Case Report

Serologic and molecular characterization of D variants in Brazilians: impact for typing and transfusion strategy

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Rh discrepancies are a problem during routine testing because of partial D or weak D phenotypes. Panels of monoclonal antibodies (MoAb) are being developed to identify D variants such as partial D and weak D when there are anomalous D typing results; however, molecular characterization offers a more specific classification of weak and partial D. The weak D and partial D phenotypes are caused by many different *RHD* alleles encoding aberrant D proteins, resulting in distinct serologic phenotypes and the possibility of anti-D immunization. We evaluated currently used serologic methods and reagents to detect and identify D variants and correlated the results with molecular analyses. A total of 306 blood samples from Brazilian blood donors and patients with discrepant results in routine D typing were analyzed. In total, 166 (54.2%) weak D, 136 (44.4%) partial D, 3 (1%) DEL, and 1 (0.3%) DHAR variants were identified. Among weak D samples, 76 weak D type 1 (45.8%), 75 weak D type 2 (45.2%), 13 weak D type 3 (7.8%), and 2 weak D type 5 (1.2%) alleles were found. Among the partial D samples, 49 type 4.0 weak partial D (36%), 9 DAR (6.6%), 24 DFR (17.6%), 6 DBT (4.4%), 1 DHMi (0.73%), 26 DVI (19%), 14 DVa (10.3%), 5 DIVb (3.7%), and 2 DVII (1.5%) were observed. Two samples identified as DEL by adsorption-elution were characterized by molecular analyses as *RHD(IVS5–38DEL4)* and one sample was characterized as *RHD(K409K)*. One sample was characterized as DHAR, a CE variant positive with some monoclonal anti-D. Our results showed that the use of different methods and anti-D reagents in the serologic routine analysis revealed D variants that can be further investigated. Molecular methods can help to differentiate between partial D and weak D and to characterize the weak D types, providing additional information of value in the determination of D phenotypes. This distinction is important for optimized management of D– RBC units and for the prevention of anti-D–related hemolytic disease of the fetus and newborn. ***Immunohematology* 2011;27:6–11.**

Key Words: *RHD* alleles, weak D phenotype, partial D, anti-D immunization, Brazilians

Rh epitopes are highly conformational, and single amino acid changes in one part of the protein, including changes within the transmembrane regions, can affect expression of epitopes or result in new epitopes. Single nucleotide polymorphisms (SNPs) and gene conversions between the *RHD* and *RHCE* genes are primarily responsible for the large number of variations in expression of the Rh antigens.[1,2]

The D antigen is the most important blood group antigen determined by a protein, because D– individuals can be easily immunized to make anti-D. A plethora of *RHD* alleles have been identified at the molecular level, including those that encode partial D, and weak D types, and a particularly weakly expressed D antigen termed DEL, that can only be demonstrated by adsorption and elution.[3–9]

Weak D phenotypes are characterized by depressed expression of the D antigen, and at the molecular level, D variants are caused by many different *RHD* alleles carrying single or multiple missense mutations in their *RHD* coding sequences encoding altered D proteins.[3,4,6] No alloanti-D has been found in individuals with the most common weak D types (1, 2, and 3); however, little is known about the anti-D immunization risk in people with the rare *RHD* alleles with lower antigen density than weak D type 2.[4,10] Weak D types generally present all epitopes albeit with some epitopes showing variability depending on the monoclonal anti-D used for testing.[11] The classification of variants as weak D does not imply that carriers will not be immunized by exposure to normal D through transfusion or pregnancy as weak D types 4.0, 4.2, 11, and 15 were described to be prone to anti-D alloimmunization and thus should be considered as partial D.[4,11–15] Partial D variants lack D antigen epitopes, and individuals who harbor partial D variant alleles have the potential to make alloanti-D.[8] Anti-D immunization was also attributed to patients transfused with red blood cell (RBC) units from DEL donors.[9]

The differentiation and identification of D variants is important for selection of blood products and to prevent anti-D-related hemolytic disease of the fetus and newborn; however, it is not always straightforward, and, occasionally, phenotype discrepancies occur between two reagents.[11,16,17]

