

ORIGINAL REPORT

Identifying obstetrics patients in whom *RHD* genotyping can be used to assess risk of D alloimmunization

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The D antigen is highly immunogenic and may cause alloimmunization to occur after blood transfusion or pregnancy. Some *RHD* variant alleles express a D antigen that is missing one or more epitopes, thus putting a presumed D+ patient at risk for alloanti-D and hemolytic disease of the fetus and newborn. It is generally accepted that individuals who have a serologic weak D phenotype due to one of three alleles common in Caucasians, *RHD*weak D types 1, 2, or 3*, are not at risk for alloimmunization. In this study, blood samples from 46 obstetrics patients from a local health system were identified based on discrepant results between automated gel and manual tube testing ($n = 20$) or based on presentation with a serologic weak D phenotype ($n = 26$). *RHD* genotyping was performed using commercial and laboratory-developed tests. Of the 26 serologic weak D samples, 18 (69.2%) were found to carry alleles *RHD*weak D type 1, 2, or 3*. The remaining eight samples (30.8%) were found to carry partial D alleles. Of the 20 samples submitted because of D typing discrepancy, 7 (35%) carried alleles *RHD*weak D type 1, 2, or 3*, while 13 (65%) carried partial *RHD* alleles. This report summarizes the findings of one hospital system and its approach to integrating *RHD* genotyping into its assessment of risk of alloimmunization in obstetrics patients. It demonstrates that individuals with partial *RHD* alleles can present with serologic weak D phenotype, such that, without *RHD* genotyping, these individuals may not be identified as candidates for Rh immune globulin. The study also demonstrates that use of two methods (automated gel and tube testing) allows for identification of partial D cases that would otherwise be missed. ***Immunohematology* 2020;36:146–151.**

Key Words: D, blood group antigen, genotyping, pregnancy, alloimmunization, RhIG prophylaxis, hemolytic disease of fetus and newborn (HDFN)

The D antigen is different from many other blood group antigens in that the antigen is not derived from one or a few amino acids, but rather from the presence of the entire protein; thus it contains many epitopes. Genetic variation within the *RHD* gene can alter expression of the antigen both qualitatively and quantitatively. A D protein that lacks one or more of the epitopes is referred to as a partial D antigen, and loss of epitopes is associated with risk of alloimmunization from exposure to D+ red blood cells (RBCs) during pregnancy

or transfusion. The International Society of Blood Transfusion (ISBT) has reported over 90 different *RHD* alleles encoding partial D antigens. Other variations in the *RHD* gene significantly alter the number of antigenic sites expressed on the RBC membrane and are often referred to as weak D alleles.

There are many alleles for which the impact on epitopes has not been assessed or the risk of alloimmunization identified. The three most common *RHD* variants in Caucasian individuals are *RHD*weak D types 1, 2, and 3*. These three variants are estimated to be responsible for 85 percent of serologic weak D phenotypes in Caucasian individuals.^{1,2} These alleles are uncommon in individuals of African descent, where a large number of variants have been identified. *RHD* genotyping of African American blood donors predicted 15.9 percent have a partial D phenotype.³

Both partial D and weak D variants can give variable serologic results, depending on the reagent and serologic method used. There are several methods and reagents commercially available for D typing by serology. Several commercial antisera, both polyclonal and monoclonal, are used in standard agglutination reactions in tube. Variable reactivity with different sources of anti-D are noted because of the specific epitopes targeted by different reagents. There are also blends of monoclonal and polyclonal antisera to broaden the specificity of the reagent.

Tubeless systems for D typing use column agglutination technology, microplate solid-phase technology, and hemagglutination microplate technology. Some automated systems use a binary classification of samples as positive or negative whereas others are capable of grading samples that react weaker than expected.

