

# On the Complexity of D Antigen Typing: A Handy Decision Tree in the Age of Molecular Blood Group Diagnostics

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## Abstract

RH is the most complex of all 29 blood group systems. New discoveries relating to the *RHD* gene, and an appreciation of its variant phenotypes such as weak D and partial D, have challenged the way that D status is assigned to both blood donors and blood product recipients. This concise review introduces the current concepts of weak D and partial D and how the identification of these variants has influenced the testing methods for the D antigen. We demonstrate how molecular tests of the *RHD* gene can and should be used in resolving serological discrepancies, in particular in pregnant women.

## Résumé

Le système Rh est le plus complexe des 29 systèmes sanguins. De nouvelles découvertes en ce qui concerne le gène *RHD* (et la compréhension des variantes de son phénotype, telles que l'antigène D faible et l'antigène D partiel) ont remis en question la façon dont le statut D est attribué aux donneurs de sang et aux receveurs de produits sanguins. Cette analyse concise présente les concepts actuels d'antigène D faible et d'antigène D partiel, et la façon dont l'identification de ces variantes a influencé les méthodes de dépistage de l'antigène D. Nous démontrons la façon dont les tests moléculaires portant sur le gène *RHD* peuvent et devraient être utilisés pour résoudre les divergences sérologiques, particulièrement chez les femmes enceintes.

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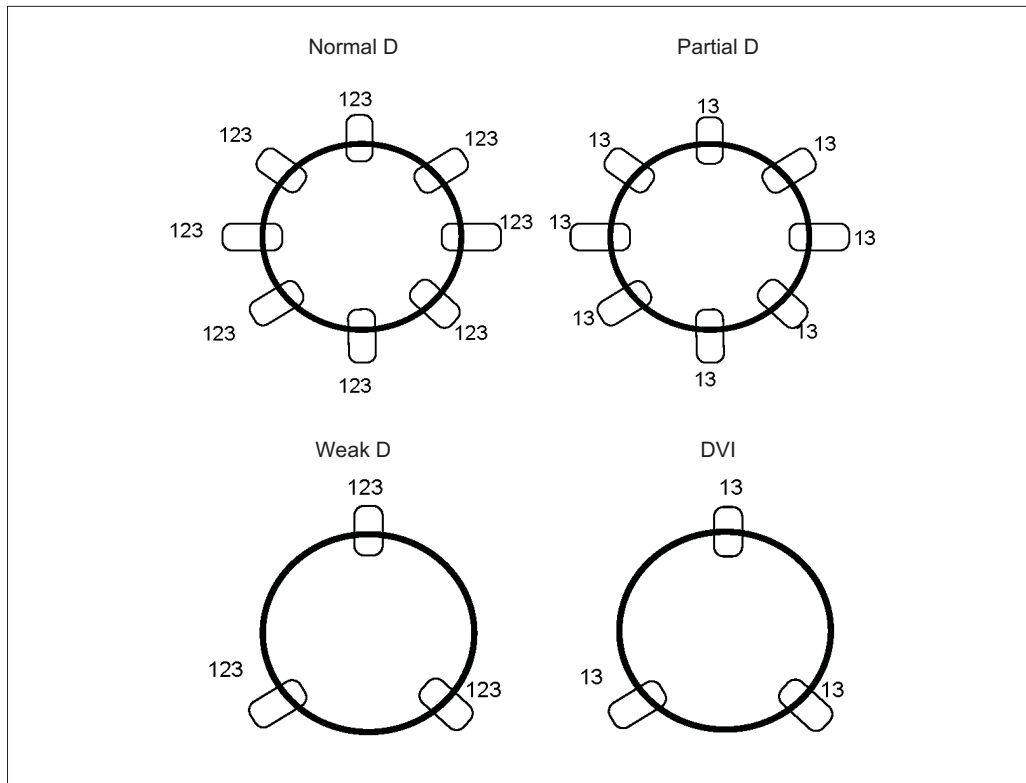
The task of determining an individual's D status seems simple enough: add commercially available anti-D reagents to a suspension of red blood cells, and if hemagglutination occurs then they should be D positive. Unfortunately it is not that simple. A myriad of different serological techniques and reagents, each with different sensitivities, and a rapidly expanding understanding of the genetics of the *RHD* gene have greatly complicated D typing. In this commentary, we will summarize the reasons for D antigen typing anomalies and describe our approach to D antigen testing with a particular focus on pregnant women.