Populations with African admixture, such as the Brazilian population, can present a high variety of *RHD* alleles.[18] A comprehensive investigation of the *RHD* alleles that encode weak D expression at the RBC surface could have a considerable impact on the typing and transfusion strategy in countries like Brazil where the prevalence of D– phenotypes ranges from 5 percent to 12 percent

approximately. The use of different methods and anti-D reagents in the routine serologic analysis has revealed some D variants that are being investigated by molecular methods. We here describe the serology and molecular analyses performed to identify such variants among Brazilian blood donors and patients with discrepant results of D typing.

Materials and Methods

Blood Samples

Blood samples, collected over a 2-year period, from 306 Brazilians (117 blood donors and 189 patients) with discrepant results of D typing with different commercial anti-D monoclonal antibodies (MoAbs), or weak reactivity (<3+ at room temperature or reactivity in indirect antiglobulin test [IAT] only) were referred by routine laboratories from different regions of Brazil to our laboratory for molecular characterization and transfusion counseling. D typing in the referring laboratories was performed with two different anti-D MoAbs in conjunction with IAT as is required in Brazil for donors and patients as a result of the deficit of D– RBC units.

Serologic Studies

D, C, E, c, and e status of all RBCs was determined by hemagglutination in gel neutral cards (DiaMed AG, Cressier sur Morat, Switzerland) using routine anti-D, anti-C, anti-E, anti-c, and anti-e monoclonal reagents (Fresenius Kabi, São Paulo, Brazil). D-antigen reactivity was analyzed by agglutination in tube and gel cards using six anti-D monoclonal reagents: anti-D IgM (clone 175–2) and anti-D IgG (clone ESD1; DiaMed AG); IgM (clone P3X61) and anti-D Blend (clones P3X290, P3X35, P3X61, P3X21223B10; Grifols, Barcelona, Spain); and anti-D IgM (clone MS201) and anti-D IgG (clone MS26; Smart Kit, Fresenius Kabi). Nonreactive samples were tested with anti-D blend (clones MS26/MS201) and anti-D IgG (clone ESD1) using the IAT in tube and two gel matrix techniques (DiaMed and Grifols). An adsorption-elution test was performed on samples that were nonreactive in the IAT and expressed the C antigen.

PCR Assays

DNA was extracted from whole blood samples using the QIAmp DNA Blood Mini-Kit (Qiagen, Valencia, CA), according to the manufacturer's recommendations. Two polymerase chain reaction (PCR) assays were used to determine the presence or absence of *RHD*-specific amplified products from sequences in intron 4 and exon 7.[19] The other assays used were a PCR system using sequence-specific primers (SSP) that detect the common weak D types,[6] a multiplex PCR[20] that detects the *RHD* gene hybrid alleles, and specific PCR–restriction fragment length polymorphisms (RFLP) to distinguish between weak D type 4.2.2 and DAR alleles.[21] Table 1 summarizes the *RHD* alleles investigated and the polymorphisms detected.

Sequence Analysis

Sequence analysis was performed to confirm the DEL phenotypes found by adsorption-elution on PCR products amplified from genomic DNA using *RHD*-specific primers as previously reported.[3] PCR products were purified by elution from 1 percent agarose gels using a Qiaex II gel extraction kit (Qiagen) and sequenced directly, without subcloning, on an ABI 373XL Perkin Elmer Biosystems (PEB) sequencer using the PEB Big Dye reagent BD Half-term (GenPak, Perkin Elmer Biosystems, Foster City, CA).

Results

During a 2-year period blood samples from 117 blood donors and 189 patients with discrepant results or weak reactivity with two monoclonal anti-D reagents in routine diagnostics were tested by hemagglutination with currently used MoAbs in Brazil and by molecular analyses. Although we have separated those two populations (patients and donors) in this study, they are comparable in terms of ethnic background in Brazil.

