testing and gel matrix IAT. Likewise, also polyclonal and monoclonal anti-D contained within gel matrix yielded

TABLE 1. Breakdown and frequency of all D variants identified in this study

Subgroups	Genetic characteristics	ISBT nomenclature	Number	%
Weak D Types 1, 2, and 3	Weak D Type 1	*RHD*01W.1 (and W.1.1)*	265	53.5
(n = 424)	Weak D Type 2	*RHD*01W.02*	146	29.5
85.7%	Weak D Type 3	*RHD*01W.03*	13	2.6
Weak D type others	Weak D Type 4.0/4.1/4.3*	*RHD*09.03, or 09.04, or 09.05*	10	2.0
(n = 22)	Weak D Type 4.2†	*RHD*09.01, not 09.02*	6	1.2
4.4%	Weak D Type 5	*RHD*01W.05*	3	0.6
	Weak D Type 11†	*RHD*11*	1	0.2
	Weak D Type 15†	*RHD*15*	1	0.2
	Weak D Type 17	*RHD*01W.17*	1	0.2
Partial D	DIIIa, or c, or III Type 4	*RHD*03.01, or 03.03, or 03.04*	1	0.2
(n = 49)	DVa	*RHD*05 (suballeles undefined)*	1	0.2
9.9%	DVI Type 1	*RHD*06.01*	10	2.0
	DVI Type 2	*RHD*06.02*	14	2.8
	DVI Type 3	*RHD*06.03*	3	0.6
	DVI Type 3 (A399T)‖	*RHD*06.03.02*‖	1‖	0.2‖
	DAR	*RHD*DAR*	4	0.8
	DHMi	*RHD*19*	2	0.4
	DAU	*RHD*10 (suballeles undefined)*	4	0.8
	RhCE-D(5)-*CE*	*RHCE*01.22*	8	1.6
	RHD(S103P)	*RHD*39*‡	1	0.2
Total			495	100

* These three weak D subtypes were not differentiated.

† Weak D types considered as partial D. Alloimmunization risk in weak D Type 4 allele carriers is being discussed controversially and clear assignments are further complicated by the usage of parallel existent terminologies and the number of different subtypes reported so far.[2,3] Recommended reading could start with reports published by Wagner et al.[4] and Hemker et al.[20]

‖ New *RHD* allele observed in this study.

TABLE 2. D epitope mapping of *RHD*(S103P) RBCs

Monoclonal anti-D	Immunoglobulin class	D epitope	Reaction in gel IAT*
LHM70/45	IgG	1.2	+++
P3x249	IgG	2.1	++++
BS227	IgG	2.2	++++
H41 11B7	IgG	3.1	++++
P3x290	IgG	3.1	++++
LHM76/55	IgG	3.1	++++
ESD1	IgG	4.1	++++
NaTH109-1G2	IgG	5.2	++++
BS229	IgG	5.4	++++
BS231	IgG	5.4	++++
P3x35	IgG	5.4	++++
P3x241	IgG	5.4	++++
MS-201	IgM	6.1	++++
RUM-1	IgM	6.1	++++
P3x61	IgM	6.1	++++
D175-2	IgM	6.1	++++
BRAD-3	IgG	6.2	++++
BS221	IgG	6.3	++++
BS228	IgG	6.3	++++
LHM169/80	IgG	6.3	++++
HIRO-9	IgG	6.3	++++
HM16	IgG	6.4	++++
BS225	IgM	6.4	++++
BS226	IgM	6.4	++++
BS232	IgM	6.4	++++
B9A4-B2A6A6A1A1	IgM	6.4	++++
HIRO-5	IgG	6.5	++++
LDM1	IgM	6.5	++++
LDM3	IgM	6.5	++++
HM10	IgM	6.6	++++
HIRO-94	IgG	6.7	++++
BRAD-5	IgG	6.8	++++
LHM59/19	IgG	8.1	++++
P3x212 11F1	IgM	8.2	++++
BRAD-2	IgG	9.1	++++
MS26	IgG	9.1	++++
P3x212 23B10	IgM	9.1	+++
LHM77/64	IgG	9.1	++++
BIRMA D6	IgG	9.1	++++
HIRO-3	IgG	16.1	++++
BIRMA D10	IgM	NA	++++

* Identical results were obtained with R1r control RBCs.
NA = not assigned.