## D Typing Methods

There are several approved D antigen testing modalities, ranging from simple saline and test tube based methods to semi-automated methods using a gel matrix or solid phase technology. There are generally two phases of testing: an “immediate spin” phase when the reagent antisera, often composed of a mixture of monoclonal IgM and IgG anti-D,<sup>1</sup> are added to the recipient's RBCs at room temperature; direct agglutination of the RBCs at this phase is mediated by the IgM component. If hemagglutination is not observed, or if it is weak (< 2+) we recommend incubating the mixture of RBCs and anti-D reagent in accordance with the manufacturer's guidelines, followed by re-centrifugation and inspection for hemagglutination. This extra step helps to identify recipients with certain weak D phenotypes (Figure) and needs to be performed only once per patient over his or her entire lifetime.

If after incubation hemagglutination is still not observed, it is sometimes appropriate to add antiglobulin reagent. This

**Figure 1.** Comparison of weak D and partial D with a normal D positive RBC. The circles represent the RBC membrane, the rectangles represent an RhD protein (antigen) and the numbers above each RhD protein are a stylized representation of different D epitopes on the protein. The D epitopes are arbitrarily numbered 1, 2, and 3. The number of antigens and epitopes, as well as the size of the RhD protein are not to scale. In this example, 8 D antigens on the RBC surface are schematically shown as normal, and each D antigen has 3 D epitopes. In reality, the number of D antigens ranges from 10 000 to 25 000 and more than 30 D epitopes are expressed on the D antigen. The weak D RBC features D antigens with the full complement of D epitopes, but the number of D antigens is reduced compared to normal. The partial D RBC demonstrates the normal number of RhD proteins (antigens) but each protein is lacking at least 1 D epitope. The partial D type DVI demonstrates both weak D and partial D features.



“AHG phase” or “weak D test,” formerly known as the “D<sup>u</sup> test,” further enhances the test’s sensitivity by causing the agglutination of RBCs coated with IgG anti-D.

### Weak D and Partial D

The vast majority of people from all ethnic backgrounds demonstrate very strong hemagglutination with modern anti-D reagents at the IS phase regardless of the testing

methodology. However, this is not always the case. “Weak D” RBCs (0.2%–1% of Caucasians)<sup>2</sup> demonstrate reduced quantities of the D antigen because of mutations in the protein’s transmembrane domains (Figure 1).<sup>3</sup> As the name implies, these RBCs tend to demonstrate either weak or no hemagglutination at IS phase, although they sometimes react more strongly in the weak D test. “Partial D” RBCs, a phenomenon less common than weak D, usually contain normal numbers of RhD protein, although the protein is mutated in an exofacial loop, eliminating at least one D-specific epitope on the RhD protein (Figure 1).<sup>3</sup> Both weak D and partial D blood recipients/pregnant women might become sensitized to the D antigen if exposed to D positive RBCs. Note that some crossover exists: the partial D variant DVI for instance expresses a D antigen that lacks numerous D epitopes (a partial D hallmark) and also features reduced numbers of RhD proteins in the RBC

### ABBREVIATIONS

AABB	American Association of Blood Banks
AHG	AntiHuman Globulin
CSTM	Canadian Society for Transfusion Medicine
RBC	red blood cells
Rhlg	Rh immune globulin

membrane (a weak D hallmark) (Figure 1). The absolute risk of sensitization is hard to quantify. There are only a small number of serologically D negative patients (agglutination at IS < 2+) with weak or partial D alleles who have been exposed to D+ RBCs and followed with repeat antibody screens. Data from the most complete collection of patients with D variant alleles who have become sensitized to anti-D can be found online at The Rhesus Site<sup>4</sup> (see also<sup>5</sup>).

