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Variant *RHD* Types in Brazilians With Discrepancies in RhD Typing

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Background: The knowledge of D variants in patients and donors is important because anti-D alloimmunization can occur in some but not all individuals who express a variant *RHD* allele. Serologic distinction of RhD discrepancies is not always straightforward, which makes molecular analysis highly desirable. *Methods:* A group of 223 subjects, 129 patients, and 94 blood donors was identified and analyzed on the basis of a D typing discrepancy. The D antigen expression was evaluated by tube and gel hemagglutination with four anti-D reagents. PCR-single specific primer (SSP), multiplex PCR, RHD BeadChip (Immucor), or sequencing were used for molecular analysis. *Results:* In total, 168/223 (75%) weak D and 55/223 (25%) partial D variants were identified. Hemagglutination results varied in methods and anti-D reagents used in this process. There was no standard serologic reactivity identified, which could predict what type of D variant would be identified. Among weak D samples, types 1–3 were the most common, while DAR and DVI were most prevalent among partial D samples. *Conclusion:* Our results show that discrepancies found in the serologic typing should be investigated by molecular methods in order to determine the D variant involved and also to distinguish between weak D and partial D. The knowledge of the distribution of weak D types and partial D among populations is important for D− patients and pregnant women management. J. Clin. Lab. Anal. **30**:845–848, 2016. © 2016 Wiley Periodicals, Inc.

Key words: hemagglutination; molecular analysis; partial D; RhD; weak D

INTRODUCTION

The D antigen is very immunogenic and most important in the Rh blood group system, because D− individuals can be easily immunized (1, 2). Anti-D is clinically significant as it causes hemolytic transfusion reactions and hemolytic disease of the fetus and newborn (HDFN) (2). Besides D+ and D− phenotypes, there is a plethora of D variants characterized as weak D, partial D, and deletion (DEL) and in recent years an increasing number of altered *RHD* alleles have been reported (*RhesusBase*) (3). The molecular mechanisms responsible for D variants predominantly include nucleotide changes in *RHD*, resulting in amino acid substitutions in the RhD protein, and genetic recombination giving rise to a hybrid RhD protein (4–6).

Serologic tests cannot distinguish between partial D and weak D types but can detect variants with weakened or altered expression, and therefore discrepancies in the serologic routine analysis should be investigated by molecular methods (7).

Weak D types 1–3 are the most frequent variants detected by serology, but frequencies vary in different populations (8–12). The populations of African admixture, such as the Brazilian population, can present a high variety of *RHD* alleles and therefore information on the

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prevalence of different variants can impact the typing and transfusion strategy.

Individuals with DEL and partial D variant alleles have the potential to form anti-D, whereas common weak D types 1–3 do not form anti-D (13–15). It has been recommended that weak D patients with the most common types may receive D+ red blood cells (RBCs) to conserve stocks of D− RBCs, and partial D patients should be given D− RBCs (8,16). The identification of D variants is important for selection of blood products and prevention of anti-D-related HDFN (2).

In the past years, we introduced in our D typing routine the use of immunoglobulin G (IgG), immunoglobulin M (IgM), and blend anti-D reagents in tube and gel, and noted a high frequency of RhD discrepancies among patients and blood donor samples. Here, we describe the serology and molecular analyses performed to identify D variants with discrepant results of D typing in order to determine the frequencies of D variants in this Brazilian population and to avoid the unnecessary use of D− blood in those patients who are not at the risk of anti-D alloimmunization. We also evaluated, retrospectively, whether patients molecularly characterized as D variant developed alloanti-D.

MATERIALS AND METHODS

Samples

A total of 21,353 patients and 51,671 blood donors from Hospital Albert Einstein, São Paulo, Brazil, were typed during a 4-year time frame. Blood samples from 223 individuals (129 patients and 94 blood donors) showed discrepant results of D typing with four commercial anti-D monoclonal antibodies (MoAbs) and were evaluated by molecular methods. This study was conducted in accordance with institutional ethical review.

Serologic Analysis

D antigen expression was evaluated by hemagglutination using four anti-D MoAbs. For gel cards, anti-D IgM (clone P3 × 61) and anti-D blend (clones P3 × 290, P3 × 35, P3 × 61, P3 × 2123B10) from Grifols, Barcelona, Spain, were used. For tube technique, anti-D IgM (clone MS201) and anti-D IgG (clone MS26) from Fresenius Kabi, São Paulo, Brazil, were used. In all nonreactive samples, a confirmatory test was performed with anti-D IgG (MS26) using the indirect antiglobulin test (IAT) in the tube. C, c, E, and e status of all RBCs was determined by hemagglutination in gel cards (Grifol) with specific MoAbs. Retrospective analysis of antibody screen results was performed on all samples.

