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RESEARCH ARTICLE

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RHD alleles among pregnant women with serologic discrepant weak D phenotypes from a multiethnic population and risk of alloimmunization

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Background: A considerable number of *RHD* alleles responsible for weak and partial D phenotypes have been identified. Serologic determination of these phenotypes is often doubtful and makes genetic analysis of *RHD* gene highly desirable in transfusion recipients and pregnant women. We analyzed the *RHD* gene in a cohort of pregnant women with doubtful D phenotypes.

Methods: *RHD* genotyping was performed on 104 cases with D typing discrepancies or with history of serologic weak D phenotype. Laboratory-developed DNA tests, *RHD* BeadChip (Bioarray Solutions, Immucor), and sequencing were used to identify the *RHD* alleles.

Results: Molecular analyses showed 23 of 104 (22%) pregnant women were *RHD*weak D types 1*, *2*, or *3* and not at risk for anti-D. Fifty-one (49%) were *RHD*weak partial 4.0*, 6 *RHD*weak D type 38* (6%), 1 *RHD*weak D type 45* (1%), 1 *RHD*weak D type 67* (1%), and potentially at risk for being alloimmunized and making anti-D. Partial D was identified in 22 of 104 (21%) patients and definitively at risk for anti-D.

Discussion: Appropriate classification of RhD phenotypes is recommended for correct indication of RhIG in pregnant women. However, the serologic distinction between RhD-negative and RhD-positive phenotypes is a difficult task in the case of D variants due to the variations in serologic testing. Our results show a great variability in *RHD* variant alleles in pregnant women from this population of high admixture. According to these results, 78% of these obstetric patients are at risk for anti-D and candidates for RhIG.

KEYWORDS

D variants, genetic counseling, partial D, pregnant women, *RHD* alleles, weak D

1 | INTRODUCTION

The D antigen is one of the most important blood group antigens because of its implication in transfusion practice and fetal maternal medicine. Anti-D is a frequent cause of hemolytic disease of the fetus and newborn (HDFN) and, as a rule, immunization occurs in D-negative pregnant women, but occasionally anti-D is also observed in carriers of D variants.[1]

Prophylaxis with Rh immune globulin (RhIG) has been highly successful in preventing RhD alloimmunization and HDFN in pregnant women whose red blood cells (RBCs) type as RhD-negative but this practice became more complex with recognition of D variants in different populations.[2,3] A number of D variants caused by hybrid genes or nucleotide polymorphisms have been identified and classified as weak and partial antigens.[4-6] Weak D antigens are characterized by amino acid changes within either the membrane-spanning domains or

the cytoplasmic loops of the RhD protein causing decreased antigen expression on the red blood cell (RBC) surface.[7] Weak D antigens have all D epitopes and are unlikely to produce anti-D. Partial D antigens have amino acid changes outside of the membrane and lack one or more D epitopes. Individuals with partial D antigens have the potential to produce alloanti-D against the part of D that they lack, and therefore, it is of clinical importance to identify D variants with potential risk of RhD alloimmunization in pregnant women and transfused patients.[5]

Serologic reagents cannot always discriminate between weak D and partial D, and reactivity patterns often reflect the characteristic of the reagent rather than the D expression on RBCs but, as the genetic basis of most common RhD variant antigens has been determined, D variants have been classified at molecular level.[6,8]

The molecular distinction of partial D and weak D alleles, and the characterization of weak D types have been applied to predict the risk for RhD alloimmunization in patients and pregnant women.[5,9] Weak D phenotypes are the most common D variants detected by serology but their occurrence varies according to race and ethnicity. In Caucasians, the majority of weak D phenotypes are associated with *RHD*weak D types 1*, *2*, and *3* genotypes in which alloimmunization has not been observed.[10,11] On the other hand, *RHD*weak partial 4.0* and partial D are the most frequent type of D variants found in individuals of African origin whose carries may develop anti-D.[12]