normal D+ reactions. Further testing demonstrated a C–c+E+e+C^{w}– phenotype. The *RHD*(S103P) allele had been reported to be associated with a G– phenotype in two individuals.[21] In fact, Sample 1 was also G– when tested with polyclonal anti-G. Moreover, these RBCs expressed apparently normal D antigens without detectable epitope loss, as evidenced by epitope mapping studies: all 41 monoclonal anti-D were strongly reactive in gel matrix IAT, comparable to *CDe*/*cde* control RBCs (Table 2). D antigen quantification of *RHD*(S103P) RBCs by flow cytometry paralleled the normal D+ serologic results, with an absolute antigen density of 14,200 D sites per RBC. For comparison, the antigen densities of *CDe*/*cde* and *cDE*/*cde* control samples amounted to 10,700 and 17,000 D sites per RBC, respectively.

In the plasma of the proposita, a woman aged 74, unexpected RBC antibodies were found: anti-D and anti-C specificity was evident in gel matrix IAT (2+), with augmented reactivity (4+) with papain-treated test cells. In contrast, only negative reactions were observed after incubation at 4°C. Anti-G specificity could be excluded by a sequential adsorption-elution procedure: no antibodies were detected in the second eluate in gel IAT after sequential adsorption-elution of plasma with C–D+ and C+D– RBCs removing anti-C and anti-D, respectively.[14] Additional anamnestic anti-Jk^{b} reactivity of the plasma of this Jk^{b}– individual could not be detected. The proposita had a negative transfusion history but at least two children of unknown Rh phenotype. Therefore, it was suspected that her anti-RBC immunization resulted from pregnancy.

Discovery of a novel partial DVI Type 3 variant

Sample 2 was genotyped because of discordant serologic typing results: it appeared negative with monoclonal anti-D in gel matrix not reactive with DVI RBCs but was strongly positive (4+) with another routine anti-D in tube test. No unexpected RBC antibodies were found in this sample. PCR-SSP genotyping with partial D-type SSP kit showed typical genetic characteristics of a *RHD***VI* Type 3 (*RHD***06.03*) allele,[3] but lacked specific amplification for *RHD* Exon 9. Therefore, it was sequenced in the respective parts of the gene: the *RHD* allele appeared to be a *RHD***VI* Type 3 (*RHD***06.03*) variant, where coding Nucleotide 1195 in Exon 9 was additionally mutated G>A. This predicts a change of Amino Acid 399 from the nonpolar alanine to the polar threonine. This amino acid substitution has previously only been described for weak D Type 45 and 45.1[2,17] but is here present in combination with the alterations of *RHD* Exons 3 to 6 of D Category VI Type 3 mutants.[22] The 1195G>A mutation prevented binding of the oligonucleotide primer in the PCR-SSP specific for *RHD* Exon 9 and caused the negative result, an observation also reported for weak D Type 45.1.[17] This novel allele was designated *RHD***VI* Type 3 (A399T) and represents a previously unreported *RHD* variant.[17] The carrier of this novel variant *RHD* allele was of Caucasoid origin. No RBCs were available for extended D antigen characterization. The new Category VI Type 3 (A399T) was assigned the name *RHD**06.03.02 by the ISBT, Working Party for Blood Group Allele Terminology.[3]

DISCUSSION

This is the first study investigating the relative frequencies of *RHD* variants in Flanders on a molecular basis. Moreover, detailed immunohematologic properties of an interesting *RHD*(S103P) with anti-D and anti-C as well as genetic characteristics of a novel *RHD***VI* Type 3 (*RHD***06.03*) with an additional amino acid exchange A399T are described.