Molecular Analyses

DNA was extracted from whole blood using the QIAmp DNA Blood Mini-Kit (Qiagen, Valencia, CA), according to the manufacture's recommendations. Molecular tests performed on all 223 discrepant samples included PCR-SSP that detects the common weak D types (14) and a multiplex PCR that detects the *RHD* gene hybrid alleles (17) for all samples; RHD BeadChip^{TM} (Bioarray Solutions, Immucor, NJ) was used for 55 samples. However, six samples could not be assigned an *RHD* allele and were subjected to direct automated sequencing of *RHD* using RHD-specific primers as previously reported (18).

RESULTS

Molecular Analyses

The molecular analyses confirmed the presence of D variants in all samples with discrepant results in serology. In total, 168 of 223 (75.4%) weak D and 55 of 223 (24.6%) partial D were characterized. Partial D was identified in 31 (24%) patients and 24 (25.5%) donors, while weak D was identified in 98 (76%) patients and 70 (75%) donors.

Among weak D samples, 70 (41.6%) weak D type 1, 44 (26.2%) weak D type 2, 26 (15.5%) weak D type 3, 25 (14.9%) weak D type 4.0, 1 (0.6%) weak D type 5, and 2 (1.2%) weak D type 38 were found. Among the partial D samples, 10 DIVa (18.2%), 7 (12.7%) DIV type 4, 3 (5.5%) DIVb, 17 (30.9%) DAR, 16 (29.1%) DVI, and 2 (3.6%) DFR were identified. Table 1 presents the D variants identified in patients and donors.

Serology

The reactivity of monoclonal anti-D reagents and methods used in this process varied among the samples with different D variants, but presented a consistent pattern among the same types of variants. Table 2 shows the serologic results found in the donor and patient samples associated with the D variant identified by molecular analyses and with the CE haplotype determined by phenotyping. Weak D types 1, 3, and 4.0 and partial D DFR showed reactivity with the four MoAbs used in the tube and gel. The other weak D types and partial D samples showed different patterns of reactivity with the four monoclonal anti-D used in this process.

Anti-D Alloimmunization

In the population of patients with D-typing discrepancies, 46 of 129 (36%) showed D variants with the potential to form anti-D. Retrospective analysis and a limited clinical data regarding the transfusion histories and antibody screen results were reviewed. Five of those patients were

TABLE 1. Distribution of D Variant Alleles Identified in Patients and Blood Donor Samples

Samples	Alleles encoding weak D phenotypes						Total
	*RHD**weak D type 1	*RHD**weak D type 2	*RHD**weak D type 3	*RHD**weak D type 4.0	*RHD**weak D type 5	*RHD**weak D type 38	
Blood donors	29 (41%)	23 (32.8%)	5 (7.1%)	11 (15.7%)	0	2 (2.8%)	70
Patients	41 (41.8%)	21 (21.4%)	21 (21.4%)	14 (14.3%)	1 (1%)	0	98
	Alleles encoding partial D phenotypes						
	*RHD**DIVa	*RHD**DIV type 4	*RHD**DIVb	*RHD**DAR	*RHD**DVI	*RHD**DFR	Total
Blood donors	4 (16.6%)	0	3 (12.5%)	8 (33.3%)	7 (29.2%)	2 (8.3%)	24
Patients	6 (19.3%)	7 (22.6%)	0	9 (29%)	9 (29%)	0	31

*RHD typing was performed by molecular tests.

transfused with RhD-positive RBCs with an average of 1.8 (range: 1–4), which included two typed weak D type 4.0, one partial DIVa, one partial DIV type 4, and one partial DVI type 1, but only the patient with the partial DIVa formed alloanti-D.

DISCUSSION

RHD genotyping is recommended when discordant RhD typing results are encountered and/or serologic weak D reactivity is observed (7, 8). The identification and differentiation of D variants can avoid the unnecessary use of RhD-negative RBCs in patients who are not at risk of alloimmunization, and therefore could have an important impact on the transfusion strategies in countries such as Brazil, where the prevalence of D− phenotypes ranges from 5% to 12%.

In this study, we used molecular assays to analyze samples of patients and donors in São Paulo, Brazil, with discrepancies in serologic RhD typing, and 12 variant *RHD* alleles were identified. Our molecular approach used for identifying the Brazilian samples with RhD antigen discrepancies recognized *RHD* variant alleles in all the investigated samples, reinforcing the need to set up a strategy for serologic discrepancies identification and resolution.