Individuals who are D– are at risk for producing alloanti-D, as well as are individuals with partial *RHD* alleles, and even some with weak D phenotypes. An individual with RBCs that express a partial D phenotype would be at risk of alloanti-D directed at RBCs expressing the D antigen containing the missing epitope. Alloimmunization can occur

via blood transfusion or pregnancy. The significance of D in pregnant women and in women of childbearing potential is well appreciated in the obstetrics community.^{4,5} Maternal alloanti-D can cause mild to severe hemolytic disease of the fetus and newborn (HDFN).⁶⁻⁸ Importantly, some partial D phenotypes may present with a serologic weak D phenotype, a fact that causes confusion based on the terminology. Without the use of extensive serologic testing with antibody panels, standard serologic tests are unable to accurately deduce alloimmunization risk. Many blood banks do not resolve a serologic weak D phenotype in a patient, but instead treat them as D-. Many of these women may not be at risk, however, and can safely be treated as D+. Conversely, some women will test strongly D+ by automated methods but actually be at risk. *RHD* genotyping can predict risk of alloimmunization based on alleles. For this reason, use of *RHD* genotyping to determine D status is recommended for hospital blood banks caring for pregnant women and women of childbearing potential.^{1,9-11}

RHD genotyping is available to blood banks and transfusion services, mostly via reference laboratories.¹ Currently, *RHD* genotyping may be performed using either laboratory-developed tests or commercially available research-use-only reagents. Test methods in use include sequence-specific primer-polymerase chain reaction (SSP-PCR), PCR-restriction fragment-length polymorphism (RFLP), Sanger sequence analysis of genomic DNA or cDNA, solid support- or bead-based single nucleotide polymorphism arrays, and mass spectrometry-based genotyping.³ In the scenario of a pregnant woman who has produced alloanti-D, noninvasive prenatal testing for *RHD* can be used to predict the D status of the fetus.^{12,13} The clinical utility of *RHD* genotyping for predicting alloimmunization risk in the obstetrics population has been appreciated in Europe for many years.^{9,14,15}

Particular *RHD* variant alleles are known to be more prevalent in different ethnic groups. The most common *RHD* variants in Caucasian individuals are *RHD*weak D types 1, 2, and 3*, where the sample is often negative in tube by direct agglutination, but positive by the indirect antiglobulin test (IAT).⁸ These alleles are not associated with alloimmunization.¹⁶ Individuals of African descent, however, are more likely to express a partial D phenotype, which can either present as strongly D+ or as serologic weak D+ and may unknowingly put a patient at risk for anti-D alloimmunization and, consequently, HDFN.^{3,17,18} D typing discrepancies or serologic weak D phenotypes can identify samples that need further testing to resolve (not all will be the same).

As per current American College of Obstetricians and Gynecology standards,¹⁹ pregnant women who present to the hospital blood bank as weak D+ are managed as D-.¹⁹ These laboratory practices are designed to help protect pregnant women and women of childbearing potential from developing alloanti-D, thus reducing the risk of HDFN in future pregnancies. They are transfused with D- blood, and administration of Rh immune globulin (RhIG) is recommended at appropriate times throughout the pregnancy and after delivery. Nevertheless, this approach is imperfect. Less than 100 percent specificity means that there is unnecessary administration of RhIG and unnecessary use of rare D- RBCs for transfusion. Less than 100 percent sensitivity means that there is a failure to administer RhIG or to provide D- RBCs when indicated. Although *RHD* genotyping was recently recommended to resolve ambiguous cases, many hospital laboratories do not have policies in place for if and when to use this method.