Molecular Analyses

In total, 166 weak D (54.2%), 136 partial D (44.4%), 3 DEL (1%), and 1 DHAR (0.3%) variants were identified. Tables 2 and 3 summarize the distribution of weak D and partial D alleles and the associated haplotypes. The weak D types 1, 2, and 3 were the most prevalent weak D types found in this population. Weak D types 1 and 3 were associated with the DCe and Dce haplotypes, and weak D type 2 was associated with DcE .

Serologic Reactivity

Six selected monoclonal anti-D (IgG, IgM, and blend) were used in tube and gel to evaluate the reactivity pattern of these monoclonal anti-D reagents with D variants. The reactivity with the monoclonal anti-D reagents showed a generally consistent pattern among the variant *RHD* alleles that occurred more than once. Table 4 summarizes the results found in the donor and patient samples studied. Weak D types 1 and 3 and weak partial D type 4.0 and partial D DBT were detected with all anti-D MoAbs in tube and gel. Weak D types 2 and 5 and partial D DAR, DFR, DHMi, and DVI were not detected with the IgM monoclonal anti-D antibodies, whereas DIVb was not detected with

Table 1. Molecular basis of D variants and polymorphisms detected in this study

RHD allele	Molecular basis	Polymorphisms detected
*RHD*weak D type 1*	809T>C	809T>C
*RHD*weak D type 2*	1154G>C	1154G>C
*RHD*weak D type 3*	8C>G	8C>G
*RHD*weak D type 4.0*	602C>G, 667T>G, 819G>A	602C>G, 819G>A
*RHD*weak D type 4.1*	48G>C, 602C>G, 667T>G, 819G>A	48G>C, 602C>G, 819G>A
*RHD*weak D type 4.2.2*	602C>G, 667T>G, 744C>T, 1025T>C	602C>G, 667T>G, 744C>T, 1025 T>C
*RHD*weak D type 5*	446C>A	446C>A
*RHD*DAR*	602C>G, 667T>G, 1025T>C	667T>G,744C>T, 1025T>C
*RHD*DFR*	505A>C, 509T>G, 514A>T (*RHCE*-like segment encompassing part of exon 4)	505A>C, 509T>G, 514A>T
RHD*DBT	667T>G, 697G>C, 712G>A, 733G>C, 744C>T, 787G>A, 800A>T, 916G>A, 932A>G, 941G>T, 968C>A, 974G>T, 979A>G, 985G>C, 986G>A, 989A>C, 992A>T, 1025T>C, 1048G>C, 1053C>T, 1057G>T, 1059A>G, 1060G>A, 106C>A (*RHCE*-like segment encompassing exons 5 to 7)	*RHD* exon scanning: exons 5 to 7 negative
*RHD*DHMi*	848C>T	848C>T
*RHD*DIVb*	104G>C, 1053C>T, 1057G>T, 1059A>G, 1060G>A, 1061C>A, 1170C>T, 1193A>T (*RHCE*-like segment encompassing part of exon 7 to exon 9)	*RHD* exon scanning: exons 7 and 9 negative
*RHD*DVa*	667T>G, 697G>C, 733G>C, 744C>T, 787G>A, 800A>T (*RHCE*-like segment encompassing exon 5)	*RHD* exon scanning: exon 5 negative
*RHD*DVI.1*	505A>C, 509T>G, 514A>T, 544T>A, 577G>A, 594A>T, 602C>G, 667T>G, 676G>C, 697G>C, 712G>A, 733G>C, 744C>T, 787G>A, 800A>T (*RHCE*-like segment of CE-allele encompassing exons 4 to 5	*RHD* exon scanning: exons 4 and 5 negative
*RHD*DVI.2*	505A>C, 509T>G, 514A>T, 544T>A, 577G>A, 594A>T, 602C>G, 667T>G, 697G>C, 712G>A, 733G>C, 744C>T, 787G>A, 800A>T, 916G>A, 932A>G (RHCE-like segment encompassing exons 4 to 6)	*RHD* exon scanning: exons 4 to 6 negative
*RHD*DVII*	329T>C	329T>C

anti-D IgG. Weak D type 2 and partial D DVI type 1 showed the same pattern of reactivity with the six monoclonal anti-D used.