Recently, a Work Group on *RHD* genotyping developed recommendations to clarify clinical issues related to RhD typing in persons with weak D phenotype.[13] This Work Group recommended that *RHD* genotyping be performed when a discrepant result or a serologic weak D phenotype is identified in patients, including pregnant women. According to this Group, patients and pregnant women with *RHD*weak D types 1*, *2*, or *3* genotypes should be managed as RhD-positive with regard to administration of RhIG and/or selection of blood components for transfusion. The benefit of this recommendation is unnecessary injections of RhIG in pregnant women and increased availability of RhD-negative RBCs for transfusion.[13,14]

This recommendation supports the use of *RHD* genotyping in obstetric patients in order to avoid confusion due to discrepant serologic results and to indicate the RhIG. Knowledge of *RHD* variant alleles and risk of alloimmunization in obstetric patients are important for prenatal management.

Knowing that our population is very ethnically diverse and that RhIG should be offered to women known to have D variants, other than *RHD*weak D types 1*, *2*, and *3*, we investigated serologic discrepancies in D typing of pregnant women at our institution in order to characterize *RHD* alleles and thereby distinguish women with variants who are capable of making anti-D and who are therefore candidates for RhIG.

2 | MATERIALS AND METHODS

2.1 | Samples

A total of 21 353 samples from pregnant women was submitted to our laboratory for D typing during 2-year period and samples showed D typing discrepancies were further analyzed. All patients had a high degree of admixture between descendants of Europeans, Africans and Native-Americans in their ethnic background. The study was conducted in accordance with our institutional ethical review.

2.2 | Serologic analysis

D antigen expression was evaluated by hemagglutination using four anti-D MoAbs. For the gel technique, anti-D reagents used were IgM, clone P3X61 and blend, clones P3X290, P3X35, P3X61, and P3X2123B10 (Grifols, Barcelona, Spain). For the tube technique, anti-D reagents used were IgM, clone MS201 and IgG, clone MS26 (Fresenius Kabi, São Paulo, Brazil). In all nonreactive samples, a confirmatory test was performed in with anti-D IgG (MS26) using the indirect antiglobulin test (IAT) in tube. C, c, E, and e status of all RBCs was determined by hemagglutination in gel cards (Grifols) with specific MoAbs. Retrospective analysis of antibody screen results was performed on all samples.

2.3 | Molecular assays

DNA was extracted from whole blood using the QIAmp DNA blood mini-kit (Qiagen, Valencia, CA, USA), according to the manufacture's recommendations.

Molecular tests performed on all 104 obstetric patient samples with discrepant serologic results included a PCR-SSP that detect the common weak D types,[11] a multiplex PCR that detects the *RHD* gene hybrid alleles[15] and the *RHD* BeadChip™ (Bioarray Solutions, Immucor, NJ, USA). Samples that could not be assigned an *RHD* allele were subjected to direct automated sequencing of *RHD* using *RHD*-specific primers as previously reported.[16]

3 | RESULTS

A total of 104 pregnant women samples 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.

3.1 | Molecular analyses

RHD genotyping showed that 23 of 104 (22%) pregnant women were *RHD*weak D types 1*, *2*, or *3*, 51 (49%) were *RHD*weak partial 4.0* and 22 (21%) were partial D (12 *RHD*DAR* and 10 *RHD*DVI.1*).

RHD sequencing identified three rare *RHD* alleles: *RHD*weak D type 38* (6%), *RHD*weak D type 45* (1%) and *RHD*weak D type 67* (1%). Table 1 summarizes the distribution of weak D and partial D alleles, their molecular alteration, the associated haplotypes and the risk of alloimmunization.

3.2 | Serologic D typing

The reactivity with the monoclonal anti-D reagents showed a generally consistent pattern among the variant *RHD* alleles that occurred

TABLE 1 Molecular basis of D variants, nucleotide changes, associated haplotypes and risk of alloimunization

RHD allele	Nucleotide changes	Haplotypes	Risk for anti-D
*RHD*weak D type 1*	809T>G	*DCe*	No
*RHD*weak D type 2*	1154G>C	*DcE*	No
*RHD*weak D type 3*	8C>G	*DCe*	No
*RHD*weak partial 4.0*	602C>G, 607A>G, 667T>G, 744C>T, 957G>A, 1025T>C	*Dce*	Yes
*RHD*weak D type 38*	833G>A	*DCe*	Yes
*RHD*weak D type 45*	1195G>A	*DcE*	Yes
*RHD*weak D type 67*	722C>T	*DcE*	Yes
*RHD*DAR1.00*	602C>G, 667T>G, 1025T>C	*Dce*	Yes
*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 4-5)	*DcE*	Yes

December 2016 access in http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/ in blood group allele tables.