### DEL

The DEL phenotype is a third group of D variants. DEL cannot be detected using routine serological reagents or the weak D test. It is, however, easily detected by genetic analysis.<sup>6</sup> DEL RBCs contain an extraordinarily low number of D antigens but, despite this paucity, can cause primary<sup>7</sup> and secondary<sup>8</sup> immune responses against the D antigen in D negative recipients. Fortunately its incidence is very low. It is found mainly amongst Asian populations where a recent study found a DEL allele in approximately 13% to 16% of serologically D negative Chinese and Japanese individuals.<sup>9</sup>

### D Typing of Transfusion Recipients and Pregnant Women

The current data suggest that the most common weak D types (1, 2, 3, 4.0, and 4.1), encompassing more than 90% of all European weak D individuals, do not appear to be susceptible to immunization to the D antigen on the basis of our current knowledge of the *RHD* gene and the immunological responses, or lack thereof, amongst people with these alleles.<sup>10</sup> These individuals could safely receive D positive blood and do not need Rh immune globulin prophylaxis during pregnancy. However, serological tests cannot discriminate between these weak D types and those that are susceptible to alloimmunization; only a molecular analysis of the *RHD* gene can distinguish between weak D types.

In addition, many of the partial D phenotypes appear to be vulnerable to alloimmunization. Partial D phenotypes are particularly difficult to characterize serologically because different subtypes react variably with different anti-D reagents.<sup>1</sup> Some of the government approved anti-D reagents feature limited D specificity; they will not cause hemagglutination with the most common clinically significant partial D type (DVI). Others have broader specificity and react with many partial D types.<sup>11</sup> Similarly, only a molecular analysis will reveal which partial D allele is present.

As molecular genotyping of the *RHD* gene is complicated by its size, propensity for rearrangement with the related *RHCE* gene, and significant variability between ethnic groups, it is best performed in a dedicated academic laboratory with expertise in interpreting the gene's many alleles.

There may be fewer than 100 such dedicated laboratories in the world.