Sequence Analysis

To confirm the DEL phenotype results obtained by adsorption-elution, we performed sequence analysis of the *RHD* from three DNA samples. Two donor samples were characterized by molecular analyses as *RHD(IVS5–38DEL4)* and one patient sample was characterized as *RHD(K409K)*.

Discussion

We report a serologic and molecular study of D variants in Brazilians who were identified because of weak or discrepant D typing results with different commercial monoclonal anti-D reagents and show that a high percentage (44%) of them are partial D, including the weak partial D type 4.0. Seventy-seven partial D variants (56.6%) were from patients, and 5 of them (3 partial D category VI type 2, 1 partial D DAR, and 1 partial D category Va) already had alloanti-D in their serum. Many partial D variants were classified as weak D owing to variable reactivity with the anti-D MoAb used.

Table 2. Weak D types and associated haplotypes found in blood donor and patient samples

		Weak D types				
Samples	Haplotypes	Type 1	Type 2	Type 3	Type 5	Total
Blood donors	*Dce*	4		2		6
	DCe	33		4	2	39
	DcE		22			22
	Total	**37**	**22**	**6**	**2**	**67**
Patients	*Dce*	3		3		6
	DCe	36		4		40
	DcE		53			53
	Total	**39**	**53**	**7**		**99**
Total of samples	*Dce*	7		5		12
	DCe	69		8	2	79
	DcE		75			75
	Total	**76**	**75**	**13**	**2**	**166**
	%	**45.8**	**45.2**	**7.8**	**1.2**	

Table 3. Partial D and associated haplotypes found in blood donor and patient samples

Samples	Haplotypes	Partial D									Total
		Type 4.0	DAR	DFR	DBT	DHMi	DVI	DVa	DIVb	DVII	
Blood donors	*Dce*	21	1					3			25
	DCe			5	3	1	12*				21
	DcE						1†				1
	Total	**21**	**1**	**5**	**3**	**1**	**13**	**3**	**0**	**0**	**47**
Patients	*Dce*	28	8		1			11		2	50
	DCe			16	2		13*		5		36
	DcE			3							3
	Total	**28**	**8**	**19**	**3**	**0**	**13**	**11**	**5**	**2**	**77**
Total of samples	*Dce*	49	9		1						59
	DCe			21	5	1	25*	14	5		71
	DcE			3			1†				4
	Total	**49**	**9**	**24**	**6**	**1**	**26**	**14**	**5**	**2**	**136**
	%	**36**	**6.6**	**17.6**	**4.4**	**0.73**	**19**	**10.3**	**3.7**	**1.5**	

*DVI type II. †DVI type I.

This 44 percent value corresponds to a prevalence study in a population with a D variant phenotype and not to the ratio between the number of *RHD* alleles known to encode a partial and weak D and the total number of weak *RHD* alleles described. That is the reason why our results differ from other results reported, that approximately 5 to 10 percent of weak D are partial D.[3,4,11,22]

In our study weak D type 2 and D category VI type 1 showed a similar reactivity pattern with the anti-D used, despite their different molecular background. The IgM anti-D used do not detect DVI, whereas the IgG anti-D detect DVI. As observed in Table 4, the IgM anti-D failed to react with weak D type 2 and DVI type 1, and the patterns of reactivity with the other anti-D used were similar in tube and in gel. One explanation for these results may be the difference in the ability of the IgG anti-D MoAb and blend IgG + IgM anti-D to detect "weak D phenotypes" compared with that of the IgM anti-D. This finding reinforces that there is no well-defined borderline between weak D and some partial D phenotypes that express the D antigen weakly (partial weak D phenotype) that have an aberrant *RHD* coding sequence,[3] and therefore a PCR screen for those variants should be recommended.