TABLE 2 *RHD* alleles and reactivity with monoclonal antibodies (MoAbs)

		MoAbs reactivity			
		Tube		Gel Grifols	
Samples (n)	*RHD* alleles	MS201	MS26 IAT	P3X61	P3X290, P3X35, P3X61, P3X2123B10
9	*RHD*weak D type 1*	2+	4+	2+	2+
12	*RHD*weak D type 2*	0	2+	0	1+
2	*RHD*weak D type 3*	2+	3+	3+	4+
51	*RHD*weak partial 4.0*	3+	4+	4+	2+
6	*RHD*weak D type 38*	0	(+)	0	0
1	*RHD*weak D type 45*	0	(+)	0	0
1	*RHD*weak D type 67*	0	(+)	0	0
12	*RHD*DAR*	0	3+	0	2+
10	*RHD*DVI*	0	2+	0	1+

more than once, which means that all weak D types samples showed a similar serological profile. Table 2 summarizes the results found in the obstetric patient samples studied.

*RHD*weak D types 1* and *3*, and *RHD*weak partial 4.0* were detected with all anti-D MoAbs in tube and gel. *RHD*weak D types 1* and *3* were associated with *DCe* haplotype and *RHD*weak D type 2* was associated with *DcE* haplotype.

*RHD*weak D type 2*, partial *RHD*DAR*, and *RHD*DVI* were not detected with the IgM monoclonal anti-D antibodies. *RHD*weak D type 2* and partial *RHD*D DVI* showed the same pattern of reactivity with the four MoAbs used. All the rare *RHD* alleles identified were only reactive in the antiglobulin confirmatory test with anti-D IgG.

4 | DISCUSSION

Appropriate classification of RhD phenotypes is ethically recommended for correct indication of RhIG in pregnant women. However, the serologic distinction between RhD-negative and RhD-positive phenotypes is a difficult task in the case of D variants due to the variations in serologic testing.[17] In the last years, RhD typing practice and recommendation of RhIG for pregnant women is changing with the introduction of molecular testing in the routine and a recent publication from a Work Group in the United States emphasizes that it is time to begin to phase in selective genotyping to promote more personalized medicine.[13]

We report a serologic and molecular study of D variants in pregnant women with a multiethnic background who were identified because of weak or discrepant D typing results with different commercial monoclonal anti-D reagents and show the effect of *RHD* genotyping for more precise decision making in obstetric practice.

Among the 104 samples studied, 23 (22%) were categorized as *RHD**weak D types 1, 2, and 3 with molecular assays, and for those weak D types no immunization events have been documented yet.[2,5,13] *RHD*weak D types* and *partial D* and the associated *RHCE* haplotypes found in this study were consistent with other studies.[7] Therefore,

the association of *RHD* variants with specific *RHCE* haplotypes could be used to predict some weak D an partial D phenotypes. Although *RHD*weak D types 1*, *2*, and *3* are the most common weak D types in Europeans,[10,11] in this cohort of pregnant women we see a higher prevalence of *RHD*weak partial 4.0* (49%). For the partial D, we also observed a higher prevalence of *RHD*DAR1.00*, reinforcing that the ethnic background of the population may govern which variants are prevalent. Interestingly, we also found three very rare *RHD* alleles encoding the weak D type 38, previously found in the Portuguese population,[18] weak D type 45, and weak D type 67 with potential risk of RhD alloimunization in this group of obstetric patients. These findings are particularly important to recommend the RhIG as anti-D in women with variant D RBCs has been responsible for severe HDFN.[19,20] It is postulated that RhIG should be offered to women known to have D variant red cells, other than *RHD*weak D types 1*, *2*, and *3*, during and after pregnancy, because the anti-D constituent that does not bind to the mothers' own variant D cells should suppress alloimmunization by binding to the normal D of the fetus.[20,21] According to our results, 78% of these obstetric patients are at risk for anti-D and candidates for RhIG. Our findings are in agreement with those obtained by Wang et al.[22] showing a higher prevalence of *RHD*weak partial 4.0* and partial *RHD*DAR* in a multiethnic prenatal population but are in contrast with the prevalence of weak D types in Central Europe where 95% of Caucasians are *RHD*weak D types 1*, *2* and *3*.[11]