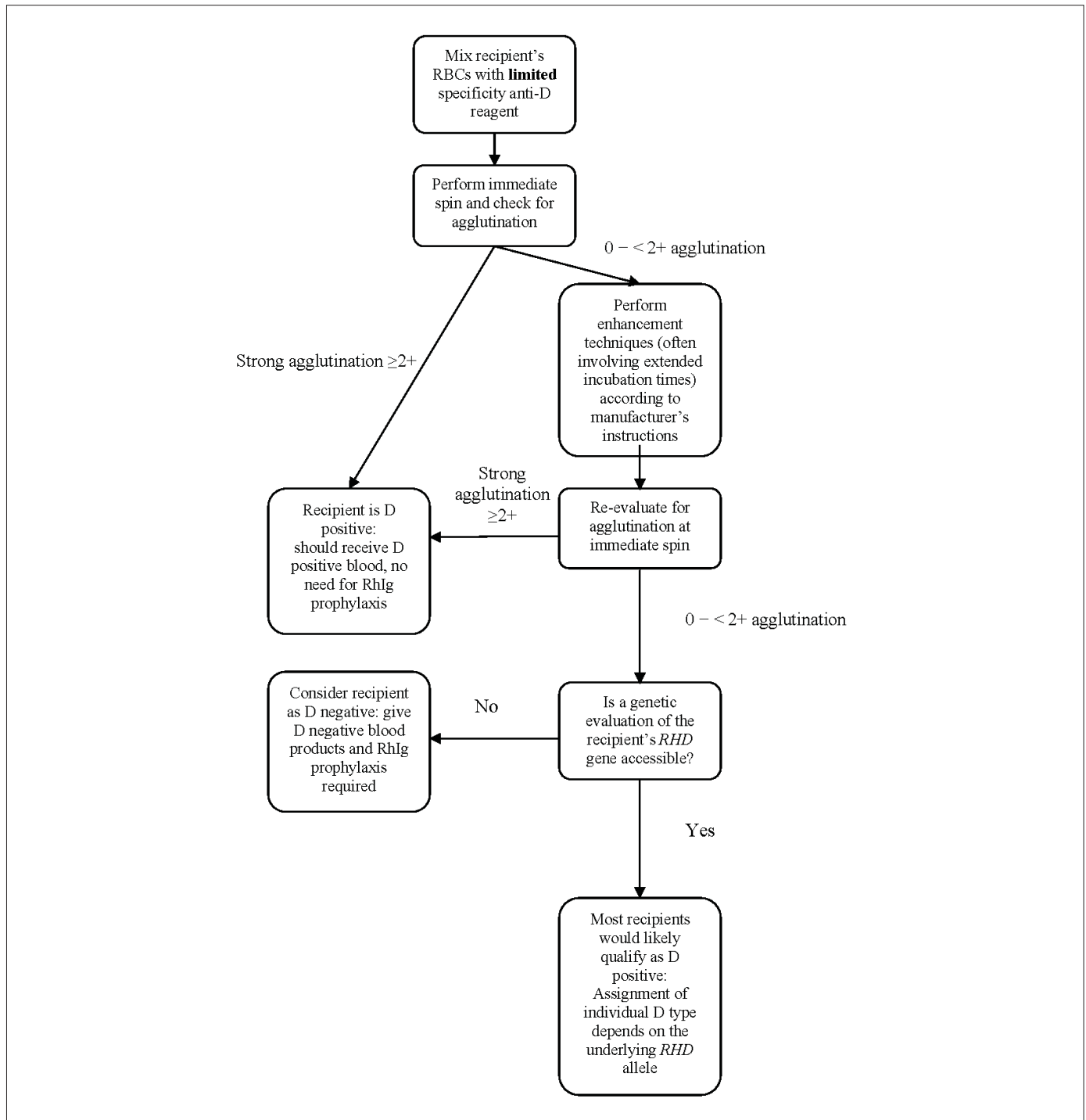
### Practical Recommendations

Because genetic *RHD* tests are not routinely available and because of the non-negligible risk of anti-D alloimmunization in weak D and partial D individuals, we recommend omitting the weak D test in the pre-transfusion testing of blood recipients and pregnant women who are D negative at IS phase, and using a typing reagent with limited D specificity to increase the likelihood that a partial D individual would be classified as D negative (Figure 2). Thus, the assignment of D status by serologic criteria alone should be made at the IS phase of testing; pregnant women who previously tested positive only in the weak D test in an earlier pregnancy (and might not have received RhIg) would require RhIg in their current pregnancy if they continue to test < 2+ at the IS phase of testing. By omitting the weak D test, any term referring to agglutination only at the weak D phase of testing (such as "D<sup>u</sup>") becomes obsolete (the recipients would be deemed D negative) and the question of whether "D<sup>u</sup> positive" pregnant women or blood product recipients require RhIg would no longer be an issue. In the standards for pre-transfusion testing, the AABB recommends not performing the weak D test in this population, which is in agreement with the forthcoming second edition of the Canadian Society for Transfusion Medicine Standards for hospital transfusion services.<sup>12,13</sup> Omitting the weak D test would also prevent the misclassification of some partial D recipients as D positive.<sup>1</sup> Obstetricians who are faced with a D<sup>u</sup> positive result should contact their blood bank medical director to discuss the patient's findings in light of the AABB and CSTM guidelines so that the appropriate action is taken to avoid sensitization to the D antigen.