In 2015, an interorganizational group of transfusion medicine physicians and molecular immunohematology experts published their findings and recommendations after reviewing the literature. They determined that if women in the United States with serologic weak D phenotypes had *RHD* genotyping performed, about 13,000 of them would be *RHD*weak D types 1, 2, or 3* and could be managed as D+.¹ This finding would eliminate unnecessary RhIG injections and the use of D- blood in these women. Based on this group's assessment, a joint statement was issued by AABB that recommends that women with D typing discrepancies, variable D reactivity, direct agglutination results of 2+ or lower grade, or a serologic weak D phenotype be provided *RHD* genotyping for determination of weak or partial D status.¹ Another study evaluated the cost-effectiveness of *RHD* genotyping as a test that can be performed once and the genotype result used to inform the management of multiple pregnancies over the course of a woman's life.²⁰

However, there is no guidance for blood banks as to how to implement this recommendation; therefore, there is no universal approach. A recent study showed one hospital's approach by using several serologic criteria to identify candidates for *RHD* genotyping.¹¹ This report illustrates the importance of *RHD* genotyping referred from a health care system comprising four separate hospitals and examines the findings of patient samples submitted for *RHD* genotyping due to serologic weak D phenotype or D typing discrepancy between automated gel and tube methods in the past 30 months.

Materials and Methods

Sample Selection

Blood samples from obstetrics patients received in the four hospital blood banks from July 2013 to January 2016 were identified based on D typing discrepancies or weak D+ phenotypes. Of the 46 samples that met these criteria, 15 were from African American individuals, 26 from Caucasian women, and 5 from individuals of unknown race.

Serologic Testing

Serologic testing was performed using Bio-Rad (Hercules, CA) (RH1) immunoglobulin (Ig)G and IgM monoclonal blend anti-D antiserum by standard tube agglutination methods and/or automated gel testing using the Ortho ProVue (Ortho Clinical Diagnostics, Raritan, NJ), which uses the ID MTS/Gel cards for direct agglutination testing. Discrepancies were defined as samples that typed D+ (1+ to 4+) using ProVue automated gel testing while typing D- at immediate spin (IS) and D+ (1+ to 3+) at antihuman globulin (AHG) phase using tube method. Serologic weak D phenotype was defined as a sample that tested D- at IS and D+ by IAT.

RHD Genotyping

Peripheral blood samples collected in EDTA anticoagulant were submitted to the National Molecular Laboratory, American Red Cross for *RHD* genotyping. Genomic DNA was isolated from mononuclear cells using DSP DNA Blood Minikit (Qiagen, Carlsbad, CA). Genotyping was performed using *RHD* BeadChip (Immucor, Norcross, GA). In cases where variants were not identified using the array, *RHD* exon 8 RFLP for *RHD* c.1136 was performed and/or Sanger sequencing of *RHD* exons and exon/intron borders (GeneWiz, South Plainfield, NJ). Sequences were aligned with the reference sequence using Sequencher (GeneCodes, Ann Arbor, MI), in which variant bases were identified and used to predict amino acid changes and assign alleles using the ISBT *RHD* allele tables²¹ or RhesusBase 2.0.²²

Results

RBC phenotyping for D was performed using standard tube method and direct agglutination testing by both IS and IAT, when indicated, for all 46 samples included in this study. The Ortho ProVue was used to test 20 of the samples. Serologic D typing using standard tube methods found 40 samples to be weak D+. Variable D typings were found in 20 samples when

the first type was performed on the Ortho ProVue. These samples were found to be D+ on the Ortho ProVue, ranging in strength from 1+ to 4+ agglutination. The second D type was performed using standard tube testing with commercially available reagent antisera Bio-Rad anti-D (RH1) Blend antiserum. Of these samples, four were found to be 1+ to 2+ reactive at IS. These four samples all typed strongly positive (3+ to 4+) on the Ortho ProVue. The other 16 samples all typed as weak D, negative at IS and positive by IAT.