RhD antigen serologic discrepancies have been associated with specific partial D and weak D types generally associated with ethnicity (10–15). Most studies on frequencies of *RHD* alleles were performed in Central Europe and show that approximately 95% of Caucasians with serologic weak D phenotype have weak D types 1–3 (14). The prevalence of weak D types 1 through 3, which we observed in this population of patients and donors with weak D expression, is much lower (approximately 63%)

TABLE 2. D Variants, CE Haplotypes, and Reactivity With Monoclonal Antibodies

Samples	*RHD* alleles	Associated CE haplotype	Serological reactivity with monoclonal antibodies				
			Tube			Gel Grifols	
			MS26	MS201	MS26 IAT	P3×61	P3 × 290, P3 × 35, P3 × 61, P3 × 2123B10
70	*RHD**weak D type 1	Ce	2+	1+	3+	2+	3+
44	*RHD**weak D type 2	cE	1+	0	3+	0	2+
26	*RHD**weak D type 3	Ce	2+	2+	4+	3+	3+
25	*RHD**weak D type 4.0	ce	2+	3+	4+	3+	3+
1	*RHD**weak D type 5	cE	0	0	1+	0	(+)
2	*RHD**weak D type 38	Ce	0	0	(+)	0	0
17	*RHD**DAR	ce	2+	0	4+	1+	2+
2	*RHD**DFR	Ce	2+	2+	3+	3+	3+
10	*RHD**DIVa	ce	0	3+	0	3+	3+
7	*RHD**DIV type 4	Ce	0	2+	0	3+	3+
3	*RHD**DIVb	Ce	0	2+	0	2+	2+
16	*RHD**DVI	Ce	1+	0	3+	0	2+

+, weak.
*RHD typing was performed by molecular tests.

and we observe a higher prevalence (15%) of weak D type 4.0. These data differ from studies performed in Caucasians but are consistent with another study performed in Brazilians (19). For the partial D, we observed a higher prevalence of DAR, DIVa, and DVI, reinforcing that the prevalence of D variants may be associated with the ethnic background of a population. Weak D and partial D, and the associated CE haplotypes found in this study were consistent with the haplotypes found in other studies (4).

It is well established that transfusion recipients with the most common weak D types 1–3 are not at risk of forming alloanti-D when exposed to conventional RhD-positive RBCs (7,9) and, according to our results, 83 of 129 (64%) patients could be managed safely as RhD-positive. Although there are few reports of anti-D in patients with weak D type 4.0 (8,9,20), it is still recommended to manage this weak D type as RhD-negative (16).

In the population of patients with D-typing discrepancies, 46 of 129 (36%) showed D variants with potential to form anti-D. The goal of RhD typing practices is to protect RhD-negative persons from inadvertent alloimmunization to the D antigen by exposure to RhD-positive RBCs, including RBCs expressing a serologic weak D phenotype. Although, in this study, two patients with weak D type 4.0 did not form anti-D, the other two patients with partial D also did not form anti-D, and therefore more evidences are necessary to determine whether weak D type 4.0 can be managed as RhD-positive.

In this study, partial D variants were initially classified as weak D owing to variable reactivity with the anti-D MoAb used in this process. Weak D type 2 and D category VI were not reactive with the two IgM anti-D (clones MS201 and P3 × 61), while the IgG anti-D (MS26) failed to react with D categories IVa, IVb, and IV type 4. Weak D types 5 and 38 were only detected with IgG anti-D by IAT. The blend anti-D in gel detected all D variants, except weak D type 38. This finding reinforces that there is no well-defined borderline between weak D and partial D phenotypes, as a negative reaction with a particular MoAb or a specific method could originate from weak expression of the D epitope (10), rather than from its absence. Although the correct classification of D variants in relation to transfusion strategies should be performed by molecular typing, the selection of anti-D reagents and sensitivity of the serologic method used in the donor routine are essential to identify donor red cells capable of immunizing a D− patient to form anti-D (21).

In conclusion, this study gives insight into the diversity, frequencies, and clinical relevance of the variant *RHD* alleles in Brazilians with discrepancies in RhD typing. Moreover, here we show that although weak D types 1 through 3 are most common in our population, their prevalence is much lower than the types found in Europeans. These findings may contribute to the development of D phenotyping and genotyping strategies in this population for more precise clinical decision making in transfusion medicine and obstetric practice.

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