Table 4. *RHD* alleles and reactivity with monoclonal anti-D reagents

Samples	*RHD* Alleles	MoAbs × reactivity								
		Tube			Gel DiaMed			Gel Grifols		
		MS201	MS26+MS201	P3X35/ P3X61 IAT	175/2	MS26	ESD1 IAT	P3X61	P3X35/ P3X61	MS26/MS201 IAT
76	*RHD*weak D type 1*	1+	2+	3+	1+	2+	3+	2+	3+	3+
75	*RHD*weak D type 2*	0	0	1+	0	(+)	1+	0	1+	2+
13	*RHD*weak D type 3*	2+	3+	3+	3+	2+	3+	3+	3+	4+
2	*RHD*weak D type 5*	0	0	(+)	0	0	1+	0	1+	2+
49	*RHD*weak partial 4.0*	2+	3+	3+	3+	2+	3+	2+	3+	3+
9	*RHD*DAR*	0	1+	2+	0	1+	2+	0	1+	2+
24	*RHD*DFR*	0	1+	2+	0	2+	3+	0	2+	3+
6	*RHD*DBT*	1+	1+	2+	2+	2+	3+	3+	2+	3+
1	*RHD*DHMi*	0	1+	2+	0	1+	2+	0	2+	3+
5	*RHD*DIVb*	2+	1+	3+	2+	0	3+	2+	1+	2+
14	*RHD*DVa*	2+	2+	3+	1+	1+	2+	2+	1+	2+
25	*RHD*DVI.1*	0	0	1+	0	1+	2+	0	1+	2+
1	*RHD*DVI.2*	0	1+	2+	0	2+	2+	0	2+	3+
2	*RHD*DVII*	0	2+	3+	1+	2+	3+	2+	3+	3+

(+) = weak. MoAbs = monoclonal antibodies.

Among the weak D samples, 164 of 306 (53.6%) were categorized as weak D types 1, 2, and 3 with the molecular assays, and for those individuals D+ transfusion could be considered safe because no immunization events have been documented yet.[4] Such a strategy is estimated to reduce the use of D– blood by 2 to 3 percent. The high prevalence of weak D types 1, 2, and 3 was consistent with other studies in Europe.[3,4] In our population we see a higher prevalence of weak partial D type 4.0 (36%), perhaps because of the marriage among Caucasians, Amerindians, and Africans that occurred in our population. For the partial D, we also observed a higher prevalence of DAR, DFR, and DVa, reinforcing that the ethnic background of the population may govern which variants are prevalent.

Weak D and partial D and the associated haplotype found in this study were consistent with those found in other studies,[3] although we have also found the Dce haplotype associated with weak D types 1 and 3 (Table 2). Unfortunately, we did not have enough DNA to sequence the full *RHD* on those samples, and further studies are necessary to confirm this finding.

The three samples identified as DEL by adsorption and elution exhibited known DEL alleles, *RHD(IVS5–38DEL4)* and *RHD(K409K)*. Donor RBC units from such individuals have been described before as inducing anti-D alloimmunization in D– patients.[5,9] Taking these results into account, we recommend performing molecular analyses on donor samples phenotyped as D– C+ but D+ by adsorption and elution to identify the DEL allele and avoid immunization.

The sensitivity of the method used to type donor and patients may depend on the anti-D reagent used and on the exact conditions of the methods. For donor typing all potentially immunogenic D+ samples should be recognized as D+, and based on this we propose the use of two anti-D reagents (one anti-D blend and one IgM anti-D) to minimize the need for the IAT on donor samples. Because anti-D immunization may pose a serious clinical problem mainly in women of childbearing age, we propose that two different anti-D reagents (one IgM anti-D that does not detect DVI and one IgG anti-D that detects DVI) be used routinely to establish the Rh status for obstetric patients and transfusion recipients. When a discrepancy occurs between the two reagents, we recommend that molecular analysis is performed to identify the *RHD* allele. This strategy of combining serologic and molecular typing can provide a better solution to accurately determine the D-antigen status .

Finally, this study is of interest from a genetic and population perspective because it gives insight into the diversity of the *RHD* alleles in Brazilians. It is important to remember that much of the recent data on weak D and partial D have come from Europe.

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