RHD Genotyping

A total of 43 samples were tested using *RHD* BeadChip, and 3 samples were tested using gel-based PCR and/or PCR-RFLP to rule out common weak and partial D alleles, due to unavailability of these *RHD* BeadChips. Twenty of the samples that were tested using *RHD* BeadChips were also tested using PCR-RFLP for *RHD* c.1136C>T to rule out presence of *RHD*DAU0*. Of the 26 samples submitted because of serologic weak D phenotype, 8 (30.8%) were determined to be due to the presence of partial *RHD* alleles. The other 18 samples (69.2%) were determined to be *RHD*weak D types 1, 2, or 3* (Fig. 1A). Of the 20 samples submitted due to D typing discrepancies and variable reactivity, 13 (65%) were predicted to be partial D+. The other seven (35%) were determined to be *RHD*weak D types 1, 2, or 3* (Fig. 1B). In all, 21 of 46 samples (45.7%) were predicted to be partial D+, and 25 of the 46 (54.3%) were *RHD*weak D type 1* ($n = 11$), *RHD*weak D type 2* ($n = 11$), and *RHD*weak D type 3* ($n = 3$). Alleles in the *RHD*DAR* family represented the most common partial D identified ($n = 9$), presenting both as typing discrepancies ($n = 4$) and as serologic weak phenotypes ($n = 5$). *RHD*weak partial D 4.0* allele was found in seven samples (five typing discrepancies and two serologic weak D). As shown in Table 1, of the 21 cases in which partial alleles were detected, *RHD*DAR* family was found in 9 cases (43%), *RHD*weak partial D 4.0* was found in 7 cases (33%), *RHD*DAU3* in 1 (5%), *RHD*DAU5* in 2 (9%), *RHD*DCS1* in 1 (5%), and *RHD*DNB* in 1 (5%).

Discussion

Of the 20 samples submitted because of D typing discrepancies and variable reactivity, 13 samples (65% of discrepancy cases) were determined by *RHD* genotyping to be partial D+. These individuals were of Caucasian ($n = 9$), African American ($n = 10$), and unknown ($n = 1$) race. These patients would best be managed as D- and be administered

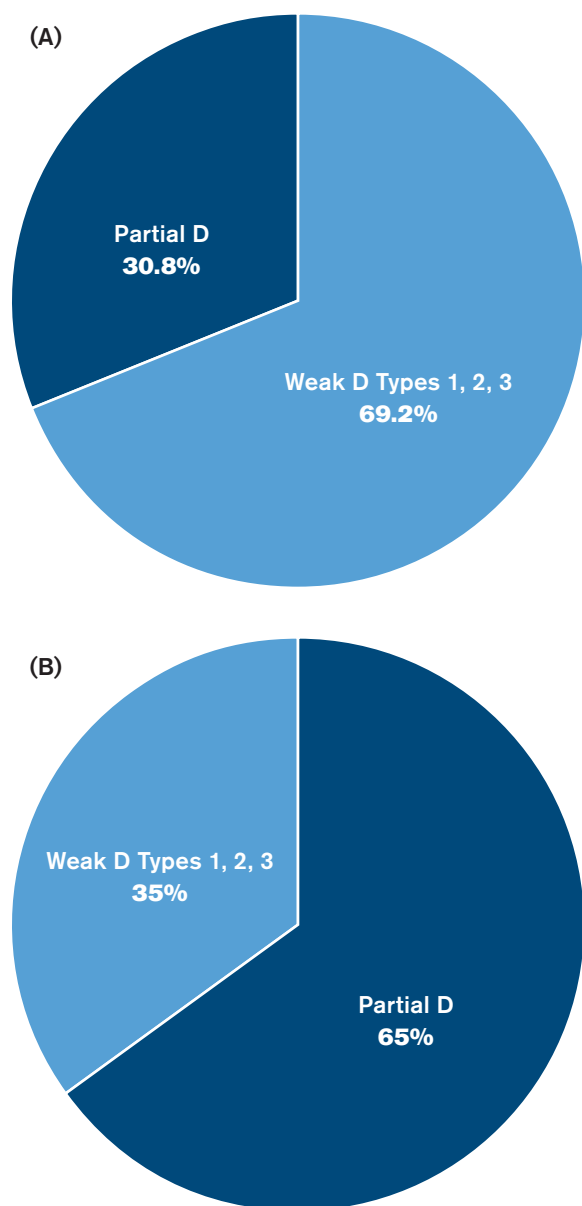


Fig. 1 (A) Samples submitted as weak D+. (B) Samples submitted for D typing discrepancy.