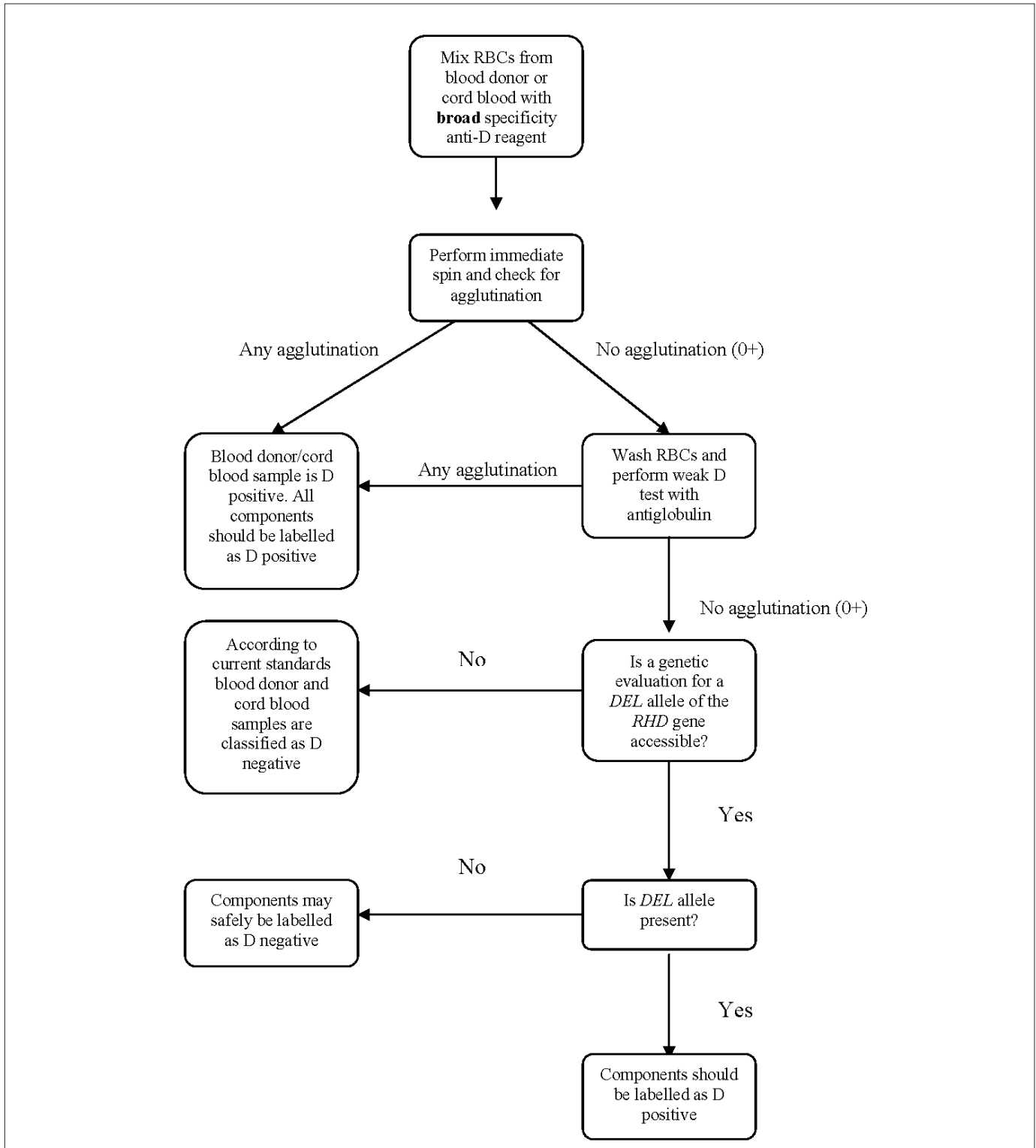
However, some weak D recipients who should be considered D positive will be classified as D negative if the weak D test is omitted; we believe that this would impose only a minor burden on the D negative RBC inventory, especially if an incubation is performed after a weak or negative IS test in accordance with the reagent manufacturer's instructions. Again, without an analysis of the *RHD* gene, it is impossible to know if a recipient with a weak D phenotype is susceptible to alloimmunization. Furthermore, RhIg has a long history of both safety and efficacy in preventing alloimmunization.