Our study shows a great variability in *RHD* variant alleles in pregnant women from this population of high admixture. Given the complexity of D antigen expression, it is concluded that some clinically important D variants identified by serologic analysis phenotype as weak D in one specific technique or with one specific reagent are potentially at risk for the development of anti-D. If we were to select candidates for RhIG based only on the serological techniques applied in this study, we would not recommend prophylaxis for pregnant women with *RHD*weak partial 4.0* as it was detected with all MoAb selected and, we would unnecessarily recommend the RhIG for the carriers of *RHD*weak D type 2* that showed the same pattern of serologic reactivity of partial D DVI. Our data show that although those variants have different molecular background they can present the same serologic pattern of reactivity. This finding reinforces the importance to perform *RHD* genotyping to identify weak D discrepancies for obstetric patient management and the recommendation that if significantly weak or equivocal results are obtained on routine D typing of a pregnant women, she should considered D-negative, until molecular testing be performed. Calculations on cost efficiency of combined introduction of noninvasive fetal *RHD* genotyping or pregnant women *RHD* genotyping and antenatal anti-D prophylaxis have been published.[23,24] The cost benefit ratios depend on tests costs and anti-D Ig prophylaxis costs; however, these parameters differ between countries. Although the present study has not aimed to compare these management strategies, the use of molecular technologies to guide RhIG prophylaxis among pregnant women with serologic weak D phenotypes may be clinically beneficial mainly in our population that have shown a high admixture. The main reduction in RhIG usage will be from noninvasive testing identifying women who carry a D-negative baby, and this will be up to 40% of cases. However, identifying women with the weak D types 1, 2, and 3 phenotypes, although a much smaller subset, will also contribute to the reduced usage. Noninvasive testing of all women will also increase the sample size to reveal detection of rare *RHD* variants in this multiethnic population.[25] We should also take into account that although it is very important to prevent Rh alloimmunization, RhIG is in short supply and produced by immunizing volunteers with RBCs presenting a risk of infectious diseases and therefore unnecessary injections of RhIG should be avoided. Regardless of the costs, it also has been argued that it is ethically unacceptable to continue routine anti-RhD prophylaxis when fetal *RHD* genotyping using maternal blood is available and could identify those women who do not need this product.[26] According to our results, using *RHD* genotyping, we could prevent unnecessary injections of RhIG in 22% of the pregnant women with a serologic weak D phenotype. Our findings also support a review on the current American College of Obstetricians and Gynecologists (ACOG) standards[27] recommending that if the woman`s test for D antigen is D-negative, a test for weak D is not required.

Serological D variants have been known since middle 1940s and their adequate characterization impact on different clinical scenarios: pregnancy, blood donors, patients, and sickle cell disease. When D antigens discrepancies arise clinicians are faced with assigning the appropriate D antigen status; moreover, in an era of informed consent, the impact of a D antigen discrepancy can create confusion over the use of RhIG. Serologic analysis alone does not resolve the issue of weak D types not at risk of making anti-D. Molecular tests that can distinguish common partial and weak D types provide best solution to the resolution of an accurate D antigen status.[8] Literature has already recommended molecular testing should be made as widely available as possible, especially for *RHD*weak D types 1*, *2*, and *3* in pregnancy.[1] Taking together our data, we can conclude that there is a diversity in *RHD* genotypes in this obstetric population and this knowledge can inform the risk for being alloimmunised. This can facilitate the prenatal management, as 22% were low risk and could avoid unnecessary injection of RhIG or prevent Rh alloimmunization on those patients with high risk.

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