TABLE 3. Overview of comparable studies with more than 30 samples included

Study	Provenience	Type all	Weak D*			Type others	Highest weak D type ID-number observed	Reference
			Type 1	Type 2	Type 3			
1	Germany, Southwest	159	95 (59.7)	43 (27.0)	7 (4.4)	14	16	Wagner et al.[23]
2	Australia	89	38 (42.7)	48 (53.9)	3 (3.4)	n.a.	3	Cowley et al.[24]
3	Austria, Tyrol	130	43 (33.1)	10 (7.7)†	65 (50.0)†	12	14	Müller et al.[25]
4	Germany, North	260	169 (65.0)†	44 (16.9)	45 (17.3)	2	21	Müller et al.[25]
5	France	68	30 (44.1)	21 (30.9)	3 (4.4)	14	39	Ansart-Pirenne et al.[26]
6	Canada, Ontario	32	16 (50.0)	8 (25.0)	1 (3.1)	7	5	Denomme et al.[27]
7	Czech Republic	169	98 (58.0)	17 (10.1)	33 (19.5)	21	NA	Araujo et al.[28]
8	Portugal	99	16 (16.2)†	63 (63.6)†	14 (14.1)	6	4	Araujo et al.[28]
9	Spain, Catalonia	43	21 (48.8)	14 (32.6)	4 (9.3)	4	4	Araujo et al.[28]
10	France, West	230	93 (40.4)	63 (27.4)	11 (4.8)	63	43	Le Marechal et al.[29]
11	Austria, North	128	72 (56.3)	29 (22.7)	19 (14.8)	8	49	Polin et al.[30]
12	France, South	141	37 (26.2)	59 (41.8)	4 (2.8)†	41	61	Silvy et al.[31]
13	Argentina	55	21 (38.2)	9 (16.4)	8 (14.5)	17	59	Brajovich et al.[32]
14	Belgium, Flanders	495	265 (53.5)	146 (29.5)	13 (2.6)	71	17	This study
	Total	2098	1014 (48.3)	574 (27.4)	230 (11.0)	280		

* Data are reported as number (%).
† Highest and lowest percentages of the observed weak D type alleles of the listed studies, respectively.

Despite the wealth of different weak D alleles known, the molecular first-line approach to genotype the Flanders samples was remarkably successful: every weak D type recognized by the weak D PCR-SSP kit was actually encountered at least once, and no additional weak D types were discovered. Obviously, the choice of allele resolution of this European kit (and similar commercially available products) seems perfectly matched to the weak D spectrum in Flanders.

Our results were put in relation to 13 comparable studies (Table 3). Summing up all of these studies resulted in a considerable number of independent observations for weak D Types 1, 2, and 3. In comparison to the average of all studies, observation frequencies in the Flanders region seem to be slightly elevated for weak D Types 1 and 2 and decreased for weak D Type 3, respectively. Of note, none of the cited studies including ours reported anti-D immunization among the weak D Types 1 (n = 1.014), 2 (n = 574), and 3 (n = 230) individuals investigated (Table 3).[2] Individuals with these weak D types may therefore be safely transfused with D+ RBCs and appear not to require anti-D prophylaxis in case of pregnancy with a D+ child.[33]

However, many other D variants have the potential for anti-D alloimmunization after exposure to normal D. For safety reasons, individuals with partial D, DEL, and weak D types other than Types 1, 2, and 3 should be treated as D– to exclude anti-D induction.[33] Molecular *RHD* typing of patients with ambiguous D typing is therefore of considerable clinical relevance, and correct *RHD* variant identification allows to reserve the D– blood supply for those that are actually in need for it. In addition, *RHD* genotyping is able to confirm D– typing of donor samples, excluding extremely weak D expression. In this study, 71.0% of all samples with unclear serologic D typing results could be assigned a safe D-matched transfusion strategy using a single PCR-SSP kit. A further 21.2% were identified as *RHD*+ or *RHD*–, mostly concerning samples with inconclusive serology consequent to recent transfusion. Only a small percentage of the *RHD*+ samples may include weak or partial D types that are not included in the kits used.

Besides the prevalent weak D Types 1, 2, and 3 with apparently complete D epitope composition, 71 samples with other genotypes were identified, including the novel *RHD*06.03.02* allele. Unfortunately, no detailed immunohematologic work-up of this partial D could be performed due to unavailability of RBCs.

Other variant *RHD* alleles known to include the Ser103Pro substitution are *RHD*03.02* and *RHD*07.02* and comparative serology could certainly have added additional information, but was not pursued, due to the unavailability of respective sample material.[34,35] However, our *RHD*(S103P) analysis is the first documented case with anti-D (and anti-C) proving the partial nature of this near-normal D phenotype. The single 307T>C substitution predicts an isolated Ser103Pro amino acid exchange in the second extracellular RhD loop. Due to proline's angled structure it may act as structural disruptor probably altering the D antigen.[36,37]

CONFLICT OF INTEREST

CG is employed by the Blood Transfusion Service Zurich, SRC, Switzerland, and acts as a consultant for Inno-Train GmbH, Kronberg im Taunus, Germany. Inno-Train GmbH produces and distributes similar products in comparison to those described for molecular *RHD* typing within this manuscript. All other authors have disclosed no conflict of interest.

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