The Society of Obstetricians and Gynaecologists of Canada, in their 2003 guidelines on the prevention of D alloimmunization, mandated the use of the weak D test in pregnant women.<sup>14</sup> This policy classifies those women who demonstrate hemagglutination with anti-D reagents *only* in the weak D test as D positive, which might unnecessarily

**Figure 2.** Suggested algorithm for D testing of blood recipients. Note the use of anti-D reagents featuring limited specificity (see Glossary) to enhance the probability that a weak D or partial D recipient would be classified as D negative. The minimum agglutination strength at immediate spin for a recipient to be considered D positive varies between laboratories but is generally  $> 2+$  or  $= 2+$ . Note that the incubation procedure only needs to be performed once on every new recipient or pregnant woman. Most people who agglutinate  $< 2+$  at IS or after enhancement techniques will have one of the prevalent weak D alleles that, based on our current understanding of the *RHD* gene, would not make them susceptible to alloimmunization if exposed to normal D+ RBCs. Some recipients who demonstrate these serological findings may have partial D (such as DIV) and one of the rare weak D alleles (such as weak D type 15) and would be at risk of alloimmunization. Thus the assignment of D status based on the underlying *RHD* allele should be done on a patient-by-patient basis.



**Figure 3.** Suggested algorithm for D testing of blood donors and cord blood samples. Note that the threshold for classifying a blood donor as D positive is generally considerably lower than for a blood recipient. This, along with the use of anti-D reagents featuring broad specificity (see Glossary) that detect many D epitopes, is done to ensure that the components from a donor with weak expression of D antigen are labelled as D positive. To further increase D antigen detection, the weak D test is employed as required by the AABB and CSA Z902 standards.<sup>12,13</sup> A D negative woman who gives birth to a baby who types positive at any phase of D typing should receive Rhlg prophylaxis.



increase the number of sensitizations. This adverse outcome could be safely and easily prevented if our recommendation of using *RHD* genotyping in resolving weak D and partial D individuals were implemented.

### The Way Forward

To avoid a possible D alloimmunizing event, many institutions classify pregnant women and transfusion recipients as D negative if their RBCs demonstrate weak (< 2+) agglutination at the IS phase of testing (including an incubation if permitted by the manufacturer) on the premise that weak agglutination might indicate the presence of a weak D or a partial D. We strongly advocate the use of *RHD* genotyping to resolve these problematic typings. The molecular assays are safe, are cost-effective as they only need to be performed once per individual, can be adapted to suit various laboratory throughput levels, and have been in use for many years in some, predominantly European, health care systems. If applied when the IS phase is equivocal, *RHD* gene analysis will identify which recipients and pregnant women harbour alleles not susceptible to D alloimmunization and can be safely classified as D positive, and which recipients should be classified as D negative. This would provide a personalized approach to determining the need for RhIg and D negative blood products.

### D Typing of Blood Donors and Cord Blood Specimens

The AHG weak D test still has its place in the blood bank. It is performed on blood donors<sup>12,13</sup> and should be performed on cord blood samples that initially type as D negative, as these are potentially sensitizing events. In these settings, we recommend the use of anti-D reagents with the broadest specificity so that weak D and partial D donor units and cord blood samples would be deemed D positive (Figure 3).

One area of confusion lies in the possibility that a weak D or partial D individual could be classified as D positive at the time of donation but as D negative if he or she required a transfusion. This apparent paradox is easily explained by the different testing requirements for blood donors and recipients. Similarly, if a pregnant woman tested positive only in the weak D test (D<sup>u</sup> positive) in an earlier pregnancy, RhIg would probably not have been administered; if in the current pregnancy her assignment of D status was made only at the IS phase and she still typed weakly, then RhIg should be recommended. This apparent change in her D status can likewise be explained by the different sensitivities of the testing modalities. Weak D and partial D RBCs are capable of stimulating an anti-D response when transfused to truly D negative recipients. Yet individuals who express partial D phenotypes, and certain rare weak D phenotypes, are themselves susceptible to anti-D alloimmunization and for this

reason should be considered D negative in pregnancy and for transfusions. However, identifying DEL donors will remain a challenge until methods for *RHD* genotyping become more mainstream; an analysis of 19 679 serologically D negative donors revealed 41 with an *RHD* allele, of whom at least 14 carried a DEL phenotype.<sup>15</sup> The potential morbidity if DEL positive RBC units from these potentially repeat donors were regularly transfused to women of childbearing age, resulting in sensitization to the D antigen, is worrisome.