the RhIG injection. The other seven (35%) were determined to be *RHD*weak D types 1, 2, or 3* and would not be predicted to be at risk for alloanti-D, and therefore would not require RhIG administration. The definition of a sample with a serologic weak D phenotype used in this study was one that tested D– at IS and weak D+ at AHG. However, the current definition also includes that the sample typing results were less than or equal to 2+ by initial testing in any method.¹ It is likely that additional samples would have been identified as candidates for *RHD* genotyping if the current definition had been used.

Table 1. Summary of serology and molecular results for the 46 samples, as well as summary of candidacy for RhIG

Automated test result (positive grade 2+ to 4+)	Tube test result, IS/IAT	Samples n	<i>RHD</i> genotype(s)	Genotype-predicted phenotype	Candidate for RhIG
Positive	0/+	4	<i>RHD*DAR</i> family	Partial	Yes
Positive	0/+	5	<i>RHD*weak partial D 4.0</i>	Partial	Yes
Positive	0/+	2	<i>RHD*DAU5</i>	Partial	Yes
Positive	0/+	1	<i>RHD*DAU3</i>	Partial	Yes
Positive	0/+	1	<i>RHD*DCS1</i>	Partial	Yes
Positive	0/+	4	<i>RHD*weak D type 1</i>	Weak	No
Positive	0/+	2	<i>RHD*weak D type 2</i>	Weak	No
Positive	0/+	1	<i>RHD*weak D type 3</i>	Weak	No
NT	0/+	5	<i>RHD*DAR</i> family	Partial	Yes
NT	0/+	2	<i>RHD*weak partial D 4.0</i>	Partial	Yes
NT	0/+	1	<i>RHD*DNB</i>	Partial	Yes
NT	0/+	7	<i>RHD*weak D type 1</i>	Weak	No
NT	0/+	9	<i>RHD*weak D type 2</i>	Weak	No
NT	0/+	2	<i>RHD*weak D type 3</i>	Weak	No

RhIG = Rh immune globulin; IS = immediate spin; IAT = indirect antiglobulin test; NT = not tested.

Of the 26 samples submitted because of serologic weak D phenotype, 8 (30.7%) were determined to have partial *RHD* alleles associated with the production of allo anti-D. These individuals were of Caucasian ($n = 17$), African American ($n = 5$), and unknown ($n = 4$) race. The other 18 (69.3%) samples submitted because of serologic weak D phenotype were determined to be due to *RHD*weak D types 1, 2, or 3*. These individuals would not be predicted to be at risk for alloanti-D and, therefore, would not require RhIG administration.

Of all 46 samples, 20 samples (43.4%) were determined to have partial *RHD* alleles and these patients are at risk for alloanti-D production. The majority of these samples (45% of partial D+) were *RHD*DAR* family alleles, which are classified as partial *RHD* alleles.⁴ Interestingly, four of these samples were submitted as D typing discrepancies, and five presented as serologic weak D+ (Fig. 2). Heterogeneity of presentation was also seen with the *RHD*weak D type 1, 2, and 3* samples, with 7 presenting as typing discrepancies and 18 as serologic weak D.

The cases compiled in this study highlight the fact that different testing methods, different monoclonal reagents, and subjective interpretations can yield discrepant results for D

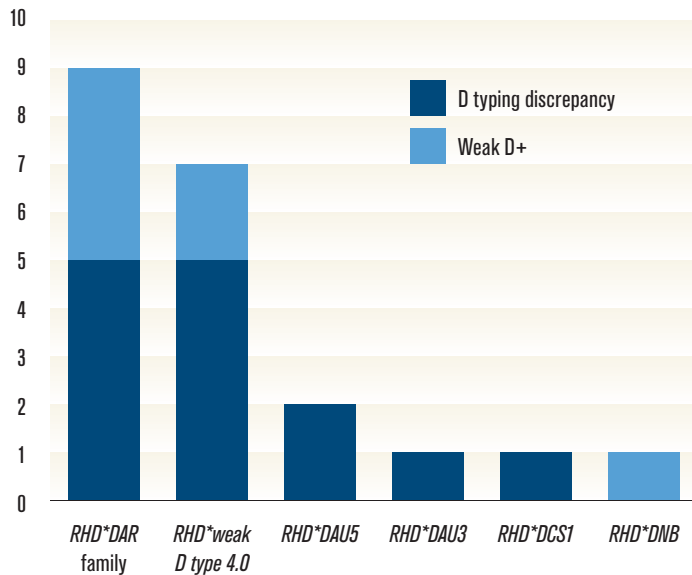


Fig. 2 Summary of partial D alleles detected by reason for submission.

antigen typing. If used as the sole test method, automated gel testing would not have identified any of these samples as weak or partial D+. Based on the Ortho ProVue results alone, 65 percent of these individuals would have been classified as D+ and no RhIG would have been administered when, in fact, they were partial D. Furthermore, this study demonstrates the advantage of using an alternate method such as tube testing to obtain the required second ABO/D type of a patient before transfusion and for purposes of determining RhIG prophylaxis. In more than half of the cases with discordant results between gel and tube testing, partial D alleles were identified such that these patients were identified as at risk for anti-D alloimmunization.

It is important for hospital blood bank physicians and obstetricians to be aware that not all samples with serologic weak D phenotype are due to weak D alleles; they can be due to a partial D phenotype. It is not widely known that partial D alleles can be associated with a serologic weak D phenotype, since generally they are thought to give strong reactivity using direct agglutination methods. However, it has been previously described that partial D antigens can present through D typing discrepancies.¹⁸

Also of note, patients presenting with a serologic weak D phenotype could be partial D+, especially if they are of African ancestry, where these alleles are common.^{3,10} In our experience, the most common partial *RHD* alleles found to be associated

with weak reactivity and/or D typing discrepancies are the *RHD*^{DAR}* family of alleles and the *RHD*^{weak}* partial D 4.0 allele, as this study also supports. These alleles are frequently identified in the African population.⁷ The *RHD*^{DAR}* family of alleles is largely associated with production of alloanti-D. In the U.S. experience, the *RHD*^{weak}* partial D 4.0 allele is also frequently identified in individuals with alloanti-D.²³ The risk of developing anti-D after transfusion or pregnancy in individuals with these alleles is not known, but there are several reports of individuals with partial D phenotypes who have developed anti-D.^{14,17,24} Recently, the interorganizational group of transfusion medicine physicians and molecular immunohematology experts published a second report that recommends use of *RHD* genotyping for all serologic weak D phenotypes and included a discussion of the lack of clear alloimmunization risk in patients carrying *RHD*^{weak}* D 4.0 and *RHD*^{weak}* D 4.1.²⁵ As of yet, the ISBT Working Party on Red Cell Immunogenetics and Blood Group Terminology has not changed the classification of these alleles from their current status as encoding partial D antigens.²¹

Determination of an accurate D status is critical to determining the appropriate management of obstetric patients. When patients are suspected of carrying a D variant, *RHD* genotyping can assign alleles and, from that information, provide information about risk of alloimmunization and candidacy for RhIG. Importantly, when patients are found to be *RHD*^{weak}* D types 1, 2, or 3, the most common D variants in Caucasian individuals, they can be treated as D+, and avoid the unnecessary use of RhIG or D- RBCs for transfusion. The approach described in this report illustrates the heterogeneity of presentation of D variant antigens, the limitations of using the serologic weak phenotype alone to predict risk of alloimmunization, the utility of using two serologic methods to identify candidates for *RHD* genotyping, and the ability of *RHD* genotyping to resolve these cases and provide recommendations as it pertains to alloimmunization risk and candidacy for RhIG.

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