### Summary of Recommendations For D-typing of Samples From Recipients and Pregnant Women and of Blood Donors and Cord Blood Samples

Recipients and pregnant women:

- Use limited specificity anti-D typing reagent (e.g., contains a single IgM monoclonal anti-D).
- Do not perform the weak D test (do not use AHG reagent).
- If negative or weak agglutination at immediate spin (IS) phase, incubate as indicated by the reagent specifications.
- Use *RHD* genotyping to resolve the underlying allele in individuals who demonstrate weak agglutination at IS phase of testing.

Blood donors and cord blood samples:

- Use broad specificity anti-D typing reagents (e.g., mix of IgM and IgG oligoclonal anti-D).
- Weak D test must be performed on blood donors<sup>12,13</sup> and should be performed on cord blood samples.
- Use *RHD* genotyping to identify clinically relevant *RHD* allele in individuals who appear D negative using the weak D test.

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### Competing Interests

Dr Flegel and his employer, the blood transfusion service of the Deutsches Rotes Kreuz Baden-Württemberg-Hesse gGmbH (German Red Cross Baden-Württemberg-Hesse Ltd.), hold patents or have patents pending on nucleotide sequences and their use in molecular diagnostics for weak D, Rhesus box, *RHD* deletion, and several DEL alleles. Dr Flegel advises Ortho-Clinical Diagnostics, and received EU project support (BloodGen consortium).

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## GLOSSARY

**Anti-D typing reagents:** Most anti-D typing reagents are a blend of IgM and IgG anti-D with specificities to different D epitopes on the D antigen.

**Antigen:** An entity that can stimulate the production of an antibody.

**Broad specificity anti-D reagent:** A serological reagent containing a mixture of a large number of different antibodies each recognizing different D epitopes. This type of reagent is suitable for typing blood donors and cord blood samples, as the blend of anti-D is designed to recognize a large number of D variants, any of which may stimulate an

immune response in D negative or partial D pregnant women or transfusion recipients.

**Epitope:** Determinant of the antigen: binding site of an antibody to its antigen.

**Immediate spin (IS) test:** In the setting of D-typing, this test involves mixing IgM anti-D with red blood cells (RBC) at room temperature, incubating this mixture as indicated by the reagent specifications, followed by centrifugation and then inspection for hemagglutination. IgM can cause direct agglutination of RBCs without requiring an antiglobulin reagent (see Weak D test).

**Limited specificity anti-D reagent:** A serological reagent composed of a single monoclonal anti-D or of a blend of anti-Ds each recognizing a different D epitope expressed on selected D variants. This type of reagent is suitable for use in typing blood recipients or pregnant women, as the formulation of anti-D is designed not to detect partial D types that are susceptible to D alloimmunization.

**Partial D RBCs:** These uncommon RBCs usually contain normal numbers of RhD protein, although the protein is mutated in an exofacial loop, eliminating at least one D-specific epitope on the protein thus leaving the individual susceptible to alloimmunization if exposed to the wild-type form of the D antigen.

**RhD protein:** A highly immunogenic protein present on the surface of approximately 85% of Caucasian, > 90% of African and nearly 100% of Asian RBCs. By convention, the term RhD refers to the protein itself, while the letter D refers to the D antigen and to the D epitopes on the RhD protein.

***RHD* gene:** The gene located on chromosome 1 encoding the RhD protein.

**Weak D RBCs:** These RBCs demonstrate reduced quantities of the D antigen due to mutations in the proteins' transmembrane or cytoplasmic domains. Generally, all D-specific epitopes are expressed on these RhD proteins.

**Weak D test:** This involves mixing RBCs with anti-D typing reagents containing an IgG component. Unlike IgM, IgG cannot directly agglutinate RBCs. After washing the RBCs to remove any unbound antibodies, the antiglobulin reagent (AHG) is added which causes the agglutination of IgG coated RBCs. This test is essentially an indirect antiglobulin test.<sup>16</sup>